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### Lipopolysaccharide-Binding Protein Is Vectorially Secreted and Transported by Cultured Intestinal Epithelial Cells and Is Present in the Intestinal Mucus of Mice<sup>1</sup> **FREE**

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# Lipopolysaccharide-Binding Protein Is Vectorially Secreted and Transported by Cultured Intestinal Epithelial Cells and Is Present in the Intestinal Mucus of Mice<sup>1</sup>

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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. *The Journal of Immunology*, 2000, 165: 4561–4566.

The acute phase protein LPS-binding protein (LBP)<sup>3</sup> 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 (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 (10–12). 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.

## Materials and Methods

### Reagents and Abs

Human rTNF- $\alpha$  was kindly provided by BASF/Knoll (Ludwigshafen, Germany); human rIL-6 by Dr. W. Sebald (Physiologisch-Chemisches Institut der Universität Würzburg, Würzburg, Germany); human rIL-1 $\beta$  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 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, Center Hospitalier Universitaire Vaudois, Lausanne, Switzerland. The serum amyloid A (SAA) immunoassay was kindly provided by Dr. P. C. Limburg, Department of Rheumatology (University of Groningen, Groningen, The Netherlands). The apoA-I and apoB immunoassay were a gift from Dr. Gavilondo, Center for Genetic Engineering and Biotechnology (Havana, Cuba).

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<sup>3</sup> Abbreviations used in this paper: LBP, LPS-binding protein; mLBP, murine LBP; SAA, serum amyloid A; TMB, tetramethylbenzidine.

### 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% CO<sub>2</sub> in DMEM (Life Technologies, Paisley, U.K.), supplemented with 10% FCS (HyClone), 1 mM sodium pyruvate (Life Technologies), 0.1 mM nonessential 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-cm<sup>2</sup> or 75-cm<sup>2</sup> flasks (Costar, Cambridge, MA). Cultures were split when they reached 80% confluence.

For experiments, 5 × 10<sup>5</sup> Caco-2 cells/cm<sup>2</sup> were seeded onto a 1-cm<sup>2</sup> or 4.7-cm<sup>2</sup> collagen-coated membrane (3 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 2 wk, 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 were 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-cm<sup>2</sup> 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 ELISAs. 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 MgCl<sub>2</sub> 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, San Francisco, CA) and tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, 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 HRP-labeled anti-SAA mAb (Reu 86.1), followed by TMB substrate.

For analysis of human apoA-I and apoB concentrations, plates were coated with specific mAbs 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-cm<sup>2</sup> 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-human LBP mAb HM-14 was cross-linked to CNBr-activated Sepharose (Pharmacia), according to the manufacturer's instructions. LBP-depleted serum contained less than 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-h 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<sup>5</sup> 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 µg LPS. Controls consisted of untreated mice. Mice were killed 24 h 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 × 1-cm filter paper (Whatmann no. 42; Maidstone, U.K.), 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 µl 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, the amount of IgG present in the buffer also was analyzed.

### Statistical analysis

Data were analyzed using SPSS (Chicago, IL) software. All values in the figures and text are expressed as mean ± 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<sub>10</sub> 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<sub>10</sub> transformation of mucus:serum ratios was performed.  $u = \text{Log}(\text{LBP ratio/IgG ratio})$  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 that provide separation of apical and basolateral compartments. For stimulation of the monolayers, a mix of IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNF-α (100 ng/ml), and 1 µM dexamethasone was administered to the differentiated cells

Table I. *Vectorial secretion of LBP by intestinal epithelial cells<sup>a</sup>*

Stimulation	Apical LBP	Basolateral LBP
None	0.19 ± 0.12	ND <sup>‡</sup>
Apical	1.12 ± 0.57*	ND <sup>‡</sup>
Basolateral	2.03 ± 0.42*†	7.22 ± 2.27*†‡

<sup>a</sup> Cells were stimulated apically or basally with a combination of IL-1 $\beta$  (5 ng/ml), IL-6 (100 ng/ml), TNF- $\alpha$  (100 ng/ml), and 1  $\mu$ M dexamethasone for 84 h. Data are expressed as the mean of total released nanograms 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 ( $p < 0.05$ ).

