Staphylococcal enterotoxin B potentiates LPS-induced hepatic dysfunction in chronically catheterized rats

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Beno, David W. A., Michael R. Uthing, Masakatsu Goto, Yong Chen, Vanida A. Jiayamapa-Serna, and Robert E. Kimura. Staphylococcal enterotoxin B potentiates LPS-induced hepatic dysfunction in chronically catheterized rats. Am J Physiol Gastrointest Liver Physiol 280: G866–G872, 2001.—Most models of liver dysfunction in sepsis use endotoxin (lipopolysaccharide; LPS) to induce a pathophysiological response. In our study published in this issue (Beno DWA, Uthing MR, Goto M, Chen Y, Jiayamapa-Serna VA, and Kimura RE. Am J Physiol Gastrointest Liver Physiol 280: G658–G665, 2001), the adverse effect of LPS on hepatic function in vivo was only significant at relatively high LPS doses despite high tumor necrosis factor-α concentrations. However, many patients with sepsis are exposed to multiple bacterial toxins that may augment the immune response, resulting in increased hepatic dysfunction. We have developed a model of polymicrobial sepsis by parentally administering a combination of staphylococcal enterotoxin B (SEB) and LPS. Using this model, we demonstrate that SEB (50 μg/kg) potentiates the effect of LPS-induced hepatic dysfunction as measured by decreased rates of biliary indocyanine green clearance and bile flow. These increases were most pronounced with doses of 10 and 100 μg/kg LPS, doses that by themselves do not induce hepatic dysfunction. This may explain the seemingly increased incidence and severity of liver dysfunction in sepsis, and it suggests that the exclusive use of LPS for replicating septic shock may not be relevant for studies of hepatic dysfunction.

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SEPSIS EVENTS THAT ARE COMPLICATED by hepatic dysfunction and cholestasis indicate a poor prognosis for survival (32). Most investigators who have studied this condition have elicited the pathophysiological response by administration of endotoxin (lipopolysaccharide; LPS) alone (22, 34). However, many septic patients are exposed to multiple bacterial toxins that may potentiate the immune response and result in even greater hepatic dysfunction than would be reflected in research using only LPS to elicit this response. This hypothesis was supported in our work with a chronically catheterized rat model reported in this issue (3), in which we demonstrated that hepatic dysfunction and cholestasis occurred only after infusion of high doses of LPS. Although lower doses of LPS (10 or 100 μg/kg) induced elevated and sustained serum tumor necrosis factor-α (TNF-α) concentrations, hepatic dysfunction did not occur. Our findings suggest that the dose of LPS required to induce hepatic dysfunction is pharmacological rather than pathophysiological and is in excess of concentrations found in septic patients (36). The fact that septic patients develop hepatic dysfunction supports our speculation that some mechanism in addition to LPS potentiates this response.

During septic events there is often exposure to Gram-positive bacteria and their toxins that may contribute to hepatic dysfunction (21). Furthermore, Gram-positive infections are responsible for 40% of sepsis cases, and 70% of sepsis-induced acute liver failure cases are caused by Gram-positive bacteria (6). Gram-positive bacteria may induce a septic response using a number of cellular components including peptidoglycans, teichoic acids, exotoxins, and superantigens. Superantigens bind the outer groove of the variable portion on the T cell receptor β-chain irrespective of clonal specificity and cross-link it to the major histocompatibility complex (MHC) class II receptor (8). This action activates a major fraction of T cells, resulting in the release of large quantities of interferon-γ (IFN-γ) (10, 23).

Staphylococcal enterotoxin B (SEB) is a soluble extracellular exotoxin and superantigen released from the Gram-positive staphylococcal family. It is a potent immunostimulatory molecule implicated in inflammatory diseases including shock syndrome and inflammatory bowel disease in addition to its association with food poisoning (14, 24, 29). SEB is used in this study as a model of superantigen-induced shock similar to the shock response associated with Staphylococcus-induced toxic shock syndrome and streptococcal pyrogenic exotoxin-induced shock (4, 27).

The shock response induced by either SEB or LPS is mediated by the release of cytokines (24). However, the induction and pattern of cytokine release by SEB-
induced T cell activation and LPS-induced activation of macrophages are quite distinct (27). Previous studies with mice have shown a synergistic cytokine response and increased lethality when LPS and SEB are given in combination (5, 26, 30). However, no previously reported research has examined the combined effect of these two bacterial toxins on hepatic function in rats. We hypothesized that administration of SEB in combination with LPS would result in greater hepatic dysfunction and cholestasis than that induced by LPS alone.

