Conjugated primary bile salts reduce permeability of endotoxin through bacteria-stimulated intestinal epithelial cells and synergize with lecithin in suppression of inflammatory cytokine production

Parlesak, Alexandr; Schaeckeler, Simone; Moser, Lydia; Bode, Christiane

Published in:
Critical Care Medicine

Link to article, DOI:
10.1097/01.CCM.0000284586.84952.FB

Publication date:
2007

Conjugated primary bile salts reduce permeability of endotoxin through intestinal epithelial cells and synergize with phosphatidylcholine in suppression of inflammatory cytokine production

Alexandr Parlesak, PhD; Simone Schaeckeler, MSc; Lydia Moser, MSc; Christiane Bode, PhD

Objective: **Endotoxemia was shown to be integral in the pathophysiology of obstructive jaundice. In the current study, the role of conjugated primary bile salts (CPBS) and phosphatidylcholine on the permeability of endotoxin through a layer of intestinal epithelial cells and the consequent activation of basolaterally cocultured human mononuclear leukocytes were measured.**

Design: **In a coculture model, a layer of differentiated, confluent Caco-2 cells was apically stimulated with growth-arrested, nonpathogenic *Escherichia coli*.**

Setting: **Basic human cell culture laboratory.**

Interventions: **The effect of CPBS (0.5 mM and 1.5 mM), phosphatidylcholine (0.38 mM), and human bile (0.5% vol/vol) on the barrier function was assessed by the measurement of transepithelial electrical resistance, by endotoxin permeability through the intestinal epithelial cell layer, and by basolateral cytokine enzyme-linked immunosorbent assay measurement (tumor necrosis factor-α, interleukins-6, -8, and -10). Micelles formed by CPBS were detected by dynamic light scattering. The association of endotoxin with CPBS micelles was tested by fluorescence resonance energy transfer.**

Measurements and Main Results: **Apical addition of CPBS suppressed the permeability of endotoxins through the intestinal epithelial cell layer significantly. In parallel, apical supplementation of CPBS dose-dependently reduced the basolateral production of all cytokines measured. Apical phosphatidylcholine supplementation enhanced this effect significantly. CPBS formed micelles (diameter, 134 ± 7 nm), which were able to bind endotoxin to their surface.**

Conclusions: **CPBS can reduce the permeation of endotoxin through intestinal epithelial cell layers by binding it to micelles. Thereby, the inflammatory processes beyond the mucosal surface are suppressed, an effect that is enhanced by phosphatidylcholine. (Crit Care Med 2007; 35:2367–2374)**

KEY WORDS: conjugated primary bile salts; phosphatidylcholine; bile; inflammation; intestinal epithelial cells; endotoxin

Invasive treatment of patients with obstructive jaundice is associated with an increased risk of death and postoperative complications, with morbidity rates of about 30% (1, 2). A considerable number of these complications has a Gram-negative septic origin and is paralleled by an endotoxemia (3, 4). Persistent systemic inflammatory complications after surgical treatment of obstructive jaundice are thought to result at least in part from this endotoxemia (5), the physiologic consequences of which are mediated by inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 (6). Ingestion of bile salts or oral supplementation with bile reduced endotoxemia in such patients (7). Furthermore, feeding of both cirrhotic rats and rats with bile duct ligation with conjugated bile salts reduced both bacterial translocation and endotoxemia (8, 9).

Etiological factors considered to be important for the development of endotoxemia are an impaired intestinal barrier (10) with a consequently elevated translocation of bacteria and endotoxins from the gut lumen (4, 11, 12). Furthermore, the reduction of bile flow may lead to an impaired ability of Kupffer cells to clear portal blood from endotoxins, leading to a spillover of these bacterial toxins into the systemic circulation (13, 14).

Although endotoxemia and elevated cytokine concentrations were demonstrated in some studies with patients (4, 15), the main source of data on pathophysiological consequences of obstructive jaundice is from animal experiments. In these experiments, organs affected by obstructive jaundice, namely the liver and the intestine, interact closely; therefore, an impaired barrier function of the gut might also result from liver dysfunction (13). Furthermore, the effects of obstructive jaundice differ between animals and humans with respect to blood concentrations of both endotoxins and cytokines (15).

The working hypothesis of the present study was that bile components such as conjugated primary bile salts (CPBS) and phosphatidylcholine, *per se*, can reduce the transmigration of endotoxins through a layer of human intestinal epithelial cells

From the Nutritional Immunology Group, Biocentrum, Technical University of Denmark, Lyngby, Denmark (AP); and the Department of Physiology of Nutrition, Institute for Biochemistry and Nutrition, Hohenheim University, Stuttgart, Germany (SS, LM, CB).

The authors have not disclosed any potential conflicts of interest.

Supported, in part, by institutional departmental funds and by a grant (12/2006) from the Institut Danone Ernährung für Gesundheit, Haar, Germany.

For information regarding this article, E-mail: alpa@biocentrum.dtu.dk

DOI: 10.1097/01.CCM.0000284586.84952.FB

(IEC), the functionality of which is not affected by liver malfunction. For this purpose, we used a coculture model that was previously established in our department (16, 17). In this model, a layer of differentiated IEC (Caco-2 cells) separated an apical compartment (luminal side) from a basolateral one (blood side). As a standard stimulus, nonpathogenic, Gram-negative bacteria (*Escherichia coli*) were applied apically. To reflect the detrimental interaction between 1) permeation of immunostimulating bacterial toxins, 2) stimulation of immunocompetent cells with the consequent shedding of proinflammatory cytokines (such as TNF-α and IL-8) (18), and 3) an increased permeability being induced by these cytokines, we added basolaterally mononuclear leukocytes from peripheral venous blood (peripheral blood mononuclear cells, PBMC).

MATERIALS AND METHODS

Human Intestinal Epithelial Cells. Cells of the human intestinal cell line Caco-2 (1×10^6; DSM Acc. No. 169, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were sown on semipermeable inserts in tissue culture plates (both from Becton Dickinson, Heidelberg, Germany) and cultured for 14–16 days in Dulbecco modified Eagle medium high glucose supplemented with L-glutamine, nonessential amino acids (PAA, Cölbe, Germany), and 17% pyrogen-free fetal calf serum (Biochrom, Berlin, Germany). Only Caco-2 layers with a transepithelial electrical resistance (TEER; Millicell-ERS, Millipore, Eschborn, Germany) higher than 380 $\Omega \cdot cm^2$ were used for experiments. The study was considered by the local ethics committee to be exempt from approval.

