Augmentation of Aflatoxin B_1 Hepatotoxicity by Endotoxin: Involvement of Endothelium and the Coagulation System

James P. Luyendyk, Bryan L. Copple, Charles C. Barton,[1] Patricia E. Ganey, and Robert A. Roth[2]

*Department of Pharmacology and Toxicology, National Food Safety and Toxicology Center, Institute for Environmental Toxicology,
Michigan State University, East Lansing, Michigan 48824-1224*

Aflatoxin B_1 (AFB_1) is a fungal toxin that causes both acute hepatotoxicity and liver carcinoma in exposed humans and animals. Previous studies have shown that exposure of rats to nontoxic doses of bacterial lipopolysaccharide (LPS) augments AFB_1 acute hepatotoxicity, resulting in enhanced injury to hepatic parenchymal cells and bile ducts. At larger doses, LPS causes damage to sinusoidal endothelial cells (SECs) and activation of the coagulation system. Accordingly, we tested the hypothesis that treatment of rats with AFB_1 and LPS damages SECs and activates the coagulation system, which is critical for potentiation of AFB_1 hepatotoxicity by LPS. Male, Sprague-Dawley rats were given 1 mg/kg AFB_1 (ip), then 4 hours later 7.4×10^6 EU/kg LPS was administered (iv). A time-dependent injury to SECs and parenchymal cells was observed in AFB_1/LPS-cotreated animals that became significant by 12 h, as estimated by increases in plasma hyaluronic acid (HA) and alanine aminotransferase (ALT) activities, respectively. Immunohistochemical analysis revealed that endothelial cell immunostaining was decreased in both centrilobular and periportal regions after AFB_1/LPS treatment. Immunohistochemical evidence of fibrin deposition was found in both centrilobular and periportal regions by 12 h, but these deposits persisted only in periportal regions by 24 h. Administration of the anticoagulant heparin to AFB_1/LPS-cotreated animals markedly attenuated increases in markers of hepatic parenchymal cell injury but provided only minimal amelioration of bile duct injury. These results suggest that AFB_1/LPS coexposure results in SEC injury and activation of the coagulation system, and that the coagulation system is required for the development of hepatic parenchymal cell injury but not bile duct injury in this model.

Key Words: aflatoxin B_1; lipopolysaccharide; coagulation system; liver; sinusoidal endothelial cell.

Aflatoxin B_1 (AFB_1) is a potent hepatotoxicant and carcinogen (Roebuck and Maxuitenko, 1994). People are commonly exposed to AFB_1 through consumption of grain products contaminated with *Aspergillus flavus* or *A. parisiticus*. Acute hepatotoxicity from AFB_1 is characterized by periportal hepatocellular necrosis, bile duct injury, and hemorrhage. In regions of the world where chronic aflatoxin exposure occurs, the incidence of hepatocellular carcinoma is increased, especially in individuals with concurrent hepatitis (Groopman *et al*., 1993). Previous studies have shown that the hepatotoxicity of AFB_1 in rats was enhanced by nontoxic doses of bacterial lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria and potent inflammagen (Barton *et al*., 2000b). In these studies, LPS exposure elicited a 10–20-fold leftward shift in the dose-response curve for AFB_1 hepatotoxicity (Luyendyk *et al*., 2002).

LPS acts at Toll-like receptors on inflammatory cells to stimulate production of a host of inflammatory mediators. In both humans and animals, large concentrations of LPS precipitate an exaggerated inflammatory response that results in injury to several organs, including the liver (Hewett and Roth, 1993). Inflammatory mediators, such as tumor necrosis factor α (TNF-α) (Hewett *et al*., 1993), neutrophils (PMNs, Hewett *et al*., 1992; Jaeschke *et al*., 1991), and thrombin (Hewett and Roth, 1995; Pearson *et al*., 1996) are involved in the liver injury produced by large doses of LPS. Human exposure to smaller amounts of LPS is commonplace and occurs through numerous modes, including bacterial infection and translocation of LPS across the GI lumen into the portal circulation (Ganey and Roth, 2001; Roth *et al*., 1997). The degree to which inflammatory cascades are activated during such exposures may not be sufficient to cause organ injury; nevertheless, exposure to small amounts of LPS can result in increased sensitivity to AFB_1 toxicity through mechanisms dependent on inflammatory mediators such as PMNs and TNF-α (Barton *et al*., 2000a, 2001).

Exposure to large, toxic doses of LPS causes injury to both parenchymal and non-parenchymal cell populations in the liver, including sinusoidal endothelial cells (SECs; Hewett and Roth, 1993). As early as three hours after LPS administration,

changes in the structure and function of SECs are evident as indicated by altered fenestrae and increases in plasma hyaluronic acid (HA) (Deaciuc *et al.*, 1994; Sarphie *et al.*, 1996). Additionally, damage to endothelial cells and early morphological alterations in hepatic sinusoids are accompanied by alterations in hepatic parenchymal cell morphology (Hewett and Roth, 1993).

An important consequence of endothelial cell injury is activation of the coagulation system (Colman *et al.*, 1994). Alterations in endothelium can cause activation of the extrinsic coagulation pathway via tissue factor expression. Additionally, loss of endothelial cell anticoagulant activity promotes coagulation system activation. Previous studies have shown that the coagulation system is activated after exposure of animals to toxic concentrations of LPS. Moreover, the hepatotoxic effects of LPS are dependent on an activated coagulation system (Pearson *et al.*, 1996).

Less is known about the effect of AFB$_1$ on SECs. Kupffer cells and SECs treated *in vitro* with AFB$_1$ are less sensitive to the damaging effects of AFB$_1$ than are parenchymal cells (Jennings *et al.*, 1994; Lafranconi *et al.*, 1986). In addition, AFB$_1$-treated nonparenchymal cells accumulate fewer AFB$_1$-DNA adducts than parenchymal cells both *in vitro* and *in vivo* (Jennings *et al.*, 1992; Schlemper *et al.*, 1991). Whether SEC injury occurs in livers of animals coexposed to AFB$_1$ and LPS at doses that cause synergistic parenchymal cell and bile duct injury remains to be elucidated.

The studies presented herein were designed to test the hypothesis that SEC injury and activation of the coagulation system accompany injury to parenchymal cells evoked by cotreatment of rats with small doses of AFB$_1$ and LPS. Furthermore, the hypothesis that activation of the coagulation system is required for LPS to augment AFB$_1$ hepatotoxicity was tested. To this end, SEC injury and coagulation system activation were evaluated using biomarkers and immunohistochemical techniques. In addition, rats were treated with the anticoagulant heparin to elucidate the importance of the coagulation system in LPS augmentation of AFB$_1$ hepatotoxicity.

MATERIALS AND METHODS

Materials. AFB$_1$ from *Aspergillus flavus* was purchased from Sigma Chemical Co. (St. Louis, MO), as was lipopolysaccharide derived from *E. coli* serotype 0128:B12 with an activity of 1.7×10^6 endotoxin units (EU)/mg. This activity was determined using a colorometric, kinetic Limulus Amebocyte Lysate (LAL) assay (Kit #50–650U) purchased from Biowhittaker (Walkersville, MD). Heparin (sodium salt) with an activity of 175.6 USP units/mg was purchased from Sigma Chemical Co. and unless otherwise noted, all chemicals were purchased from Sigma Chemical Co.