**MATERIALS AND METHODS**

**Reagents**

LPS (Escherichia coli 0127:B8; Sigma Chemical, St. Louis, MO) and SEB (Toxin Technology, Sarasota, FL) were prepared in sterile saline. Indocyanine green (ICG) and recombinant (r)IFN-γ were obtained from Akorn Industries (Deactur, IL) and R&D Systems (Minneapolis, MN), respectively.

**Animals**

A total of 56 adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 325–350 g served as subjects in this study. Rats were housed singly in standard cages and were fed chow and water ad libitum. The environment was temperature and humidity controlled, with lights on and off at 0630 and 1630, respectively. The Institutional Animal Care and Use Committee of Rush University approved all procedures.

**Operative Procedures**

Operative procedures were performed as previously described (2). Briefly, the animals were anesthetized with 60 mg/kg ketamine and 5 mg/kg xylazine intramuscularly. Catheters were placed in the aorta, inferior vena cava (IVC), and duodenum under sterile conditions. A bile catheter was made by inserting the 0.5-cm tip of a 24-gauge Insyte catheter (Becton Dickinson Vascular Access, Sandy, UT) into a 3-cm segment of Silastic tubing. A 0.5-cm segment of PE-60 tubing (Clay-Adams, Parsippany, NJ) was inserted into the other end of the Silastic tubing, and the entire catheter was placed over a 24-gauge Insyte needle. After placement of the IVC and aortic catheters, the tip of the 24-gauge Insyte needle was introduced in a retrograde fashion into the common bile duct ~2 cm distal to the liver. The bile catheter was then advanced over the Insyte needle 0.25 cm into the bile duct, the Insyte needle was removed, and the catheter was secured with cyanoacrylate glue. The distal PE-60 tubing was inserted into 3.5-in. intermittent infusion set tubing (no. 4871; Abbott Laboratories, North Chicago, IL) that had been passed from a cervical incision into the abdominal cavity as previously described for the aortic, IVC, and duodenal catheters (33). During this procedure, the bile duct was not ligated and bile flow was not obstructed. The abdominal cavity was closed with 4-0 silk suture. The infusion sets exiting the cervical incision were sutured securely to the back of the rat with 2-0 silk suture and were glued postoperatively with silicon to form a single unit. To maintain patency, all catheters were flushed daily.

**Measurement of Maximum Biliary ICG Excretion**

Bile was obtained through the bile catheter by inserting a 23-gauge infusion set needle (Abbott Laboratories), from which all but 0.5 in. of tubing had been removed, with ~24 in. of saline-filled PE-60 tubing attached. A sterile 1.5-ml sampling tube was attached to the tubing end, and bile was withdrawn by suspending the sampling tube below the cage using negative pressure. Bile was collected into tared sampling tubes at 5-min intervals over a 90-min period during which ICG was infused into the IVC at 50 μg/min. ICG (1 ml/h) was delivered by a Razel Scientific (Stamford, CT) infusion pump with a 3-ml Becton Dickinson (Franklin Lakes, NJ) syringe.

Bile flow rate was calculated using the net sampling tube weight assuming a bile density of 1 g/ml. A total of 10 μl of bile from each sample tube was aliquoted to determine ICG concentration. The remainder of the bile was infused into the duodenal catheter to maintain enterohepatic circulation of bile acids. Biliary ICG concentrations were measured spectrophotometrically at a wavelength of 805 nm. Biliary ICG excretion was calculated for each 5-min interval by multiplying the bile flow rate by the biliary ICG concentration.

**Experimental Design**

Experiments were performed at least 4 days postoperatively because our previous work (2) showed that the effects of surgical stress on the LPS-induced cytokine response are no longer present at this time. At this time, the animals had achieved at least 95% of their preoperative weight.

**Effect of LPS and SEB on hepatic function and cytokine induction.** Rats were assigned to receive one of four doses of LPS with or without one dose of SEB (50 μg/kg) infused into the IVC over 30 s: 0 (control, n = 7), 10 (n = 4), 100 (n = 9), or 1,000 (n = 8) μg/kg without SEB or 0 (control, n = 4), 10 (n = 6), 100 (n = 7) or 1,000 (n = 7) μg/kg with SEB. The dose of SEB used in these studies was selected from preliminary studies indicating that 50 μg/kg was the lowest SEB dose that provided maximal synergistic cytokine induction with LPS.