Mononuclear Leukocytes/Bacteria Cultivation. Mononuclear cells (PBMC: ~74% lymphocytes, ~15% monocytes, ~9% natural killer cells, <2% neutrophils) (19) were isolated from peripheral venous blood (Dr. Luz, Institute of Blood Transfusion Medicine, Katharinenhospital, Stuttgart, Germany) of eight healthy men (age, 44.0 ± 2.6 yrs) by density gradient centrifugation, as described previously (17). The nonpathogenic, commensal strain *E. coli* K12 was cultured for two subsequent breeding periods (12 hrs and 24 hrs) in liquid Luria broth (1% tryptone: Oxoid, Wesel, Germany; 0.5% yeast extract: Difco, Heidelberg, Germany; 0.5% NaCl). Gentamicin (120 μg/mL; GIBCO BRL/Life Technologies, Grand Island, NY) was added to all apical media to arrest growth of *E. coli*.

Cocultivation of Enterocytes, Leukocytes, and Bacteria. Cocultivation of differentiated, polarized IEC (Caco-2 cells) and leukocytes was performed as described previously (17). Briefly, 4×10^6 PBMC in 2 mL of completed Dulbecco modified Eagle medium with gentamicin (20 μg/mL) were contained in the basolateral compartment beneath the inserts with the differentiated and confluent enterocyte layer. The apical compartment contained either 2 mL of completed Dulbecco modified Eagle medium (negative control) or it was spiked with *E. coli* (2×10^7 colony-forming units, positive control). In further experimental settings, CPBS (0.5 mM or 1.5 mM), CPBS (1.5 mM) together with purified phosphatidylcholine (0.375 mM L-α-phosphatidylcholine) from egg yolk (Sigma, Taufkirchen, Germany), or human bile (0.5% vol/vol) were added apically. Human bile was obtained from nine human volunteers receiving a cholecystectomy due to bile stones in the bile duct. Patients gave informed consent on offering their bile for the experiments. The applied CPBS, glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, and taurochenodeoxycholic acid, were provided by Dr. Teuschel (Falk Pharma, Freiburg, Germany). According to the average composition of human bile (20, 21), the molar concentration ratio of the glycine conjugates to those of taurine was 4:1, whereas that of cholic acid to chenodeoxycholic acid was 1:1. All incubations lasted for 24 hrs. Viability of both cell types was determined to be ≥94% in the presence of all compounds applied (trypan blue exclusion test).

Release of Cytokines. To assess stimulation of leukocytes, the basolateral concentrations of TNF-α and IL-6 were measured after 12 and 24 hrs, respectively. IL-8 and IL-10 were determined after 24 hrs (17). All cytokine concentrations were measured with OptEIA enzyme-linked immunosorbent assay sets (BD Pharmingen, Heidelberg, Germany) following the instruction of the manufacturer.

Concentration and Permeability of Endotoxin. The concentration of endotoxin was measured with a chromogenic limulus amebocyte lysate test (Endochrome-K, Charles River, Charleston, SC) using isolated endotoxin from *E. coli* K12 (22). Purity of the extracted endotoxins was verified by electrophoresis (23). As also shown previously (24), CPBS at concentrations applied in the current study did not interact with the limulus amebocyte lysate test (data not shown).

Permeability of Polar Macromolecules. To investigate whether the treatment of IEC was associated with changes in paracellular permeability for polar soluble macromolecules, we applied a FITC-labeled dextran (Mr 4400, 1 mg/mL, Sigma) to the apical compartment. The relative permeability of this macromolecule was calculated from the concentration in the basolateral compartment after 24 hrs (Fluorstar, SLT, Freiburg, Germany).

High-Performance Liquid Chromatography Analysis of CPBS. CPBS were analyzed by applying slightly modified methods previously described (25, 26). Before extraction of CPBS, 60 μL of an internal standard solution [4-(1,1,3,3-teramethyl-butyl)-phenol (Sigma-Aldrich, Steinheim, Germany), and 0.5 mM in isopropanol-H_2O (70:30)] was added to the samples (800 μL each). CPBS were extracted from the medium by adding 200 μL of isopropanol-H_2O (70:30) for 1 hr at 4°C and evaporated. The residue was redissolved in 60 μL of isopropanol-H_2O (70:30). For each sample, 10 μL was separated on a reversed-phase column (LiChrosphere 100RP18 EC-5 μM, 125 × 3 mm, 0.5 mL/min) with a gradient system: 0 min: 100% solvent A (methanol/10 mM aqueous ammonium formate, 40:60); to 6 mins: 100% solvent B (methanol/10 mM aqueous ammonium formate [pH 5.0], 80:20; Merck, Darmstadt, Germany); to 14 mins: 100% solvent A (15 mins) to the end of the run (22 mins). CPBS were detected at 202 nm, and the area under the curve was calculated from standard serial dilution sequences of all CPBS of interest regarding the recovery of the internal standard [4-(1,1,3,3-teramethyl-butyl)-phenol] (Chromeleon software package, Dionex, Idstein, Germany).

Detection of Micelles Formed by CPBS. The (hydrodynamic) diameter of micelles was measured by dynamic light scattering with the particle size analyzer HPPS 3.1 from Malvern Instruments (Worcestershire, UK) (n = 8) and calculated by algorithms included in the software package ALV-NIBS/HPPS V.3.0.0.13, which are based on the Stokes-Einstein relation.

Fluorescence Resonance Energy Transmission. To investigate whether endotoxin (FITC-labeled) incorporates into micelles formed by CPBS, a probe for fluorescence resonance energy transfer (octadecyl rhodamine B chloride, Invitrogen, Karlsruhe, Germany) was applied. This compound showed its suitability to identify colocalization with fluorescein-labeled constructs in previous experiments (27). Florescence spectra (excitation, 492 nm [excitation maximum of FITC endotoxin]; emission, 500–640 nm) were recorded for solutions of FITC-labeled endotoxin of *E. coli* O55:B5 (3 μg/mL, 3 μg FITC/mg endotoxin; Sigma) in phosphate-buffered saline alone, together with octadecyl rhodamine B (8.5 μg/mL in dimethyl sulfoxide), and after addition of 0.5 mol/L and 1.5 mmol/L CPBS. Neither dimethyl sulfoxide nor CPBS affected the fluorescence spectra (excitation, 554 nm) of octadecyl rhodamine B solutions in the absence of FITC-labeled endotoxin (data not shown).

