Infection by Gram-Negative Organisms via the Biliary Route Results in Greater Mortality than Portal Venous Infection

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Cholangitis requires bile duct obstruction and infection. Patients with cholangitis are often more affected than those with infections that reach the liver through the portal vein. We will attempt to study the influences of (i) route of entry and (ii) presence of bile duct obstruction on hepatic infection. C57BL/6 mice received injections of Escherichia coli or lipopolysaccharide into the obstructed bile duct or portal vein and were monitored for survival. Livers were assayed for bacteria, and cytokine mRNA was measured. In order to examine the effect of biliary obstruction on hepatic infection, animals were subjected to bile duct ligation 1 day prior to portal vein injection and were monitored for survival. The 50% lethal dose (LD50) for E. coli injected into the bile duct was 50 CFU/animal; the LD50 for E. coli injected into the portal vein was 5 × 103 CFU/animal. Initial hepatic delivery of bacteria was equivalent 1 h after injection into the bile duct or portal vein. However, by 24 h, a significantly greater amount of bacteria was recovered from the livers of the bile duct-injected group. Interleukin 10 (IL-10) and IL-1RA mRNA was expressed at greater levels in the bile duct-injected group. Prior bile duct ligation followed by portal vein injection resulted in a higher incidence of death than when sham operation was performed prior to portal vein injection. Our data suggest that the increased mortality from cholangitis, compared with that from other hepatic infections, is related to the different route of delivery of pathogen and the maladaptive response (possibly involving IL-10 and IL-1RA) to biliary obstruction itself.

Despite antibiotics and biliary drainage, either surgically or endoscopically, patients with ascending cholangitis have greater mortality and morbidity than patients with other appropriately treated intra-abdominal infections. In contrast to portal vein entry of bacteria into the liver, for example, after appendicitis or diverticulitis, entry via the biliary ducts has higher morbidity and mortality (4, 7). Indeed, small numbers of bacteria from the gastrointestinal tract may continually shower the liver via the portal vein without ill effect (11). This study reports a murine model that compares infection via the biliary and portal venous routes. This model reproduces the extraordinary mortality seen in patients after the former. Our model differs from others (9, 19). It focuses on early events within days of biliary infection, as opposed to late events after biliary obstruction has resulted in chronic liver injury.

Our model suggests that the host response to infection via the bile duct is fundamentally different from the response to infection via the portal vein. We will show that one manifestation of this difference is the pattern of cytokines elicited by the infection. These cytokines affect the outcome in models of infection and injury and are supported by the literature. For example, levels of interleukin 6 (IL-6) in serum correlate with survival in critically ill patients; manipulation of IL-10 alters outcome in many models of injury and infection (1, 3, 5, 18, 24–26, 28). Furthermore, our data suggest that the hepatic response to biliary obstruction determines the host defense against infection. Such obstruction is a prerequisite for clinical cholangitis. It also suggests that the obstruction does more than allow the retrograde movement of bacteria into the liver but elicits a hepatic cytokine response that results in a maladaptive response to bacterial infection. (This research was presented at the Surgical Infection Society International Meeting in Spain, April 2002.)

MATERIALS AND METHODS

Animals. Six- to 8-week-old C57BL/6 male mice were obtained from Jackson Laboratory (Bar Harbor, Maine) and were housed according to institutional guidelines.

Cholangitis model. A midline laparotomy was performed under sterile conditions. Bile duct ligation (BDL) was performed by dissecting the common bile duct below the entrance of the cystic duct. Care was taken to stay above the level of the entrance of the pancreatic duct to avoid ligation of the pancreatic duct and pancreatitis. Common bile duct was ligated at this level by using 6-0 silk suture. The gallbladder was grasped, and P10 tubing was inserted into the gallbladder and was secured by using 6-0 silk tie. Slow injection of 0.25 ml of Escherichia coli, lipopolysaccharide (LPS), or saline was then performed. The gallbladder was ligated upon withdrawal of the P10 tubing, to prevent spilling. Animals were closed in two layers by using 6-0 silk suture.

Portal vein injection. A midline laparotomy was performed by using sterile techniques. The portal vein was isolated by reflecting intestinal contents to the left of the animal. Freehand injection of E. coli, LPS, or saline was performed by using a 0.25-ml volume and 30-gauge needle. Hemostasis was achieved by direct compression.

Bacteria and LPS. E. coli (ATCC 25922, known to be pathogenic in rodents) was grown in broth overnight. Turbidity was measured, and dilutions were prepared from previously delineated growth curves. To ensure a correct count of bacterial CFU and no contamination, E. coli was plated, prior to injection, at a given dose. LPS (O111:B4; Sigma Chemical) was diluted to an appropriate concentration, and injections were administered in a 0.25-ml volume.