Rates of biliary ICG excretion and bile flow were measured at baseline (time 0) and then 6 and 24 h after LPS administration. Aortic blood samples (0.2 ml) were collected at 0, 1.5, and 4 h for determination of corticosterone, TNF-α, and IFN-γ concentrations, respectively. Timing of LPS-induced IFN-γ peak was determined from preliminary studies in which blood was obtained every 0.5 h for 6 h after toxin administration. Experimental samples were aliquoted and stored at ~80°C until analyzed. TNF-α and IFN-γ were measured by ELISA (Biosource International, Camarillo, CA). Corticosterone was measured by RIA (ICN Biomedicals, Costa Mesa, CA).

**Effect of rIFN-γ on hepatic function.** In a separate group of animals, we tested the speculation that IFN-γ is the primary mediator of the LPS-induced inhibition of bile flow rate. For these experiments, we measured rates of biliary ICG excretion and bile flow rate after bolus infusion of 20 μg/kg rIFN-γ (n = 4). This rIFN-γ dose resulted in a mean peak serum concentration of 29.4 ± 6.3 ng/ml and was selected to surpass the peak serum IFN-γ concentrations observed in this study after infusion of 1,000 μg/kg LPS and 50 μg/kg SEB (20.6 ± 3.0 ng/ml). Rates of biliary ICG excretion and bile flow were measured immediately before rIFN-γ administration (baseline) and again at 2 and 20 h after rIFN-γ administration. Because LPS-induced peak IFN-γ was observed at 4 h after LPS infusion, we measured hepatic function at 2 and 20 h after rIFN-γ administration. These measures simulated the timing of LPS-induced IFN-γ effects on hepatic functions at 6 and 24 h. Aortic blood samples were obtained immediately.
before and 1 min after rIFN-γ administration for determination of corticosterone and IFN-γ concentrations, respectively.

Statistical Analysis

All results are expressed as means ± SE. For kinetic studies, differences were compared by two-way repeated-measures ANOVA with Bonferroni post hoc correction.

RESULTS

Effect of LPS and SEB on TNF-α and IFN-γ Cytokine Production

Mean peak TNF-α and IFN-γ serum concentrations occurred at 90 min and 4 h after toxin administration, respectively. The effect of LPS dose (0, 10, 100, or 1,000 μg/kg) with and without SEB on peak serum concentrations of TNF-α and IFN-γ is shown in Fig. 1. For each LPS dose, coadministration of SEB resulted in significantly greater mean serum concentrations of TNF-α and IFN-γ, with the exception of the TNF-α response to 1,000 μg/kg LPS, which did not differ significantly between SEB and non-SEB groups. As noted in Fig. 1, SEB in the absence of LPS did not induce a change from baseline for TNF-α or IFN-γ. Thus these findings reveal that SEB potentiated the effect of LPS on TNF-α and IFN-γ production. The mean baseline corticosterone concentration for all rats reported throughout this study was 34.2 ± 19.7 ng/ml (range 10–89 ng/ml).

Effect of LPS and SEB on Bile Flow Rate

For rats who received only LPS, mean bile flow rates at 6 and 24 h were unchanged from baseline values after infusion of 100 μg/kg LPS (Fig. 2B). However, infusion of 1,000 μg/kg LPS resulted in a reduction in mean bile flow rate of 42% (P < 0.001) at 6 h and 25% (not significant) at 24 h compared with baseline values (Fig. 2C). No differences in mean bile flow rate at 6 and 24 h were noted in the control group compared with baseline values or in rats infused with either 10 μg/kg of LPS or 50 μg/kg of SEB alone (Fig. 2A). When 50 μg/kg SEB was coinfused with 10 (Fig. 2A), 100 (Fig. 2B), or 1,000 (Fig. 2C) μg/kg LPS, mean bile flow rate was decreased significantly (P < 0.01) to 36–38% of baseline values at 6 h and to 24–29% of baseline values at 24 h. The only significant reduction from baseline at 24 h was for the combination of 1,000 μg/kg LPS with 50 μg/kg SEB, which resulted in mean bile flow of 29% below baseline (P < 0.05; Fig. 2C).