Immunomodulating Effect of CPBS and Phosphatidylcholine on Lipopolysaccharide-Stimulated PBMC. In microtiter plates with 96 wells, 1.0×10^5 PBMC (n = 8) were stimulated directly with 200 pg/mL lipopolysaccharide isolated from *E. coli* K12 (as described above) in a total of 240 μL of completed medium. Final total concentrations between 2.1 and 1500 μmol/L CPBS were added to the medium. In separate experiments, the addition of phosphatidylcholine (2.5–600 μmol/L) on the release of TNF-α was investigated. The TNF-α concentration after 12 hrs was mea-

sured by an enzyme-linked immunosorbent assay method as described above.

Statistics. All values are given as mean ± SEM. One-way analyses of variance with the consequent *post hoc* test of Tukey were applied for the determination significance levels. Progressively, the differences were considered as significant if the p value was $<.05$. Coefficients and significance of correlations between the concentrations of endotoxin and cytokines were calculated with the nonparametric test of Spearman.

RESULTS

Permeability of Endotoxin and Dextran Mr 4400 Through the IEC Layer. After 6 hrs, about 0.007% (0.14% at 24 hrs) of the endotoxin content in the apical medium (1.4 ± 0.4 μg/mL) permeated through the enterocyte layer. Addition of CPBS to the apical medium pronouncedly reduced the permeability of endotoxin through the enterocyte layer both after 6 hrs and 24 hrs (Fig. 1). Although moderately lower, the permeability of endotoxin after 24 hrs was not further reduced by phosphatidylcholine addition compared with experiments with the addition of CPBA only. Human bile also reduced significantly the transmigration of endotoxin through the IEC layer, comparable with the effect of 0.5 mM CPBS. In contrast to endotoxin, the permeability of dextran was not affected by the addition of CPBS, phosphatidylcholine, or bile. After 24 hrs, the relative permeability ranged between 0.9% ± 0.3% (positive control) and 1.3% ± 0.4% (1.5 mmol/L CPBS). With respect to dextran Mr 4400 permeability, analysis of variance revealed no significant differences among the single-treatment groups.

Release of Cytokines in the Basolateral Compartment of the Coculture. The addition of *E. coli* K12 to the apical compartment of the coculture model resulted in a pronounced and highly significant increase of all cytokines measured. The average concentration of TNF-α and IL-6 increased about 100-fold, that of IL-8 increased about 13-fold, and that of IL-10 was about 50-fold higher (Table 1).

Supplementation of the apical medium with CPBS induced a dose-dependent and significant reduction of concentration of TNF-α (12 hrs) and IL-10 (24 hrs) (Fig. 2). At a concentration of 1.5 mmol/L, the basolateral production of IL-6 (12 hrs) and IL-8 (24 hrs) was also reduced by apical CPBS addition, but to a lesser extent. Phosphatidylcholine supplementation to the apical medium containing 1.5 mM CPBS reduced the TNF-α concentration (Fig. 2) nearly to the level of the nonstimulated control (Table 1). A significant but less pronounced reduction in cytokine production in experiments with phosphatidylcholine was also measured for IL-8, IL-10, and IL-6 (Fig. 2). Human bile (0.5%) also significantly reduced the production of TNF-α and IL-10 to an extent comparable with the 0.5 mM CPBS supplementation (Fig. 2). The basolateral concentration of permeated endotoxin correlated closely with the amount of produced TNF-α (Fig. 3) and other cytokines (data not shown).

Transepithelial Electrical Resistance. After 24 hrs, apical challenge of IEC with *E. coli* (positive control) resulted in a significant decrease of the TEER (530 ± 30 vs. 659 ± 44 $\Omega \cdot cm^2$, $p = .010$ vs. positive control). None of the TEER values of the other treatments, which ranged after 24 hrs between 487 ± 54 $\Omega \cdot cm^2$ (0.5 mM CPBS, $p = .945$ vs. positive control) and 637 ± 42 $\Omega \cdot cm^2$ (0.5% bile, $p = .169$), differed significantly.

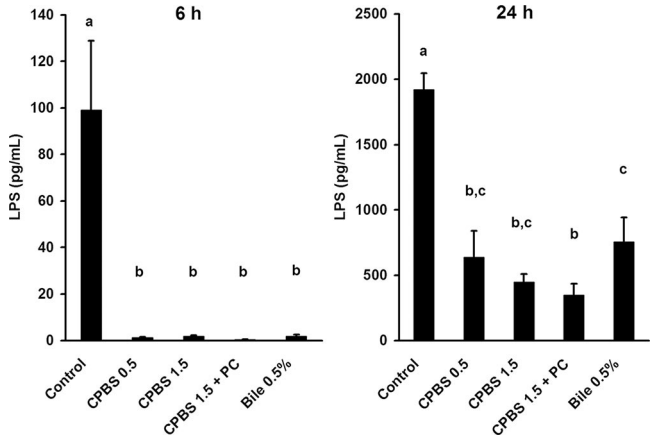

Figure 1. Concentration of endotoxin (lipopolysaccharide, *LPS*; mean ± SEM) in the basolateral compartment of the coculture model (6 hrs and 24 hrs) after the apical addition of conjugated primary bile salts (*CPBS*), phosphatidylcholine, and pooled human bile. Enterocytes were stimulated apically by *Escherichia coli*. Different letters above the columns assign statistically different values. Levels of significance (p values) range from .014 (control–bile 0.5%) to <.001. *CPBS 0.5/CPBS 1.5*, CPBS at an apical concentration of 0.5/1.5 mM; *PC*, phosphatidylcholine at an apical concentration of 0.375 mM. Columns without a common letter differ significantly (Tukey's *post hoc* test, $p < .05$).

Table 1. Effect of apical challenge of enterocytes that were cocultured with human leukocytes with 2.0×10^7 colony-forming units of *Escherichia coli* on concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, and IL-10 after 12 or 24 hrs, respectively, in the basolateral medium of the coculture model

	TNF-α (pg/mL) 12 Hrs		IL-6 (ng/mL) 12 Hrs		IL-8 (ng/mL) 24 Hrs		IL-10 (pg/mL) 24 Hrs	
E. coli	—	+	−	+	−	+	−	+
	45 ± 15	5538 ± 799[a]	0.4 ± 0.1	33 ± 3.0[a]	30 ± 7	379 ± 56[a]	14 ± 3	747 ± 108[a]

−/+, with/without *E. coli*.
[a] $p < .001$.