Animals. Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR; Charles River, Portage, MI) weighing 250–350 grams were used for these studies. Animals were fed standard chow (Rodent Chow/Tek 8640, Harlan Teklad, Madison, WI) and allowed access to water *ad libitum*. They were allowed to acclimate for 1 week in a 12-h light/dark cycle prior to use.

Treatment protocol and heparin administration. Rats fasted for 24 h were given 1 mg/kg AFB$_1$ or its vehicle, 8% dimethylsulfoxide (DMSO) in sterile water, by intraperitoneal (ip) injection. Food was returned after AFB$_1$ administration; 4 h later, animals were given 7.4×10^6 EU/kg LPS or sterile saline by tail vein injection. At various times up to 24 h after AFB$_1$ administration, they were anesthetized with sodium pentobarbital (50 mg/kg, ip). For serum collection, blood was drawn from the aorta, allowed to clot, and centrifuged to separate serum. Plasma was collected by drawing blood into a syringe containing sodium citrate (final concentration, 0.38%). For anticoagulant studies, heparin (2000 U/kg) or sterile saline was given in the tail vein 1.5 h after LPS or saline treatment.

Assessment of hepatic injury. Hepatic parenchymal cell injury was estimated by increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in serum or plasma. Injury to intrahepatic bile ducts was estimated by measuring serum activities of γ-glutamyl transferase (GGT) and 5′-nucleotidase (5′ ND). All reagent kits used to measure markers of liver injury spectrophotometrically (infinity-ALT, infinity-AST, GGT, 5′ ND) were purchased from Sigma Chemical Co. Additionally, a midlobe section of the left lateral lobe was processed for light microscopy. Paraffin-embedded sections were cut at 5 μm, stained with hematoxylin and eosin (H&E), and evaluated using a light microscope.

Measurement of plasma fibrinogen and HA. Plasma fibrinogen was determined from thrombin clotting time of diluted samples by using a fibrometer and a commercially available kit (Sigma Kit 886-A). Plasma HA concentration was measured using an enzyme-linked immunosorbent assay (ELISA; Corgenix Medical Corporation, Westminster, CO).

Immunohistochemistry. A 1-cm^3 block of liver cut from the left lateral lobe was frozen for 8 min in isopentane immersed in liquid nitrogen. For liver endothelial cell immunostaining, 8 μm-thick sections of frozen liver were fixed in acetone (4°C) for 5 min. Sections were incubated in a blocking solution consisting of PBS with 10% goat serum (Vector Laboratories, Burlingame, CA) for 30 min, then incubated overnight at 4°C in blocking solution containing diluted (1:20) mouse antirat RECA-1 (rat endothelial cell antigen-1, Serotec, Inc., Raleigh, NC) antibody. The RECA-1 antibody binds to rat endothelium but not to other cell types (Duijvestijn *et al.*, 1992). In the liver, this antibody stains both SECs and endothelial cells of larger vessels. After incubation with the RECA-1 antibody, sections were incubated for 3 h with goat antimouse secondary antibody conjugated to Alexa 594 (1:1000; Molecular Probes, Eugene, OR) in blocking solution containing 2% rat serum. Sections were washed three times, 5 min each, with PBS and visualized using a fluorescent microscope.

For fibrin immunostaining, 8 μm-thick sections of frozen liver were fixed in 10% buffered formalin containing 2% acetic acid for 30 min at room temperature. This fixation protocol solubilizes all fibrinogen and fibrin except for cross-linked fibrin; therefore, only cross-linked fibrin stains in sections of liver (Schnitt *et al.*, 1993). Sections were blocked with PBS containing 10% horse serum (i.e., blocking solution; Vector Laboratories) for 30 min, and this was followed by incubation overnight at 4°C with goat antirat fibrinogen antibody diluted (1:1000, ICN Pharmaceuticals, Aurora, OH) in blocking solution. Next, sections were incubated for three h with donkey antigoat secondary antibody conjugated to Alexa 594 (1:1000; Molecular Probes) in blocking solution. Sections were washed three times, 5 min each, with PBS and visualized using a fluorescent microscope.

For both protocols, no staining was observed in controls in which the primary or secondary antibody was eliminated from the staining protocol. All treatment groups that were compared morphometrically were stained immunohistochemically at the same time.

Morphometric quantification. Endothelial cells and fibrin deposition in the liver were quantified morphometrically by the methods described by Copple *et al.* (2002b). A decrease in the area of staining for endothelial cells suggests a loss of these cells in the liver. An increase in the area of staining of fibrin in the liver indicates fibrin deposition. These were quantified in two ways. First, morphometric analysis was performed to quantify the area of endothelial cell or fibrin staining in randomly chosen, low-power fields encompassing all regions of the liver sections. This was done to determine if the total area of endothelial cells or fibrin deposition in sections of liver changed
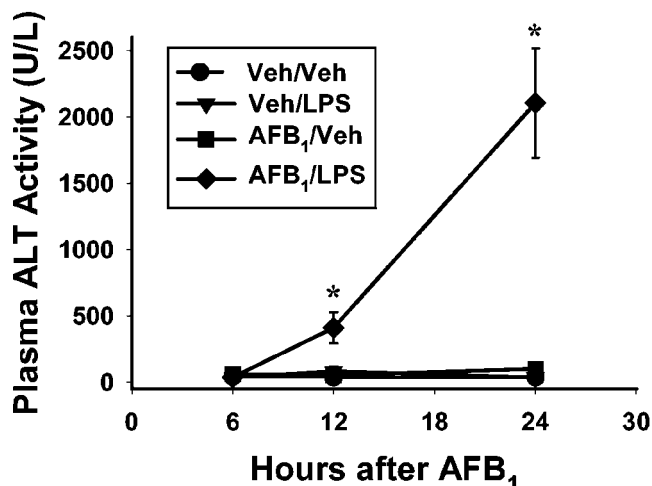
FIG. 1. Hepatic parenchymal cell injury after AFB$_1$/LPS cotreatment. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then 4 h later were given 7.4 × 10^6 EU/kg LPS or its vehicle (iv). Hepatic parenchymal cell injury was estimated from increases in plasma alanine aminotransferase (ALT) activity 6, 12, or 24 h after AFB$_1$ administration; n = 6–8 rats at each time. Data are expressed as mean ± SEM. *Significantly different from all other groups at the same time.

after AFB$_1$/LPS treatment. Secondly, the area of endothelial cells or fibrin deposition was determined in randomly chosen, centrilobular and periportal regions separately. This was done to determine if zonal differences in staining occurred after AFB$_1$/LPS treatment.

Fluorescent staining in sections of liver was visualized with an Olympus AX-80T microscope (Olympus, Lake Success, NY). For morphometric analysis of the total area of endothelial cells or fibrin deposition in a liver section, digital images of 10 randomly chosen fields per tissue section (magnification ×100) were captured using a SPOT II camera and SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI). Samples were coded such that the evaluator was not aware of treatment, and the same exposure time was used for all captured images. Each digital image encompassed a total area of 1.4 mm^2 and contained several centrilobular and periportal regions. For morphometric analysis of the area of staining within centrilobular or periportal regions, digital images of 5 randomly chosen fields (magnification ×100) that contained a centrilobular region and 5 randomly chosen fields that contained a periportal region were captured. The same exposure time was used to capture all images.