Effect of LPS and SEB on Biliary ICG Clearance Rate

For rats receiving LPS only, infusion of 1,000 μg/kg LPS resulted in a reduction in mean biliary ICG clearance of 50% (P < 0.01) at 6 h and 29% (P < 0.01) at 24 h compared with baseline values (Fig. 3C). Mean biliary
ICG clearance rates at 6 and 24 h were unchanged from baseline values after infusion of 100 μg/kg (Fig. 3B). No differences in biliary ICG clearance rates at 6 and 24 h were noted in the control group compared with baseline values or in rats infused with either 10 μg/kg of LPS or 50 μg/kg of SEB alone (Fig. 3A).

When SEB was administered with LPS, mean biliary ICG clearance rates were significantly (P < 0.01) reduced to 39–57% of baseline values at 6 h and to 32–41% of baseline values at 24 h (P < 0.01) for LPS doses of 10 (Fig. 2A), 100 (Fig. 2B), or 1,000 (Fig. 2C) μg/kg LPS.

**DISCUSSION**

Our data revealed a dose- and time-dependent LPS-induced decrease in the rates of biliary ICG excretion and bile flow. However, the dose (1,000 μg/kg) of LPS necessary to induce hepatic dysfunction was pharmacological rather than pathophysiological and in great excess of the LPS concentration observed in portal blood during sepsis (36). We therefore modified our model to include a Gram-positive toxin in addition to LPS. Our data demonstrate a dose- and time-dependent decrease in the rates of biliary ICG excretion and bile flow after coadministration of SEB and LPS that lowers the concentration of LPS necessary to induce hepatic dysfunction by 100-fold. The findings of this study suggest that models of sepsis using only LPS may underestimate hepatic dysfunction and cholestasis because Gram-positive as well as Gram-negative bacteria can be recovered from septic patients (21). The increasing clinical relevance of coinfections, including secondary nosocomial infections, is presumed to be
much greater because coinfections are very difficult to detect (29). Furthermore, Gram-negative and -positive bacteria and their respective toxic cell wall components are ubiquitous contaminants in septic patients regardless of the primary infection source (4, 16).

In view of the increasing clinical awareness of contaminating toxins during sepsis, the aim of this study was to examine hepatic dysfunction and cholestasis induced by multiflora bacterial toxins. Although LPS has long been known to be a mediator of Gram-negative sepsis, several cell wall components from Gram-positive bacteria that may induce cytokine release include peptidoglycan, lipoteichoic acid, exotoxins, and superantigens (13, 27). We chose SEB for coinfection with LPS because of the unique SEB-induced immunologic response.

SEB and LPS target distinct signaling mechanisms on different cell populations to initiate cytokine release. LPS binds its receptor (CD14) and, via the TOLL receptor, triggers macrophages to release a variety of cytokines including TNF-α (7, 13, 29). Peptidoglycan and lipoteichoic acid from Gram-positive bacteria also use this mechanism to induce the release of cytokines, leading to potential redundancy in the response to multiflora sepsis (5). In contrast, SEB, a superantigen, nonspecifically cross-links the T cell receptor from up to 20% of all T cells to antigen-presenting cells expressing MHC class II molecules (8). The linking of the T cell receptor by SEB to MHC class II molecules occurs along conserved regions outside the antigenic groove and therefore requires neither internalization nor processing of the SEB (9). This interaction results in activation, proliferation, and the release of cytokines (11, 12, 25). Because these two toxins induce cytokine release using independent pathways and cellular targets, we hypothesized that the combination of LPS and SEB may produce a synergistic release of cytokines.

The SEB- and LPS-induced synergistic release of IFN-γ has been demonstrated in murine studies (5, 26, 30). These studies have suggested that the primary mediator of the SEB- and LPS-induced response is IFN-γ because of its significant synergistic increases. Blank et al. (5) showed that neutralizing anti-IFN-γ antibody protected mice from LPS- and SEB-induced death. In our study, LPS and SEB synergistically induced serum concentrations of IFN-γ and TNF-α in a dose-dependent manner in rats, although the effect of these cytokines on hepatic function is unclear.

Because of the synergistic IFN-γ response, we wanted to determine whether IFN-γ itself mediated hepatic dysfunction. We therefore infused rIFN-γ without toxins and measured mean rates of biliary ICG excretion and bile flow. Similar to our previous studies using rTNF-α infusion, we found that rIFN-γ alone did not alter biliary ICG excretion and bile flow rate (3). Although we observed no measurable changes from mean baseline rates of biliary ICG clearance or bile flow, we recognize that a bolus infusion of rIFN-γ may not accurately reflect the toxin-induced IFN-γ response, which is sustained for a longer duration than that which resulted from our bolus rIFN-γ infusion.