Transport of CPBS Through the IEC Layer. In experiments in which CPBS were supplemented to the apical medium, the basal concentrations of glycine conjugates (glycocholic acid and glycochenodeoxycholic acid) were about four-fold higher than those of the taurine conjugates (taurocholic acid and taurochenodeoxycholic acid), which paralleled the apically applied ratio of these compounds (Fig. 4). After 6 hrs of incubation, the concentration of CPBS in the basolateral compartment was below the detection limit (<5 μmol/L). After 24 hrs, about 20% of the apically applied CPBS were detected in the basolateral medium. The relative transport of the CPBS did not differ significantly either with respect to the absolute apical concentration or with respect to their chemical composition.

As measured with high-performance liquid chromatography, the pooled human bile contained 7.2 mM taurocholic acid (final concentration in the apical medium, 0.036 mM), 8.7 mM (0.044 mM) taurochenodeoxycholic acid, 15.6 mM (0.078 mM)

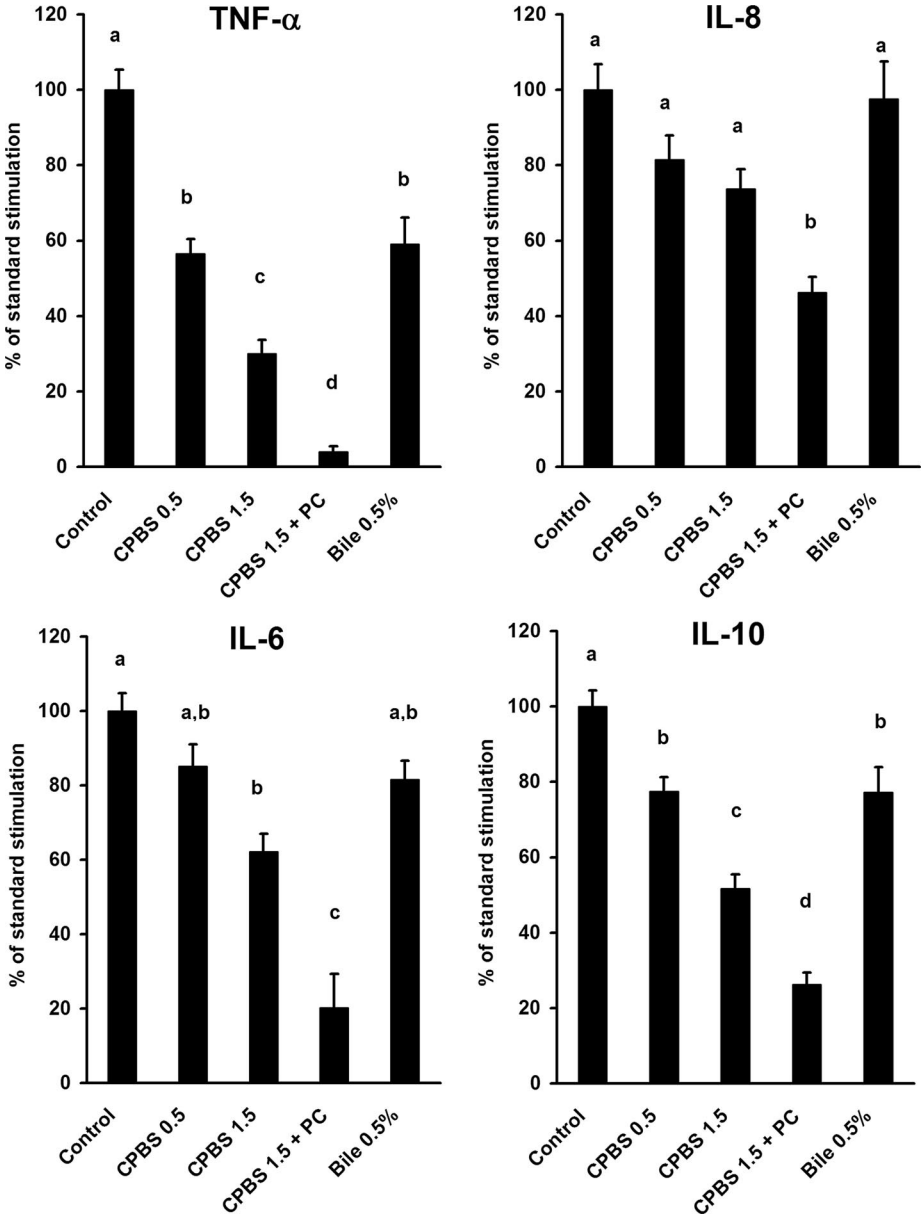

Figure 2. Influence of apically added conjugated primary bile salts (*CPBS*), phosphatidylcholine (*PC*), and pooled human bile on the concentration (mean ± SEM) of tumor necrosis factor (*TNF*)-α (12 hrs), interleukin (*IL*)-8 (24 hrs), IL-6 (12 hrs), and IL-10 (24 hrs) in the basolateral compartment of the coculture model. Enterocytes were stimulated apically with *Escherichia coli*. Columns without a common letter differ significantly (Tukey's *post hoc* test, $p < .05$).

glycocholic acid, and 15.8 mM (0.079 mM) glycochenodeoxycholic acid. Hence, the sum concentration of CPBS in the apical medium supplemented with bile was 0.24 mM. The relative transport of the single CPBS through the layer of IEC did not differ significantly from that measured in experiments with 0.5 mM and 1.5 mM CPBS or the pooled bile (Fig. 4).

CPBS Micelles: Formation in Aqueous Solution and Adsorption of Endotoxin. Already at concentrations as low as 50 μmol/L, CPBS formed micelles with an average diameter of 134 ± 6.8 nm (n = 8) (Fig. 5*A*). The diameter of these micelles did not change significantly with increasing concentrations of CPBS (125 ± 8.5 nm at 1.5 mM, n = 8).

In the absence of CPBS, solubilized FITC-labeled endotoxin and octadecyl rhodamine B did not colocalize. Therefore, no fluorescence resonance energy transfer from FITC-labeled endotoxin (and therefore no emission of octadecyl rhodamine B at 584 nm) occurred (Fig. 5*B*). First after the addition of micelle-forming CPBS, a colocalization of the FITC-labeled endotoxin and octadecyl rhodamine B took place, enabling fluorescence resonance energy transfer from endotoxin-bound fluorescein to octadecyl rhodamine B. This resulted in an increase in fluorescence of octadecyl rhodamine B (emission maximum at 584 nm), which is dose-dependent on the concentration of CPBS (Fig. 5*B*).