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  $\pm$  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  $\pm$  0.57 ng LBP,  $p < 0.05$ ), while no LBP was detectable in the basolateral medium. Control experiments demonstrated that rLBP traversed the naked filters and appeared in both compartments (data not shown). 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  $\pm$  2.27 ng) and the amount of LBP in the apical compartment increased markedly (4.06 ng/ml absolute 2.03  $\pm$  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.

#### Vectorial secretion of SAA and apolipoproteins by Caco-2 cells

During inflammation, the concentration of several plasma proteins other than LBP is 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.

#### 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 bioassay, LBP in both apical and basolateral medium was demonstrated to enhance the endotoxin-induced cell activation

Table II. *Vectorial secretion of SAA and apolipoproteins by intestinal epithelial cells<sup>a</sup>*

Stimulation	SAA		apoA-I		apoB	
	Apical	Basolateral	Apical	Basolateral	Apical	Basolateral
None	ND	ND	1.1 $\pm$ 0.2	3.3 $\pm$ 1.3 <sup>‡</sup>	3.6 $\pm$ 1.7	13.9 $\pm$ 4.0 <sup>‡</sup>
Apical	184.2 $\pm$ 69.8*	48.3 $\pm$ 15.3*†	0.9 $\pm$ 0.1*	2.1 $\pm$ 1.1 <sup>‡</sup>	2.2 $\pm$ 0.7	8.5 $\pm$ 3.4 <sup>‡</sup>
Basolateral	217.5 $\pm$ 74.4*	583.3 $\pm$ 132.3*†‡	0.9 $\pm$ 0.2	1.3 $\pm$ 0.5*	1.8 $\pm$ 0.2*	5.7 $\pm$ 3.0*†

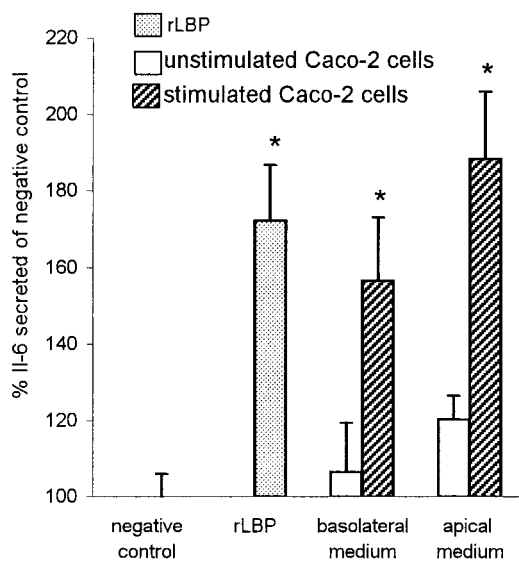
<sup>a</sup> Cells were stimulated apically or basally with a combination of IL-1 $\beta$  (5 ng/ml), IL-6 (100 ng/ml), TNF- $\alpha$  (100 ng/ml), and 1  $\mu$ M dexamethasone for 84 h. Data are expressed as the mean of total released nanograms SAA, micrograms apoA-I, and apoB  $\pm$  SD per compartment ( $n = 7$ ). 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 ( $p < 0.05$ ).



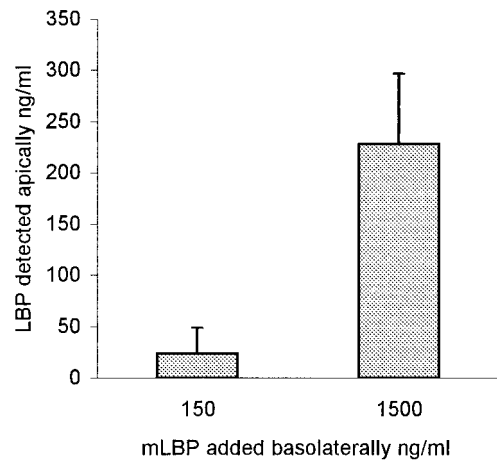
compared with LBP-deprived conditions ( $p < 0.05$ ) (Fig. 1). Apical and basolateral media from untreated monolayers did not significantly affect the endotoxin-induced cytokine release.