The area of immunohistochemical staining (number of pixels) was quantified using Scion Image software (Scion Corporation, Frederick, MD). For endothelial cell quantification, a density slice was taken from an inverted, gray-scale digital image of a liver section from a vehicle/vehicle (veh/veh)-treated rat. A density slice allows analysis of pixels in a defined range of gray values (i.e., densities). Next, the threshold was adjusted such that background staining was eliminated from the analysis, and only endothelial-cell staining was counted. The same threshold value was used to analyze digital images from sections from all treatment groups. For quantification of fibrin deposition, the threshold was selected so that little positive staining was present in veh/veh-treated animals. The same threshold value was used to analyze digital images from all treatment groups. For quantification of endothelial cells or fibrin in a field (magnification ×100) of liver, the area of positive staining was measured and divided by the total area of the image.

For analysis of endothelial cells or fibrin deposition in centrilobular or periportal regions, a 145 μm-diameter circle was drawn around the central vein or periportal region. The circumference of the circle was approximately 4–5 hepatocytes away from the central vein or portal region. This area was arbitrarily defined as the centrilobular or periportal region. The total area analyzed was 16,512 μm^2. The area of endothelial-cell or fibrin staining in that region was measured as described above and divided by the area of the circle.

For both, the staining is expressed as a fraction of the total area. The random fields analyzed for each liver section were averaged and counted as a replicate, i.e., each replicate represents a different rat. Results from veh/veh-treated rats at different time-points were combined into one group for statistical analysis, since no differences occurred among veh/veh-treated animals.

Statistical analysis. Results are presented as mean ± SEM. For the time-course study and heparin study, n = 6–8 rats. A one-way or 2 × 2, multifactorial, completely randomized ANOVA was used when appropriate. Data were log-transformed if they were not homogenous. Comparisons of group means were made using Tukey's test. The criterion for significance was $p <$ 0.05 for all studies.

RESULTS

Hepatic parenchymal cell injury after AFB$_1$/LPS cotreatment. Development of hepatocellular injury after AFB$_1$/LPS treatment was evaluated by increases in plasma ALT activity. Increases in plasma ALT activity after AFB$_1$/LPS treatment were time-dependent and significant by 12 h (Fig. 1), confirming earlier results (Barton *et al.*, 2000b). The lesions associated with this treatment regimen were similar to those described in detail previously and consisted of periportal hepatocellular necrosis with associated injury to intrahepatic bile ducts (Barton *et al.*, 2000b). No injury was evident in animals treated with AFB$_1$ or LPS alone.

Sinusoidal endothelial cell injury after AFB$_1$/LPS cotreatment. To determine if SECs were injured after AFB$_1$/LPS cotreatment, plasma HA was measured. Ordinarily 90% of plasma HA is cleared by SECs in the liver (Kobayashi *et al.*, 1999). Increases in plasma HA are indicative of impaired SEC
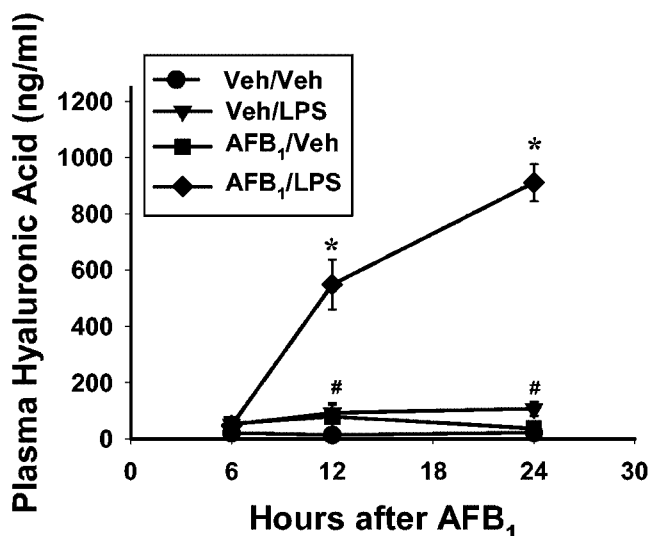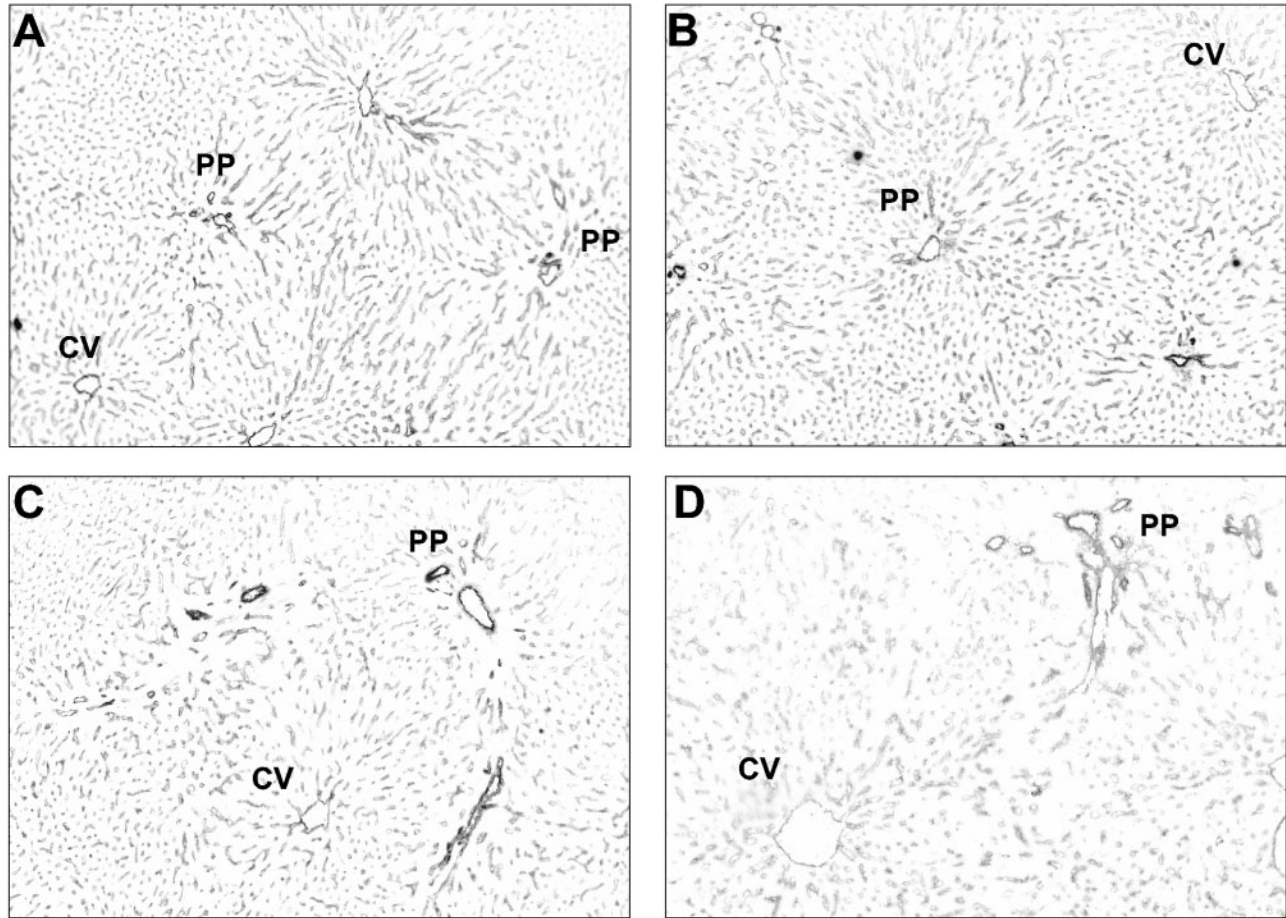

FIG. 2. Effect of AFB$_1$/LPS cotreatment on plasma HA concentration. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then 4 h later were given 7.4 × 10^6 EU/kg LPS or its vehicle (iv). Plasma HA was measured 6, 12, and 24 h after AFB$_1$ administration, using a commercially available ELISA; n = 6–8 rats at each time. Data are expressed as mean ± SEM. *Significantly different from all other groups at that time; #significantly different from veh/veh-treated rats.