Inhibition of TNF-α Release in PBMC by Exposition to CPBS and Phosphatidylcholine. Basal production of TNF-α by PBMC after lipopolysaccharide stimulation was 1220 ± 72 pg/mL (unstimulated cells, 29.5 ± 2.7 pg/mL; both values corrected for DNA of vital cells). An increasing concentration of CPBS in the incubation medium resulted in a dose-dependent decrease of TNF-α production by PBMC through lipopolysaccharide stimulation (Fig. 6). The half-maximum inhibitory dose was calculated to be 169 μM. Phosphatidylcholine in the incubation medium did not affect the production of TNF-α significantly (data not shown).

DISCUSSION

In the applied model, Caco-2 cells formed a confluent, differentiated, and polarized cell layer with tight junctions, desmosomes, and microvilli (28), the suitability of which for permeability studies was demonstrated previously (29). As found in former experiments (16, 17), apical stimulation of Caco-2 cells by nonpathogenic bacteria leads to an enhanced production of cytokines in the basolateral compartment of the coculture model. In parallel with conditions in the intestine, bacteria and leukocytes are separated from leukocytes by a layer of IEC. The leukocytes in this model act as a bioindicator, proving that the permeating amounts of bacterial toxins are sufficient to evoke an activation of human immunocompetent cells. Due to the different phenotypes of mononuclear leukocytes found in the lamina propria and the peripheral blood, the profile of cytokines might rather correspond to that which occurs during sepsis than that occurring during inflammation of the intestine.

For the first time, we conclusively demonstrate in the current study a reduction in permeability of endotoxin through an IEC layer of human origin by CPBS, without the collateral influence of other organs, such as the liver. The primary bile salts of cholic acid and chenodeoxycholic acid are formed in the liver and are conjugated to either gly-

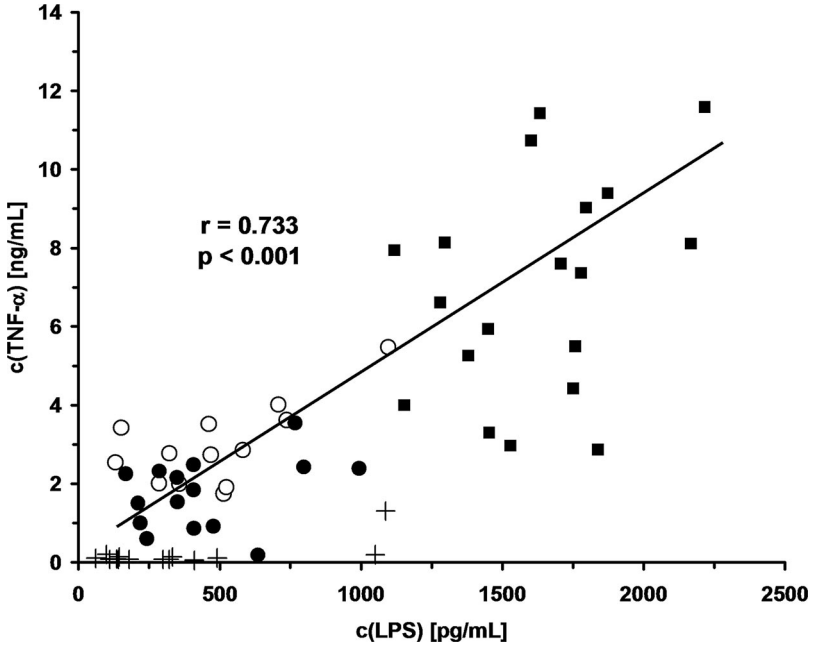

Figure 3. Correlation between the endotoxin (lipopolysaccharide, *LPS*) content and the concentration (*c*) of tumor necrosis factor (*TNF*)-α (12 hrs) in the basolateral medium of the coculture model. Correlation coefficient and significance of correlation were calculated with Spearman's test. *Squares*, control; *open circles*, 0.5 mmol/L conjugated primary bile salts; *filled circles*, 1.5 mmol/L conjugated primary bile salts; +, 1.5 mmol/L conjugated primary bile salts + 0.38 mmol/L phosphatidylcholine.

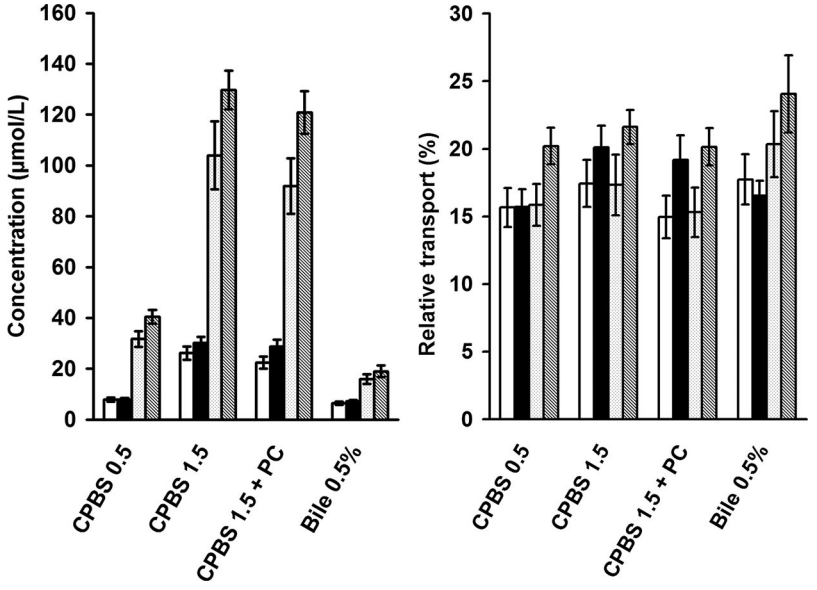

Figure 4. Absolute (*left*) and relative (*right*) concentrations of single conjugated primary bile salts (*CPBS*) in the basolateral medium of the coculture model after 24 hrs. *Open bars*, taurocholic acid; *filled bars*, taurochenodeoxycholic acid; *dotted bars*, glycocholic acid; *hatched bars*, glycochenodeoxycholic acid. *PC*, phosphatidylcholine. Relative concentrations are expressed as percentage of initial apical concentration of the corresponding CPBS.

cocholic acid, glycochenodeoxycholic acid, taurocholic acid, or taurochenodeoxycholic acid. In healthy subjects, these four CPBS comprise between 75 and 90 molar percentage of the bile salts in bile from the gallbladder, making them dominant components of the bile acid fraction in this organ (30, 31). Both the absolute concentrations and the concentration ratios of the single CPBS applied in the current study can be presumed to correspond to those occurring in the jejunum and ileum of humans (32–34).