Survival studies. Animals were treated with E. coli or LPS that was injected into the bile duct or portal vein as outlined above. BDL was performed on day −1, and portal vein injection was performed on day 0 upon some animals. Animals were monitored for survival.
Bacterial growth in infected livers. In order to investigate initial delivery of *E. coli* via the bile duct and portal vein, animals were injected with a known number of CFU of *E. coli* into the bile duct or portal vein. The left lobe of the liver was harvested 1 h after injection. Liver was homogenized and was plated on soy agar (Remel, Lenexa, Kans.), and the number of CFU recovered was counted after overnight incubation. The total number of CFU recovered from each liver was calculated. Similarly, the numbers of CFU that had been injected into the bile duct or portal vein and were recovered from livers of animals were determined at 24 h postinjection.

Examination of cytokine profiles by RPA and ELISA. Liver tissue was harvested 24 h after infection with $5 \times 10^4$ CFU of *E. coli* in the bile duct or portal vein. RNA was prepared by using the standard guanidinium isothiocyanate extraction kit technique (Rneasy-Qiagen). RNase protection assay (RPA) with mck2b or mck3 probe (Pharmingen) was performed according to the manufacturer’s instructions. Briefly, probes were manufactured and hybridized to cellular RNA. Annealed probe/RNA complexes were protected to subsequent digestion. Samples were then run on a polyacrylamide gel, and protected bands were identified. The gels were analyzed by using a Molecular Dynamics Phosphor-Imager and ImageQuant software. Densitometry was calculated as a ratio of the cytokine mRNA to a housekeeping mRNA (L32). IL-10 protein was measured in the serum of animals 24 h after injection of *E. coli* into the bile duct or portal vein with the enzyme-linked immunosorbent assay (ELISA) (endogen).

**RESULTS**

*E. coli* or LPS via the bile duct is more fatal than via the portal vein. We chose to examine the role of LPS and *E. coli* in our model of cholangitis and portal venous infection. *E. coli* is a proliferating bacterium that is a common pathogen in cholangitis (4). LPS is a nonproliferating product of infection in cholangitis. The survival rate of bile duct-injected animals was significantly less than that for portal vein-injected animals ($P = 0.004$ and 0.0009 by log rank for panels A and B, respectively).
by gram-negative organisms. Experiments with LPS were performed to examine the role of hepatic response to endotoxin compared with the hepatic response to live bacteria. E. coli or LPS injected into the obstructed bile duct resulted in significantly greater mortality than E. coli or LPS injected into the portal vein (Fig. 1 and 2A and B). Portal vein-injected animals required $5 \times 10^7$ to cause 50% mortality ($n = 20$). Lower doses of bacteria injected into the portal vein resulted in no deaths. Bile duct-injected animals had 100% mortality with doses as low as 50 CFU/animal. Injection of 2.0 mg of LPS/100 g of body weight per mouse resulted in 100% mortality in portal vein-injected animals within 24 h ($n = 5$). Saline injected into the portal vein or bile duct did not result in any fatalities ($n = 5$ in each group).

**Kinetics of bacterial growth in the liver after bile duct and portal vein infection.** Colony counts of E. coli recovered in the liver after injection of $5 \times 10^6$ or $5 \times 10^5$ CFU of bacteria are shown in Fig. 3. Initial counts recovered were similar whether bacteria was injected into the bile duct or the portal vein (Fig. 3A and B). Despite similar bacterial counts at 1 h in the bile duct and portal venous groups, there were more bacteria by greater than 3 orders of magnitude in the former group at 24 h (Fig. 3C).

The presence of BDL at the time of portal vein injection altered the response to E. coli. Animals receiving BDL 1 day prior to portal vein injection of $10^6$ CFU of E. coli suffered in 100% mortality. Animals that received the sham operation 1 day prior to portal vein injection of $10^6$ CFU of E. coli experienced 0% mortality (Fig. 4). This result suggests that the
presence of BDL affects the subsequent hepatic response to pathogen delivered via the portal vein.

**Cytokine response to bile duct- versus portal vein-injected *E. coli*** RPA of liver tissue after bile duct or portal vein injection is shown in Fig. 5. Increased IL-10, IL-1RA, and IL-6 were noted in bile duct-injected animals compared to levels found in portal vein-injected animals. Densitometric analysis confirmed these findings: IL-10 in portal vein-injected animals (IL-10-PV) = 0.0323 ± 0.0025; IL-10 in bile duct-injected animals (IL-10-BD) = 0.0577 ± 0.0130; IL-1α-PV = 0.1074 ± 0.0079; IL-1α-BD = 0.1032 ± 0.0363; IL-1β-PV = 0.1011 ± 0.0255; IL-1β-BD = 0.2916 ± 0.1443; IL-1RA-PV = 0.3151 ± 0.0312; and IL-1RA-BD = 3.0484 ± 1.1747. IL-6 was detected only in the bile duct-injected animals. IL-18 was detected only in the portal vein-injected animals. IL-12 mRNA was not detected in bile duct- or portal vein-injected animals. Similarly, tumor necrosis factor alpha, LT-β, and other proinflammatory cytokine mRNA were not detected by using other probe sets (mck3; Pharmingen) (data not shown.) Sham-operated liver tissue did not show significant expression of any cytokines examined (data not shown).