FIG. 3. Effect of AFB$_1$/LPS cotreatment on RECA-1 immunostaining. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then 4 h later with 7.4 × 10^6 EU/kg LPS (iv) or its vehicle. Livers were removed 6, 12, or 24 h after AFB$_1$ administration and processed for RECA-1 immunohistochemistry as described in Materials and Methods. (A) Representative liver section from a veh/veh-treated rat, indicating strong RECA-1 staining (black) along hepatic sinusoids and the intima of periportal (PP) and centrilobular (CV) vessels. Liver sections from AFB$_1$/LPS-cotreated rats at (B) 6h, (C) 12 h, and (D) 24 h show a time-dependent decrease in RECA-1 staining intensity.

function and therefore have been used as a marker of SEC injury after toxic insult (Deaciuc *et al.*, 1993, 1994). AFB$_1$/LPS cotreatment resulted in a time-dependent increase in HA that became significant by 12 h (Fig. 2). Interestingly, veh/LPS-treated animals showed a slight, but statistically significant increase in HA at 12 h that persisted through 24 h (Fig. 2).

SECs in sections of liver were stained immunohistochemically for RECA-1. This protocol selectively stains rat endothelial cells. RECA-1 staining in livers from veh/veh-treated animals was apparent along the sinusoids and other vessels (Fig. 3A). RECA-1 staining was not altered by treatment with AFB$_1$ or LPS alone (data not shown). Modest decreases in RECA-1 staining were observed at 12 h, and these subsequently progressed to a marked, panlobular decrease in RECA-1 staining intensity by 24 h in liver sections from AFB$_1$/LPS-cotreated animals (Fig. 3).

Quantitative morphometry confirmed that AFB$_1$ or LPS treatment alone did not cause a decrease in RECA-1 staining (data not shown). Analysis of whole field (magnification ×100)

images (Fig. 4A) revealed a time-dependent decrease in RECA-1 staining that appeared to begin by 12 h and was statistically significant by 24 h. To quantify zonal RECA-1 staining distribution in the liver, centrilobular and periportal regions were analyzed individually. Figure 4B shows that RECA-1 staining was slightly decreased in both periportal and centrilobular regions at 12 h and was decreased 50–60% by 24 h.

Activation of the coagulation system after AFB$_1$/LPS cotreatment. Coagulation system activation results in the conversion of soluble fibrinogen to insoluble fibrin clots, thereby decreasing plasma fibrinogen levels. Accordingly, a decrease in plasma fibrinogen is indicative of coagulation system activation. Plasma fibrinogen concentration was not significantly altered at any time by either AFB$_1$ or LPS compared to vehicle treatment (Fig. 5). However, in rats cotreated with AFB$_1$/LPS a significant decrease (~7-fold) in plasma fibrinogen was observed between 6 and 12 h that remained depressed at 24 h (Fig. 5).
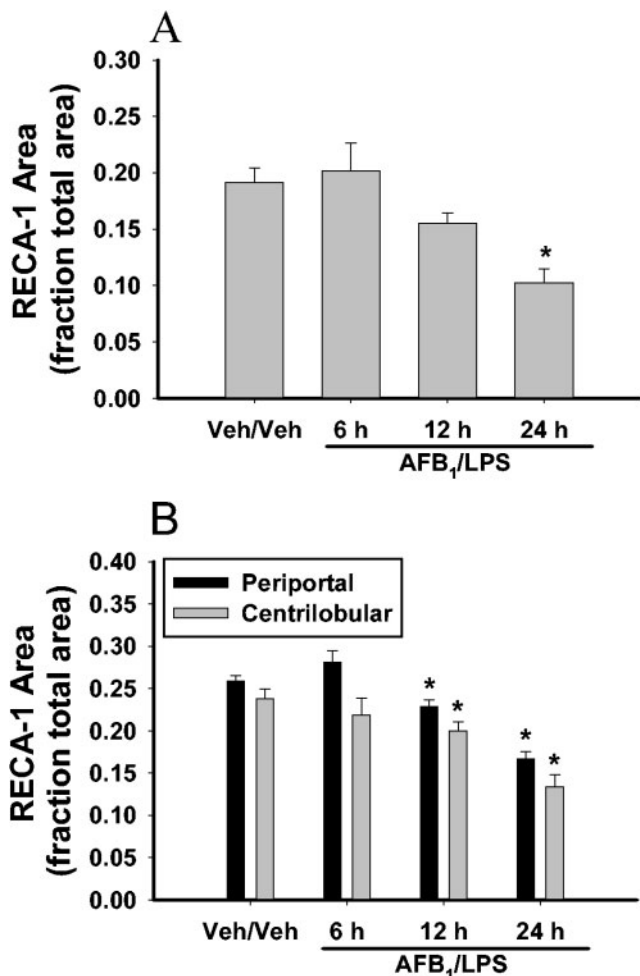
FIG. 4. Quantitative morphometry of RECA-1 staining in the liver after AFB₁/LPS treatment. Rats were given 1 mg/kg AFB₁ (ip), then 4 h later were given 7.4×10^6 EU/kg LPS (iv). Livers were removed 6, 12, or 24 h after AFB₁ administration and processed for RECA-1 immunohistochemistry as described in Materials and Methods. Veh/Vveh animals from all time points were combined, since no difference in RECA-1 staining was observed at any of the times; $n = 5$ rats per group at each time. For (A), the total area of positive RECA-1 staining in 10 randomly chosen fields ($\times 100$) per tissue was analyzed morphometrically as described in Materials and Methods. *Significantly different from veh/veh-treated rats. For (B), the area of RECA-1 staining in 5 randomly chosen fields that contained a centrilobular region and 5 randomly chosen fields that contained a periportal region were analyzed. Data are expressed as mean $\pm$ SEM. *Significantly different from the same region (centrilobular or periportal) of veh/veh-treated rats.

To determine if the decrease in plasma fibrinogen was accompanied by hepatic fibrin deposition, sections of liver were stained immunohistochemically for cross-linked fibrin. Liver sections from veh/veh-treated animals showed very little fibrin staining in tissue, with some initial staining of the larger vessels (Fig. 6A). This staining was most likely due to fibrin deposition during animal sacrifice and could be prevented by perfusing livers from control animals with heparin before organ removal (data not shown). Fibrin deposition after treatment

with AFB₁/LPS began by 6 h (Fig. 6B) and became greater in both centrilobular and periportal regions by 12 h (Fig. 6C). Twenty-four h after AFB₁ exposure in AFB₁/LPS-cotreated animals, fibrin clots remained evident in periportal regions, whereas a marked decrease in fibrin staining occurred around central veins (Fig. 6D).