*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 toward 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 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



**FIGURE 1.** Bioactivity of apical and basolateral secreted LBP. Caco-2 cells were grown on Transwell-Col and stimulated basally with IL-1 $\beta$  (5 ng/ml), IL-6 (100 ng/ml), TNF- $\alpha$  (100 ng/ml), and 1  $\mu$ 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-well 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,  $1 \times 10^5$  PBMC and 0.5 ng LPS/ml were added to the wells. Supernatants were harvested after 20 h, and IL-6 released into the supernatant was analyzed. Results shown represent percentage of IL-6 secreted compared with negative controls, and are presented as means  $\pm$  SD of three wells. \*, Significant difference from negative control ( $p < 0.05$ ).



**FIGURE 2.** Transport of LBP across Caco-2 monolayers is directed toward the apical surface. A total of 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 72-h incubation in the apical compartments. Presented are the means  $\pm$  SD of the mLBP concentration in the apical compartment of three wells.

demonstrated the presence of LBP in the apical and not the basolateral medium after apical stimulation of the cells and in quiescent cells.

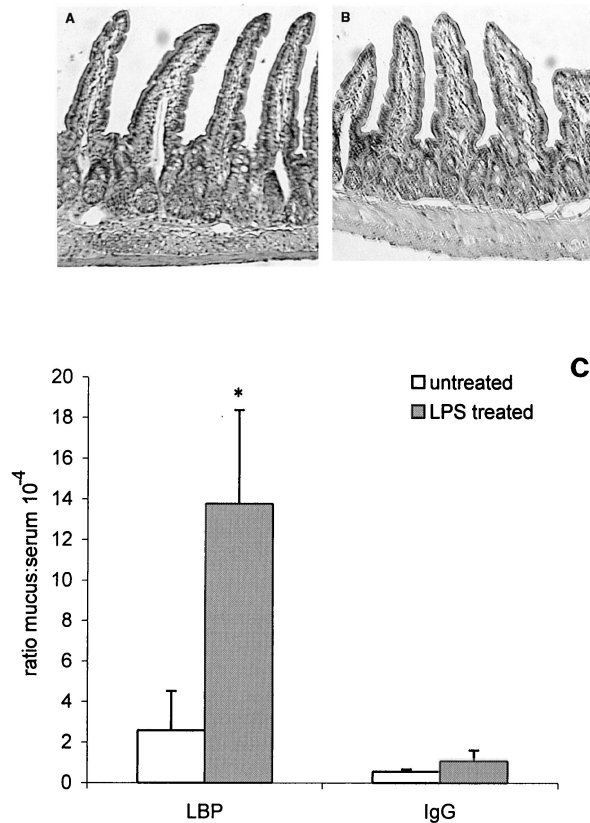
*The LBP content of mucus is enhanced during endotoxemia*

The data obtained in this study to date suggest that cytokines enhance the constitutive secretion of LBP toward the luminal surface of intestinal epithelial cells. Subsequently, 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).

Intraperitoneal administration of endotoxin to mice resulted in a significantly increased serum concentration of LBP ( $20.9 \pm 7.3 \mu\text{g/ml}$ ,  $p < 0.05$ ) as compared with the controls ( $5.4 \pm 2.6 \mu\text{g/ml}$ ). The amount of LBP absorbed from 0.5-cm<sup>2</sup> 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. 3, A and 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  $6 \times 10^{-5}$  and  $1 \times 10^{-4}$  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<sup>2</sup> mucus to the concentration of LBP in serum of untreated mice was  $2.6 \times 10^{-4}$ , and of mice injected i.p. with endotoxin,  $1.4 \times 10^{-3}$  (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 of LBP in mucus to serum corrected for IgG leakage was significantly enhanced five times ( $p < 0.05$ ) compared with 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



**FIGURE 3.** LBP presence in mucus of mice is enhanced during endotoxemia. Swiss mice were injected i.p. with 100  $\mu$ 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 four and three mice, respectively. \*, Significant enhanced compared with controls after correction for IgG leakage ( $p < 0.05$ ).

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 (10–12), 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 toward 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 overlying 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 toward 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 (10–12) 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 in which its function can contribute to the defense of the gut, comparable with other proteins that 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 microenvironment. 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 that demonstrated that an i.p. 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 catalyze the lipoprotein neutralization of LPS and lipoteichoic acid, a component of the cell membrane of Gram-positive bacteria (31). Low nonharmful amounts of endotoxin are thought to leak continuously across the intact intestinal epithelium. LBP has a role in both sensitizing the immune system 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 work 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 cryptabscesses, cellular infiltrates within the bowel wall, and bacterial translocation demands further investigation.

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