ELISA measurement of IL-10 24 h after injection of *E. coli* confirmed increased protein production of IL-10 in bile duct-injected animals compared to that found in portal vein-injected animals (n = 3 per group): bile duct group level = 1,337.00 ± 2,118.70 pg/ml; portal vein group level = 4,697.83 ± 1,313.80 pg/ml (P < 0.014 by t test).

**Liver histology in animals that received bile duct or portal vein *E. coli*** Hematoxylin and eosin staining of livers from animals receiving *E. coli* via the bile duct or portal vein is shown in Fig. 6. This demonstrates cholangitis with portal neutrophil infiltration 24 h after bile duct injection (Fig. 6A) compared to that found after portal vein injection (Fig. 6B). Forty-eight hours after bile duct injection, abscesses were seen in the liver (Fig. 6C). Normal liver tissue is shown in Fig. 6D.

**DISCUSSION** By using a novel model of cholangitis and portal venous infection, we have shown that there is significantly greater mortality with bacteria delivered into the bile duct. This finding is not related to unequal delivery of pathogen: bacterial counts recovered 1 h after injection into the bile duct or portal vein were similar. Despite these similar initial counts, there were several logs more bacteria after bile duct infection. The greater mortality after infection via the biliary route was not due to bacterial proliferation within the closed space of the bile duct: (i) experiments in which LPS, a nonproliferating product, was used caused increased mortality when it was delivered through the biliary route compared to that found when it was delivered through the portal vein; and (ii) biliary obstruction 1 day prior to portal vein injection resulted in greater mortality.

Further investigation is necessary to explain the increased mortality after biliary infection. One explanation is that the associated acute biliary obstruction itself elicits a cytokine response that prevents an effective host defense. It has been reported (M. L. Kielar, D. R. Jeyarajah, B. J. Wright, and C. Y. Yu, abstr. J. Am. Soc. Nephrol. 11:A3121, 2000) that acute biliary obstruction, in the absence of *E. coli* injections, increases the mRNA abundance for IL-10 and IL-1RA.

These cytokines are known to affect the outcome in models of injury and infection. The anti-inflammatory effects of IL-10 have been found to be detrimental after injection of live bacteria (6, 8, 17, 26, 27). Manipulation of IL-10 in models of injury and infection has resulted in alterations of the outcome (Y. Kohda, H. Chiao, P. McLeory, L. Craig, and R. A. Star, abstr., J. Am. Soc. Nephrol. 9:580A, 1999; 20, 24, 25). IL-6 levels are predictive of outcome in severely injured patients (18). Similarly, IL-1RA has been found to decrease the inflammatory response to viral and autoimmune injury (13, 21).

Consistent with this explanation is the marked difference between the cytokine responses to biliary infection and to portal venous infection shown in Fig. 5. Animals infected by the biliary route produced more IL-10 and IL-1RA mRNA than did portal vein-injected animals. The production of a more immunomodulatory cytokine profile in response to biliary infection may be maladaptive to clearance of bacteria and hence survival.

Our model differs from other models of bile duct obstruction in the literature (2, 9, 10, 19, 22, 23). These models have focused on late time points (1 to 3 weeks) after bile duct obstruction. It is likely that chronic liver injury has developed by this time. Our model focuses on the hepatic response to infection in the absence of established liver disease, a scenario more common in clinical cholangitis.

Our future goals are to further characterize the hepatic response to cholangitis and portal venous infection. Initial response to infection involves the innate immune system (15). Hepatic response to injury and cold ischemia involves recruit-
FIG. 6. Histology of C57BL/6 mice that underwent injections of *E. coli* into the bile duct or portal vein. Hematoxylin and eosin staining of livers from animals injected with *E. coli* into the bile duct (A) or portal vein (B) 24 h prior. Figure 6C shows livers from bile duct-injected animals 48 h after injection. Figure 6D shows a normal liver for comparison.
ment of nonclassical T and NK cells (12, 14, 16, 29). We will attempt to characterize the cellular response to hepatic infection via the bile duct and portal vein. Furthermore, by using knockout technology, we plan to study the impact of various cytokines, specifically IL-10, on hepatic infection.

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REFERENCES