Morphometric analysis of fibrin staining revealed that AFB₁- or LPS-treatment alone did not cause a significant increase in fibrin staining as compared to veh/veh-treated animals (data not shown). In animals cotreated with AFB₁/LPS, analysis of whole field (magnification $\times 100$) images indicated an increase in fibrin staining at 12 and 24 h (Fig. 7A). The magnitude of staining decreased between these times. Zonal distribution of fibrin staining was examined (Fig. 7B). A treatment-related increase in staining in both centrilobular and periportal regions occurred by 12 h, with staining greater in periportal than in centrilobular regions. By 24 h, centrilobular fibrin staining was no longer elevated, whereas periportal fibrin staining remained significantly greater (Fig. 7B).

Effect of heparin on AFB₁/LPS hepatotoxicity. Heparin was used to prevent activation of the coagulation system. To confirm heparin's anticoagulant activity, quantitative morphometry was performed to measure liver fibrin staining. Twenty-four h after AFB₁/LPS, analysis of fibrin staining in whole field images (magnification $\times 100$; Fig. 8A) and zonal analysis (Fig. 8B) showed that heparin markedly attenuated fibrin deposition in livers of AFB₁/LPS-treated animals.

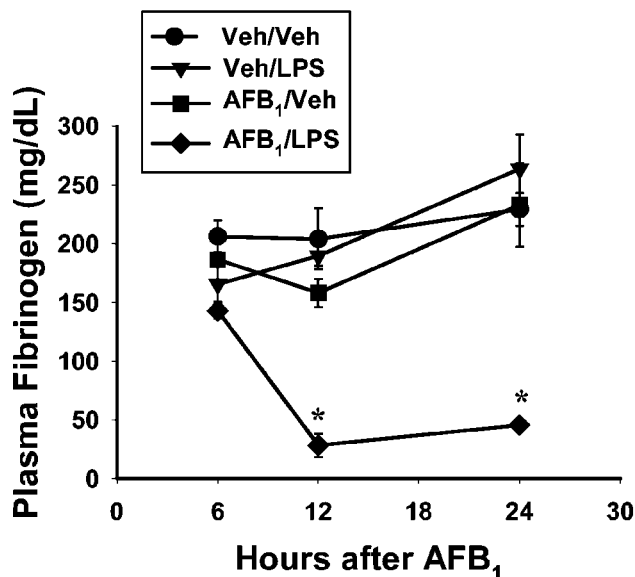The effect of heparin on the manifestation of hepatic paren-

FIG. 5. Effect of AFB₁/LPS on plasma fibrinogen concentration. Rats were treated with 1 mg/kg AFB₁ (ip) or its vehicle, then 4 h later were given 7.4×10^6 EU/kg LPS or its vehicle via the tail vein. Plasma fibrinogen was measured 6, 12, or 24 h after AFB₁ administration using a commercially available kit, as outlined in Materials and Methods; $n = 6–8$ rats at each time *Significantly different from all other groups at the same time.
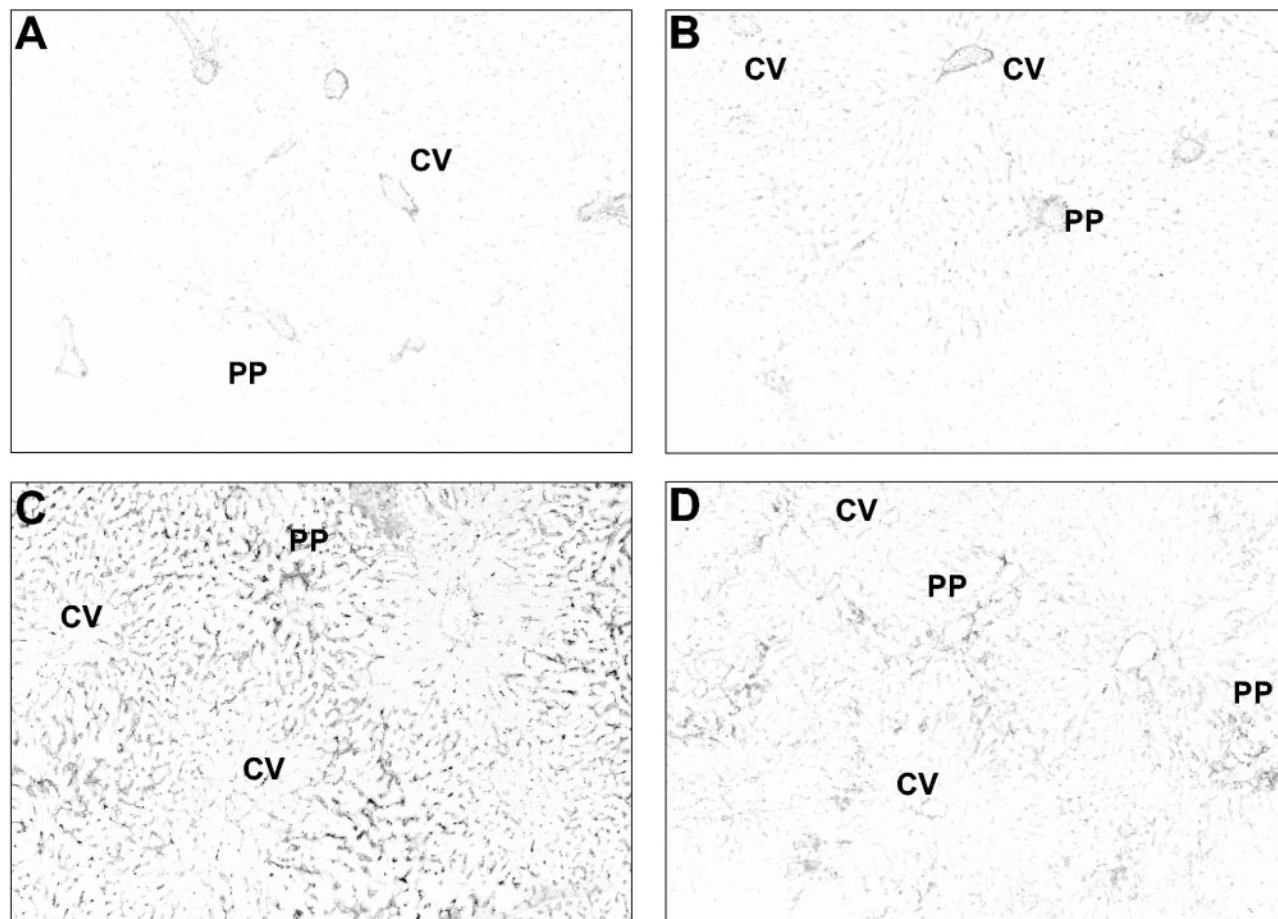
FIG. 6. Effect of AFB$_1$/LPS cotreatment on liver fibrin deposition. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then 4 h later were given 7.4 × 10^6 EU/kg LPS (iv) or its vehicle. Livers were removed 6, 12, or 24 h after AFB$_1$ administration and processed for fibrin immunohistochemistry, as described in Materials and Methods. (A) Representative liver section from a veh/veh-treated rat showing minimal fibrin staining (black). Liver sections from AFB$_1$/LPS-cotreated animals 6 h (B) and 12 h (C) after AFB$_1$ administration show a time-dependent increase in fibrin staining in both periportal (PP) and centrilobular (CV) regions. (D) Liver from AFB$_1$/LPS-treated rat 24 h after AFB$_1$ showing fibrin staining mainly in PP regions.

chymal cell injury was determined by measuring serum activities of ALT and AST twenty-four h after AFB$_1$ administration (Fig. 9). Histopathologic analysis revealed that periportal hepatocellular necrosis and bile duct epithelial cell injury in AFB$_1$/LPS-treated rats were pronounced and similar to lesions described previously (Barton *et al.*, 2000b). Livers from AFB$_1$/LPS-cotreated animals that received heparin showed only occasional, small, necrotic foci consisting of 2–4 hepatocytes in periportal regions (data not shown). Thus, inhibition of the coagulation system markedly attenuated hepatic parenchymal cell injury caused by AFB$_1$/LPS.