Animals with bile duct ligation develop both an endotoxemia and high levels of circulating TNF-α and IL-6 (6, 13). Inactivation of endotoxin with bactericidal–permeability-increasing protein (35) or with polymyxin B (36) improved mortality and morbidity in bile duct–ligated rats. Hence, from animal models of obstructive jaundice, a causative role of endotoxin in postoperative complications can be concluded. This hypothesis is confirmed by experiments with bile-deprived rats with a chronic cannulation of their common bile duct, showing a considerable intestinal endotoxin translocation (37), which was reversible by oral supplementation with both conjugated and nonconjugated bile salts (38, 39).

Although some authors suggest that the presence of bile or (nonconjugated) bile salts, *per se*, avoids transmigration of endotoxins through the epithelium (4, 40, 41), others believe that the filter function of Kupffer cells is the crucial factor in the prevention of systemic endotoxemia (13). However, in the current study, a significant reduction of both the permeability of endotoxin through IEC and the production of proinflammatory cytokines by leukocytes after addition of bile and its components became evident. Therefore, an alleviation of endotoxemia and modulation of endotoxemia-associated inflammation by luminal presence of compounds from bile seems likely. This effect can be enhanced by prevention of intestinal overgrowth with Gram-negative bacteria (42). Hence, the supplementation of bile components seems useful also in other stages of clinical care that are prone to an elevated translocation of bacteria or endotoxins, namely, total parenteral nutrition (43), postoperative state of heart surgery (44), alcohol-induced liver disease (45), or endotoxemia itself (46).

From the current data, the mechanism underlying the permeability inhibition for endotoxin by bile salts is due to the incorporation of endotoxin into the CPBS micelles. The anchoring of the lipopolysaccharides on the micelle surface seems to parallel the noncovalent anchoring of the endotoxin molecule in the lipid bilayer membrane of the outer bacterial cell wall, which is sufficient to enable the biological functions of endotoxin for Gram-negative bacteria (47). This incorporation is likely to reduce the concentration of free endotoxins, thereby preventing their permeation through the barrier of IEC. According to the results of the current study, and comparable with

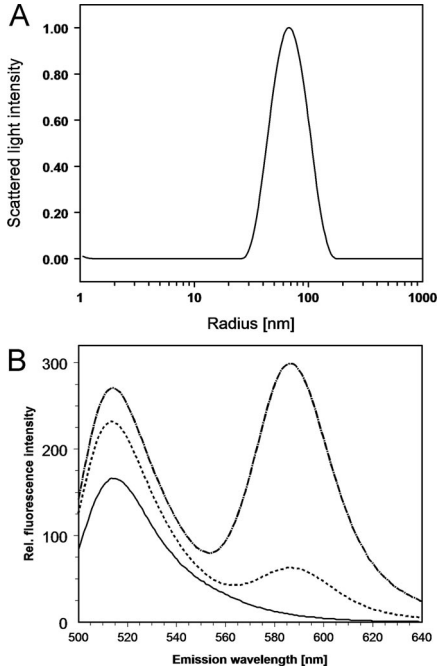

Figure 5. Micelle formation by conjugated primary bile salts (CPBS) and endotoxin binding by these micelles. *A*, particle size distribution of a 0.05 mM solution of a CPBS mixture. The average radius based on the thermodynamic motility of the micelles was 62.4 ± 4.3 nm (n = 8). *B*, fluorescence emission spectra of fluorescein-isothiocyanate (FITC)–labeled endotoxin from *Escherichia coli* (0.2 mg/mL with 3 μg FITC/mg endotoxin; *solid line*; emission maximum at 515 nm) in the presence of 0.5 mM CPBS (*dashed line*) and 1.5 mM CPBS (*dashed* and *dotted lines*). Only in the presence of CPBS micelles, the lipophilic micelle-bound rhodamine conjugate Rho-C18 received energy by fluorescence resonance energy transfer from FITC, which is bound to endotoxin and starts to emit light at a maximum wavelength of 584 nm. Excitation wavelength, 492 nm (excitation maximum of FITC endotoxin). *Rel.*, relative.

other endotoxin-binding molecules such as polymyxin B (36), CPBS micelles "capture" endotoxin molecules, thereby preventing their permeation.

The bile acid–sensitive farnesoid X receptor regulates a number of genes involved in enteroprotection, thereby preventing mucosal injury in ileum and bacterial translocation caused by bile duct ligation (48). Although the applied bile salts might have evoked activation of this receptor with the consequent regulation of farnesoid X receptor–dependent proteins, neither paracellular permeability of nonamphiphilic macromolecules (dextran Mr 4400) nor TEER were affected by CPBS, phosphatidylcholine, or bile. Therefore, an altered expression of proteins maintaining the intestinal barrier, such as tight junction proteins, might contribute only little to the observed effects of bile components, at least during the first few hours after stimulation in cocultures of human IEC and leukocytes. Nevertheless, some long-term farnesoid X receptor–mediated effects might be of importance for intestinal integrity (48).

Phosphatidylcholine synergized with CPBS in the reduction of inflammatory cytokine production. Phosphatidylcholine was shown to be effective in the treatment of both ulcerative colitis (49) and alcohol-induced liver disease (50), the development of which has been associated with an increased intestinal permeability of endotoxins (45). However, this effect is mediated by direct phosphatidylcholine-leukocyte interaction, as evident from the experiments with leukocytes challenged directly with endotoxin in the presence of phosphatidylcholine. How phosphatidylcholine mediates this effect needs attention in future studies.

The presence of proinflammatory cytokines such as TNF-α and IL-8 is detrimental to the integrity of the intestinal epithelium (18). The production of these cytokines in the present model might therefore be, at least in part, responsible for the significant decrease in TEER after bacterial challenge of the IEC, which indicates a loss in epithelial integrity. On the other hand, even in experimental conditions in which the shedding of these cytokines is abolished or at least strongly reduced (1.5 mM CPBS + phosphatidylcholine), both the TEER and the permeability of dextran Mr 4400 fail to improve compared with the control experiments. Therefore, the integrity of the IEC layer, which has been suggested to play an integral role in the development of endotoxemia in obstructive jaundice (40), seems not to be a major regulating factor for the transmigration of endotoxins in the current model.