More likely, however, is that a combination of cytokines or other factors is required to induce hepatic dysfunction (31). Ours is not the first model of polymicrobial sepsis-induced hepatic dysfunction. Multiple investigators have used cecal ligation and puncture as a model of polymicrobial sepsis-induced peritonitis (1, 18, 35). Unfortunately, that model is limited by the uncontrolled variability of the peritonitis induced by cecal contents, surgically induced alterations in the inflammatory response, and the fact that the pathophysiological response is so severe that the animals universally die within 18–24 h. Furthermore, these models have not included direct measurement of bile flow rate and biliary ICG excretion. The difficulties of the cecal ligation and puncture model have led other investigators to develop bacterial toxin-based models.

The majority of animal investigations of SEB-induced shock use murine models. However, these studies have provided limited information because the affinity of SEB for rodent MHC class II molecules is considerably lower than that in humans (10, 30). Lower immunologic responsiveness has caused the development of many models to study the SEB response in mice. They include d-galactosamine treatment (26), pretreatment with low doses of LPS (5), pretreatment with Gram-positive bacteria like Propionibacterium acnes (17, 37), or injection of LPS 4 h before SEB injection (28).

Huang and Koller (15) are among the few to study the effects of SEB infusion on rats. They found SEB-induced increases in serum concentrations of interleukin (IL)-2, IL-6, and IFN-γ in the picogram per milliliter range but no increases in serum TNF-α concentrations using bioassays for cytokine determination (15). Despite intraperitoneal injection of 50 or 500 μg SEB in each Long-Evans rat, there was not a cytokine dose-response curve. Unfortunately, direct comparisons between their study and ours are difficult because they did not report rat weight.

In our rat model we did not observe any increases in mean peak serum concentrations of TNF-α or IFN-γ using ELISA-based assay systems sensitive to picogram per milliliter concentrations or decreases in rates of ICG excretion or bile flow after infusion of 50 μg/kg SEB. These data are in contrast to the studies described above, which use greater SEB doses, up to 500 μg/rat, and require poisoning or priming of the immune response. We showed previously (2) that our model system is sensitive to very low concentrations of toxins because we can detect significant increases in mean peak TNF-α serum concentrations after LPS administration at doses as low as 10 ng/kg.

Several investigators have investigated LPS-induced changes in ICG excretion. Although not measured in our research, the patterning of the transporter regulation after LPS infusion served as the basis for our choosing to examine bile flow rate and ICG excretion at 6 and 24 h after LPS infusion. Others’ work has suggested that bile transporters at both the basolateral and canalicular membranes may regulate bile flow rate.
and biliary transport (19, 32, 34). These processes may be mediated by changes in mRNA or protein as well as by the intracellular compartmentalization of the transporters. The organic anion transporting protein 1 ( oatp1) is thought to be the primary transporter of ICG across the basolateral membrane, whereas cytoplasmic multispecific organic anion transporter (cMOAT), also known as mrp2, is thought to be the primary ICG canalicular transporter (20, 32, 34). Whether these two transporters are the exclusive transporters for ICG at their respective membrane sites is unknown.

Our study did not attempt to identify dysfunction of individual transporter proteins. Instead, we have focused this study on end point measures of hepatic dysfunction and cholestasis by directly examining bile to measure bile flow rate for determining cholestasis and biliary ICG excretion for determining dysfunction of anionic (bile acid independent) transport. Therefore, although the exact mechanism of transporter regulation and therefore function is unknown, it is likely related to the inflammatory response and may include cytokinemia, cellular aggregation of inflammatory cells, thrombus formation, and oxidative state.

This study is this first to demonstrate that SEB synergistically enhances LPS-induced hepatic dysfunction and cholestasis (reduced rates of biliary ICG excretion and bile flow) and cytokine release. These findings are the first to isolate the effects of SEB- and LPS-induced hepatic dysfunction in a nonstressed animal model. This study suggests that because superantigens are ubiquitous contaminants, low superantigen concentrations are able to synergize with toxins associated with sepsis to enhance the shock response, thereby leading to increased hepatic dysfunction that may be underestimated with models of sepsis using LPS as the sole toxin insult.

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