The effect of heparin on injury to bile ducts was evaluated by measuring serum activities of GGT and 5′-ND twenty-four h after AFB$_1$ administration (Fig. 10). Heparin did not significantly reduce the increases in serum GGT or 5′-ND activities. These results suggest that prevention of coagulation system activation has little effect on bile duct injury in this model.

The effect of heparin on SEC injury was assessed by evaluating serum HA and by quantitative morphometry 24 h

after AFB$_1$ administration. Heparin caused a modest ($\sim$30%), but statistically significant, attenuation of serum HA concentration after AFB$_1$/LPS cotreatment (Fig. 11). Analysis of images (magnification ×100) of RECA-1-stained liver sections showed that heparin did not prevent decreases in RECA-1 staining after AFB$_1$/LPS treatment (Fig. 12A). This was true in both periportal and centrilobular regions, as indicated by zonal analysis of RECA-1 staining (Fig. 12B).

DISCUSSION

Previous work demonstrated that LPS coexposure increases the sensitivity of livers to AFB$_1$-induced hepatic parenchymal cell and bile duct injuries (Barton *et al.*, 2000b; Luyendyk *et al.*, 2002). Increases in serum markers of liver injury were consistent with histopathological observations of periportal necrosis and bile duct injury and loss (Barton *et al.*, 2000b). In the present study, we show that SEC injury also occurs after AFB$_1$/LPS cotreatment, as indicated by increases in plasma
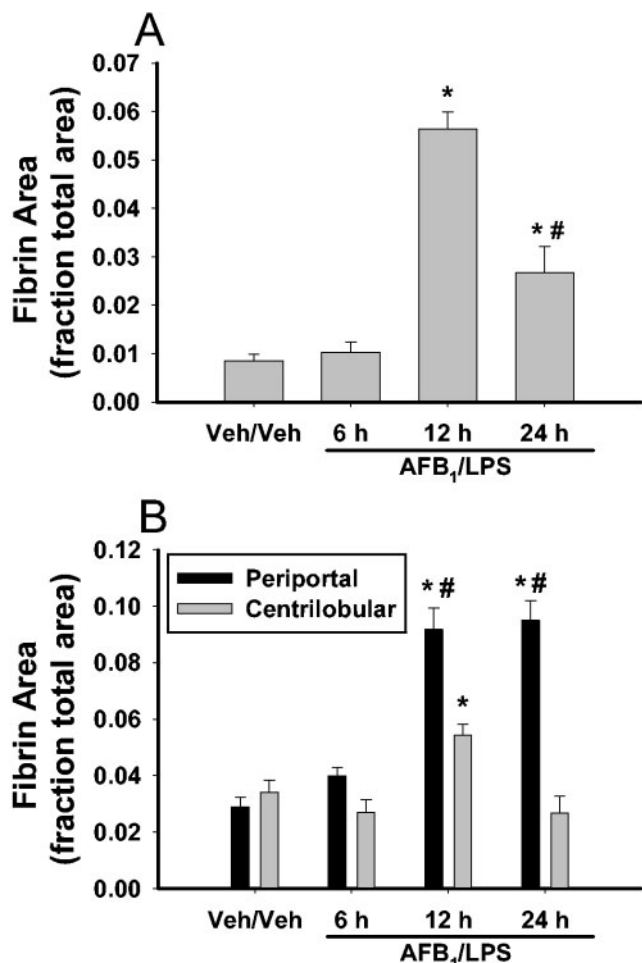
FIG. 7. Quantitative morphometry of fibrin staining in livers after AFB$_1$/LPS treatment. Rats were given 1 mg/kg AFB$_1$ (ip), then 4 h later were given 7.4×10^6 EU/kg LPS (iv). Livers were removed 6, 12, or 24 h after AFB$_1$ administration and processed for fibrin immunohistochemistry, as described in Materials and Methods. Veh/Veh animals were combined, since no difference in fibrin staining was observed at any of the times; $n = 5$ rats per group at each time. For (A), the total area of fibrin staining in 10 randomly chosen (magnification $\times100$) fields per liver, was analyzed morphometrically, as described in Materials and Methods. *Significantly different from veh/veh-treated rats; #significantly different from 12-h AFB$_1$/LPS-treated rats. For (B), we analyzed the area of fibrin staining in 5 randomly chosen fields that contained a centrilobular region and 5 randomly chosen fields that contained a periportal region *Significantly different from veh/veh-treated rats in the same liver region. #Significantly different from the centrilobular region of AFB$_1$/LPS-treated rats at the same time.

HA and decreased RECA-1 immunostaining (Fig. 2). Livers from animals cotreated with AFB$_1$ and LPS showed a pan lobular decrease in RECA-1 staining, which was apparent by 12 h (Fig. 4B). Thus, SECs are injured in both centrilobular and periportal regions, whereas parenchymal cell necrosis is evident only in periportal regions. This difference suggests that SEC injury is not sufficient to cause parenchymal cell injury in this model, and that an additional insult is required in periportal regions for expression of parenchymal cell necrosis. Alterna-

tively, qualitative differences in regional SEC injury or function not detected by RECA-1 staining but nevertheless toxicologically significant may have occurred. Hemorrhage is evident in periportal regions, but not in centrilobular areas in this model, suggesting that SEC disruption is more pronounced periportally (Barton *et al.*, 2000b).