In parallel with previous studies (51), the Caco-2 cell layer in the actual study transported conjugated bile salts from the apical into the basolateral compartment, leading to a direct contact of the CPBS with the leukocytes. A reduced production of inflammatory cytokines (IL-6, TNF-α) by monocytes after addition of non-CPBS to endotoxin-stimulated monocytes was reported (24). Evidently, CPBS can also effectively reduce activation of PBMC, which were directly stimulated with endotoxin. However, endotoxin-induced activation of leukocytes in the coculture model occurs during a time period when only very low concentrations of CPBS are present in the basolateral compartment (<5 μM CPBS at 6 hrs). Therefore, only a minor part of the reduction in TNF-α production after 12 hrs can be explained by a direct immunosuppressive effect of the CPBS. The very close correlation between basolateral concentrations of endotoxin and cytokines supports the assumption that the reduction of endotoxin permeability through the IEC layer is the most important regulating factor of inflammatory cytokine production beyond the intestinal barrier.

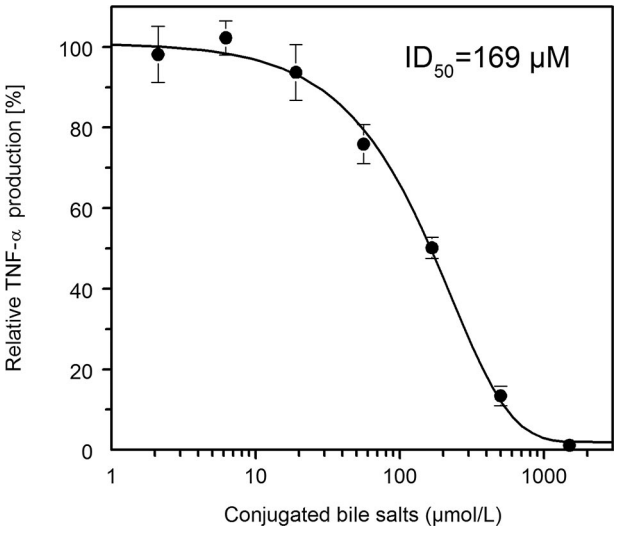

Figure 6. Production of tumor necrosis factor (*TNF*)-α by peripheral blood mononuclear cells (*PBMC*) that were directly stimulated with endotoxin (200 pg/mL) is plotted against the concentration of conjugated primary bile salts in the incubation medium. ID_{50}, half-maximum inhibitory dose.

CONCLUSION

Both CPBS and lecithin synergize in suppression of inflammation in the intestine, an effect that is at least in part due to capturing bacterial toxins and thereby

preventing their permeation through the intestinal barrier.

ACKNOWLEDGMENTS

We thank Ramona Jenske for the measurement of the concentrations of conjugated primary bile salts, Qing Heng for the measurement of endotoxin concentrations, and J. Ch. Bode for constructive remarks.