One possible mechanism for enhanced SEC injury in this model is that SECs that have been exposed to AFB$_1$ are rendered more sensitive to the action of one or more inflammatory mediators produced in response to LPS exposure. LPS
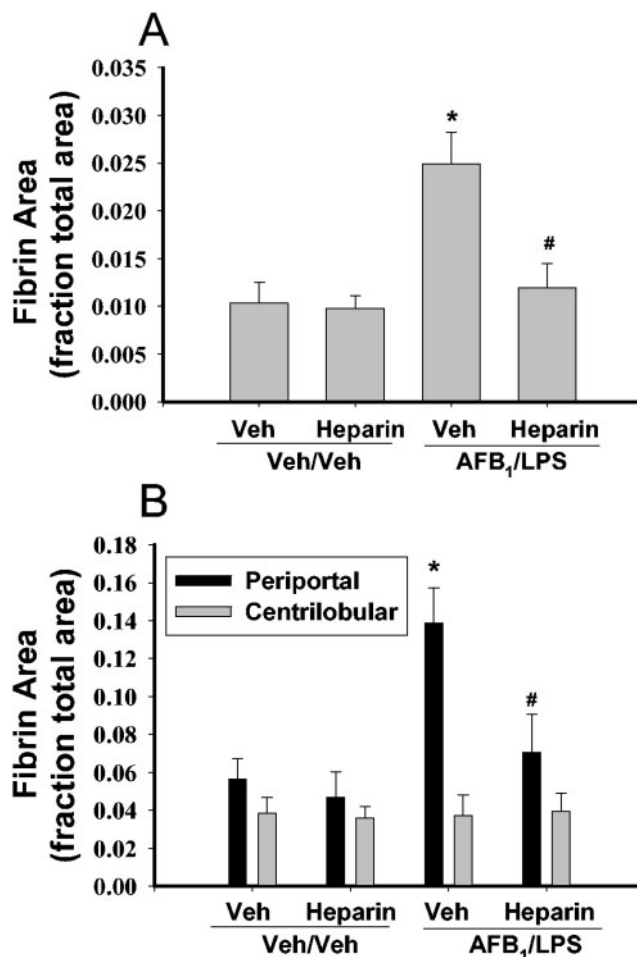

FIG. 8. Effect of heparin on liver fibrin staining after AFB$_1$/LPS cotreatment. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then 4 h later were given 7.4×10^6 EU/kg LPS or its vehicle (iv). Heparin (2000 U/kg, iv) or its vehicle was administered 1.5 h after LPS. Livers were removed 24 h after AFB$_1$ administration and processed for fibrin immunohistochemistry, as described in Materials and Methods; $n = 5$ rats per group. For (A), we analyzed the total area of fibrin staining in 10 randomly chosen fields (magnification $\times100$) per tissue. *Significantly different from respective veh/veh-treated rats. #Significantly different from AFB$_1$/LPS/veh-treated rats. For (B), the area of fibrin staining in 5 randomly chosen fields that contained a centrilobular region and 5 randomly chosen fields that contained a periportal region were analyzed. *Significantly different from the same region (centrilobular or periportal) of respective veh/veh-treated rats; #Significantly different from the periportal region of AFB$_1$/LPS/veh-treated rats.
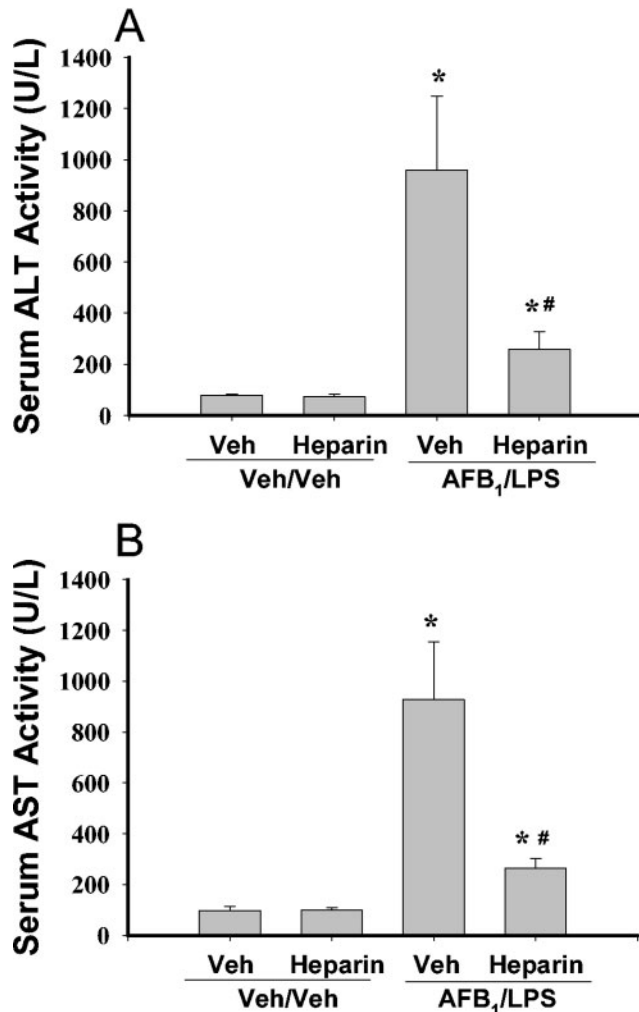
FIG. 9. Effect of heparin on hepatic parenchymal cell injury after AFB$_1$/
LPS cotreatment. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then
4 h later with 7.4×10^6 EU/kg LPS or its vehicle (iv). Heparin (2000 U/kg, iv)
or its vehicle was administered 1.5 h after LPS; $n = 6$–8 rats per group.
Hepatic parenchymal cell injury was estimated 24 h after AFB$_1$ administration
by increases in (A) serum alanine aminotransferase (ALT), and (B) aspartate
aminotransferase (AST) activities. *Significantly different from veh/veh/veh-
treated animals. #Significantly different from AFB$_1$/LPS/veh-treated animals.

causes activation of inflammatory cells implicated in tissue
injury. For example, Kupffer cells are important for manifes-
tation of SEC injury after exposure to large, hepatotoxic doses
of LPS (Deaciuc *et al*., 1994; Sarphie *et al*., 1996). Addition-
ally, PMNs and TNF-α have been shown to cause SEC toxicity
in vitro (Smedly *et al*., 1986; Takei *et al*., 1995). The dose of
LPS used in this study did not appear to cause overt SEC injury
as measured by RECA-1 immunostaining, but it did result in a
small increase in plasma HA levels, suggesting a change in
SEC homeostasis (Fig. 2). The dose of AFB$_1$ used did not
result in significant changes in either plasma HA concentration
or liver RECA-1 immunostaining. Nevertheless, nonparenchy-
mal cells isolated from rats treated with a similar dose of AFB$_1$

have been shown to contain AFB$_1$-DNA adducts, albeit at a
markedly lower level compared to parenchymal cells (Jennings
et al., 1992). Thus, SECs may be primed by AFB$_1$ to become
more sensitive to insult from inflammatory products released
from activated PMNs or from Kupffer cells stimulated by LPS.

PMNs accumulate early after AFB$_1$/LPS cotreatment and are
critical for development of hepatic parenchymal cell injury in
this model (Barton *et al*., 2000a). Interestingly, hepatic PMN
accumulation does not differ in animals treated with LPS alone
and those cotreated with AFB$_1$ (Barton *et al*., 2000a). This
suggests that a second insult is required for PMN-dependent
tissue injury in AFB$_1$-cotreated animals. Such an insult could
arise via a PMN-hepatocyte interaction facilitated by SEC
injury. *In vivo*, injury to hepatic parenchymal cells by PMNs
requires several steps: PMN accumulation in hepatic sinusoids,
migration across the SEC barrier into the parenchyma, and
contact with parenchymal cells (Jaeschke and Smith, 1997). In
models of endotoxin shock, the PMN transmigration and cy-
totoxicity to parenchymal cells depends on cell-surface adhe-
sion molecules, including Mac-1 on PMNs and ICAM-1 on
SECs and hepatocytes (Essani *et al*., 1995; Jaeschke *et al*.,
1991, 1996). However, in models of liver injury in which
endothelium has been damaged, neutralizing antibodies di-
rected against some of these adhesion molecules, including
ICAM-1, have proven to be less effective in preventing hepa-
tocellular necrosis (Farhood *et al*., 1995). This suggests that if
the SEC barrier is severely damaged, the requirement for
transmigration may be circumvented, allowing direct hepato-
cyte-PMN interaction and thereby facilitating PMN-induced
hepatocellular injury (Jaeschke *et al*., 1996). Thus, SEC injury
in AFB$_1$/LPS-treated animals may promote PMN–hepatocyte
interactions and injury to parenchymal cells by PMN-derived
products.