REFERENCES

1. Lai EC, Chu KM, Lo CY, et al: Surgery for malignant obstructive jaundice: Analysis of mortality. *Surgery* 1992; 112:891–896
2. Yeo CJ, Cameron JL, Sohn TA, et al: Six hundred fifty consecutive pancreaticoduodenectomies in the 1990s: Pathology, complications, and outcomes. *Ann Surg* 1997; 226:248–260
3. Wardle EN, Wright NA: Endotoxin and acute renal failure associated with obstructive jaundice. *BMJ* 1970; 4:472–474
4. Bailey ME: Endotoxin, bile salts and renal function in obstructive jaundice. *Br J Surg* 1976; 63:774–778
5. Ballinger AB, Woolley JA, Ahmed M, et al: Persistent systemic inflammatory response after stent insertion in patients with malignant bile duct obstruction. *Gut* 1998; 42:555–559
6. Bemelmans MH, Gouma DJ, Greve JW, et al: Cytokines tumor necrosis factor and interleukin-6 in experimental biliary obstruction in mice. *Hepatology* 1992; 15:1132–1136
7. Tripathy U, Dhiman RK, Attari A, et al: Preoperative bile salt administration versus bile refeeding in obstructive jaundice. *Natl Med J India* 1996; 9:66–69
8. Ogata Y, Nishi M, Nakayama H, et al: Role of bile in intestinal barrier function and its inhibitory effect on bacterial translocation in obstructive jaundice in rats. *J Surg Res* 2003; 115:18–23
9. Lorenzo-Zuniga V, Bartoli R, Planas R, et al: Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. *Hepatology* 2003; 37:551–557
10. Parks RW, Clements WD, Pope C, et al: Bacterial translocation and gut microflora in obstructive jaundice. *J Anat* 1996; 189:561–565
11. Slocum MM, Sittig KM, Specian RD, et al: Absence of intestinal bile promotes bacterial translocation. *Am Surg* 1992; 58:305–310
12. Clements WD, Erwin P, McCaigue MD, et al: Conclusive evidence of endotoxaemia in biliary obstruction. *Gut* 1998; 42:293–299
13. Diamond T, Dolan S, Thompson RL, et al: Development and reversal of endotoxemia and endotoxin-related death in obstructive jaundice. *Surgery* 1990; 108:370–375
14. Clements WD, Parks R, Erwin P, et al: Role of the gut in the pathophysiology of extrahepatic biliary obstruction. *Gut* 1996; 39:587–593
15. Kimmings AN, van Deventer SJ, Obertop H, et al: Endotoxin, cytokines, and endotoxin binding proteins in obstructive jaundice and after preoperative biliary drainage. *Gut* 2000; 46:725–731
16. Haller D, Bode C, Hammes WP, et al: Nonpathogenic bacteria elicit a differential cytokine response by intestinal epithelial cell/leucocyte co-cultures. *Gut* 2000; 47:79–87
17. Parlesak A, Haller D, Brinz S, et al: Modulation of cytokine release by differentiated Caco-2 cells in a compartmentalized coculture model with mononuclear leucocytes and nonpathogenic bacteria. *Scand J Immunol* 2004; 60:477–485
18. Zareie M, McKay DM, Kovarik GG, et al: Monocyte/macrophages evoke epithelial dysfunction: Indirect role of tumor necrosis factor-alpha. *Am J Physiol* 1998; 275:C932–C939
19. Parlesak A, Negrier I, Neveux N, et al: Arginine does not exacerbate markers of inflammation in cocultures of human enterocytes and leukocytes. *J Nutr* 2007; 137:106–111
20. Stiehl A, Raedsch R, Rudolph G: Ileal excretion of bile salts: Comparison with biliary bile composition and effect of ursodeoxycholic acid treatment. *Gastroenterology* 1988; 94:1201–1206
21. Hofmann AF: The continuing importance of bile salts in liver and intestinal disease. *Arch Intern Med* 1999; 159:2647–2658
22. Westphal O, Jann K: Endotoxins: Extraction with phenol-water and further applications of the procedure. *In:* Methods in Carbohydrate Chemistry. New York, Academic Press, 1965, pp 83–91
23. Tsai CM, Frasch CE: A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 1982; 1119:115–119
24. Greve JW, Gouma DJ, Buurman WA: Bile salts inhibit endotoxin-induced release of tumor necrosis factor by monocytes: An in vitro study. *Hepatology* 1989; 10:454–458
25. Rossi SS, Converse JL, Hofmann AF: High pressure liquid chromatographic analysis of conjugated bile salts in human bile: Simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile salts. *J Lipid Res* 1987; 28:589–595
26. Cantafora A, Di Biase A, Alvaro D, et al: Improved method for measuring the glycine and taurine conjugates of bile salts by high-performance liquid chromatography with tauro-7 alpha,12 alpha-dihydroxy-5 beta-cholanic acid as internal standard. *J Chromatogr* 1987; 386:367–370
27. Carraway KL III, Koland JG, Cerione RA: Location of the epidermal growth factor binding site on the EGF receptor: A resonance energy transfer study. *Biochemistry* 1990; 29:8741–8747
28. Pinto M, Robine-Leon S, Appay MD, et al: Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell* 1983; 47:323–330
29. Hidalgo IJ, Raub TJ, Borchardt RT: Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 1989; 96:736–749
30. Lin DS, Connor WE, Phillipson BE: Sterol composition of normal human bile: Effects of feeding shellfish (marine) sterols. *Gastroenterology* 1984; 86:611–617
31. Honda A, Yoshida T, Tanaka N, et al: Increased bile acid concentration in liver tissue with cholesterol gallstone disease. *J Gastroenterol* 1995; 30:61–66
32. Tangerman A, van Schaik A, van der Hoek EW: Analysis of conjugated and unconjugated bile salts in serum and jejunal fluid of normal subjects. *Clin Chim Acta* 1986; 159:123–132
33. Carey MC, Duane WC: Enterohepatic circulation. *In:* The Liver: Biology and Pathobiology. Third Edition. Arias IM, Boyer JL, Fausto N, et al (Eds). New York, Raven Press, 1994, pp 719–768
34. Black RB, Hole D, Rhodes J: Bile damage to the gastric mucosal barrier: The influence of pH and bile acid concentration. *Gastroenterology* 1971; 61:178–184
35. Kimmings AN, van Deventer SJ, Obertop H, et al: Treatment with recombinant bactericidal/permeability-increasing protein to prevent endotoxin-induced mortality in bile duct-ligated rats. *J Am Coll Surg* 1999; 189:374–379
36. Ingoldby CJ: The value of polymixin B in endotoxaemia due to experimental obstructive jaundice and mesenteric ischaemia. *Br J Surg* 1980; 67:565–567
37. Kocsar LT, Bertok L, Varteresz V: Effect of bile salts on the intestinal absorption of endotoxin in rats. *J Bacteriol* 1969; 100:220–223
38. Pain JA, Bailey ME: Prevention of endotoxaemia in obstructive jaundice: A comparative study of bile salts. *HPB Surg* 1988; 1:21–27
39. Ding JW, Andersson R, Soltesz V, et al: The role of bile and bile salts in bacterial translocation in obstructive jaundice in rats. *Eur Surg Res* 1993; 25:11–19
40. Parks RW, Cameron CHS, Gannon CD, et al: Changes in gastrointestinal morphology associated with obstructive jaundice. *J Pathol* 2000; 192:526–532
41. Greig JD, Krukowski ZH, Matheson NA: Surgical morbidity and mortality in one hundred and twenty-nine patients with obstructive jaundice. *Br J Surg* 1988; 75:216–219
42. Deitch EA, Sittig K, Li M, et al: Obstructive jaundice promotes bacterial translocation from the gut. *Am J Surg* 1990; 159:79–84
43. Deitch EA, Xu D, Naruhn MB, et al: Elemental diet and IV-TPN-induced bacterial translocation is associated with loss of intestinal mucosal barrier function against bacteria. *Ann Surg* 1995; 221:299–307
44. Oudemans-van Straaten HM, Jansen PG, Hoek FJ, et al: Intestinal permeability, circulating endotoxin, and postoperative systemic responses in cardiac surgery patients. *J Cardiothorac Vasc Anesth* 1996; 10:187–194
45. Parlesak A, Schafer C, Schutz T, et al: Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. *J Hepatol* 2000; 32:742–747
46. O'Dwyer ST, Michie HR, Ziegler TR, et al: A single dose of endotoxin increases intestinal permeability in healthy humans. *Arch Surg* 1988; 123:1459–1464

47. Rietschel ET, Brade H, Holst O, et al: Bacterial endotoxin: Chemical constitution, biological recognition, host response, and immunological detoxification. *Curr Top Microbiol Immunol* 1996; 216:39–81
48. Inagaki T, Moschetta A, Lee YK, et al: Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proc Natl Acad Sci U S A* 2006; 103:3920–3925
49. Stremmel W, Merle U, Zahn A, et al: Retarded release phosphatidylcholine benefits patients with chronic active ulcerative colitis. *Gut* 2005; 54:966–971
50. Cao Q, Mak KM, Lieber CS: Dilinoleoylphosphatidylcholine decreases LPS-induced TNF-alpha generation in Kupffer cells of ethanol-fed rats: Respective roles of MAPKs and NF-kappaB. *Biochem Biophys Res Commun* 2002; 294:849–853
51. Hidalgo IJ, Borchardt RT: Transport of bile salts in a human intestinal epithelial cell line, Caco-2. *Biochim Biophys Acta* 1990; 1035:97–103