Another consequence of injury to endothelium is activation
of the coagulation system (Colman *et al*., 1994). SEC injury or
loss might be required for activation of the coagulation system
in this model. Indeed, after AFB$_1$/LPS-cotreatment, coagula-
tion system activation was evident as a decrease in plasma
fibrinogen (Fig. 5) and deposition of insoluble fibrin in liver
sections (Figs. 6 and 7). Additionally, morphometric quantifi-
cation confirmed significant fibrin staining in periportal and
centrilobular zones of liver lobules, where SEC injury oc-
curred. However, SEC injury by itself is not sufficient to cause
parenchymal cell injury, since the latter was only observed in
periportal regions, whereas SEC injury occurred in both peri-
portal and centrilobular areas. Interestingly, centrilobular fibrin
deposition was transient, clearing by 24 h, whereas fibrin
deposits persisted in periportal regions. The persistence of
fibrin deposits only in the periportal regions where hepatocel-
lular injury localized suggests the possibility that persistent
fibrin plays an important role in the progression to necrosis of
hepatocytes homeostatically altered by AFB$_1$ exposure. Alter-
natively, fibrin may persist in periportal regions as a result of
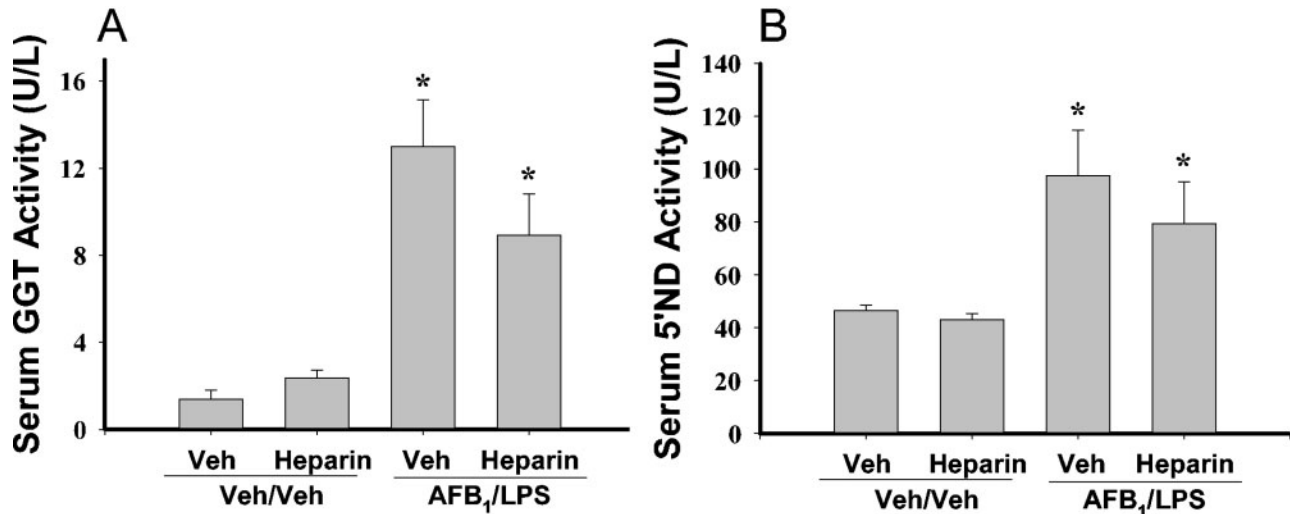parenchymal cell death.

FIG. 10. Effect of heparin on biliary injury after AFB$_1$/LPS cotreatment. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then 4 h later with 7.4 × 10^6 EU/kg LPS or its vehicle (iv). Heparin (2000 U/kg, iv) or its vehicle was administered 1.5 h after LPS; n = 6–8 rats per group. Bile duct injury was estimated 24 h after AFB$_1$ administration by increases in (A) serum γ-glutamyl transferase (GGT) and (B) 5′-nucleotidase (5′ND) activities. *Significantly different from Veh/Veh/Veh-treated animals.

Hepatic fibrin deposition has been noted in livers of animals treated with other hepatotoxicants, such as monocrotaline or carbon tetrachloride, and the location of fibrin deposition correlated with that of parenchymal cell injury (Copple *et al*., 2002a; Neubauer *et al*., 1995). Indeed, activation of the coagulation system influences the development of liver injury from dimethylnitrosamine, monocrotaline, and toxic doses of LPS (Copple *et al*., 2002b; Fujiwara *et al*., 1988, Pearson *et al*.,

FIG. 11. Effect of heparin on serum HA concentration after AFB$_1$/LPS cotreatment. Rats were treated with 1 mg/kg AFB$_1$ (ip) or its vehicle, then 4 h later with 7.4 × 10^6 EU/kg LPS or its saline vehicle (iv); n = 6–8 rats per group. Heparin (2000 U/kg, iv) or its vehicle was administered 1.5 h after LPS. Serum HA was measured 24 h after AFB$_1$ administration, using a commercially available ELISA. *Significantly different from Veh/Veh/Veh-treated animals. #Significantly different from AFB$_1$/LPS/Veh-treated animals.

1996). Inasmuch as SEC injury and coagulation occurred after AFB$_1$/LPS cotreatment, we examined whether coagulation system activation contributed to AFB$_1$/LPS hepatotoxicity. Anticoagulation with heparin markedly attenuated hepatic parenchymal cell injury but had little effect on bile duct or SEC injury after AFB$_1$/LPS treatment. Overall, the data suggest that activation of the coagulation system in response to SEC injury is required for the development of hepatic parenchymal cell injury after AFB$_1$/LPS coexposure, but SEC and bile duct injury occur predominantly by other mechanisms.

The mechanism by which the coagulation system contributes to parenchymal cell injury in this model is not known. One possibility is that formation of fibrin clots causes disruption of hepatic blood flow and consequent hypoxia. If all parenchymal cells are similarly sensitive to hypoxia, and hypoxia alone were responsible for cell injury, then shunting of blood around injured periportal regions must occur in order to maintain flow and oxygen delivery to uninjured, downstream areas. Homeostatic alterations in periportal parenchymal cells by AFB$_1$ may render them more sensitive to hypoxic injury, whereas hepatocytes unaffected by AFB$_1$ may not be sufficiently sensitized to hypoxia to undergo cell death. Alternatively, thrombin produced during coagulation system activation might mediate liver injury independently of its ability to form fibrin clots. For example, thrombin is critical for parenchymal cell injury from large, hepatotoxic doses of LPS, but its ability to cause cell death derives from activation of a protease-activated receptor rather than from fibrin formation (Copple *et al*., 2000; Moulin *et al*., 2001). Additional investigation is required to elucidate how coagulation system activation functions to promote parenchymal cell injury in LPS-potentiated AFB$_1$ hepatotoxicity.
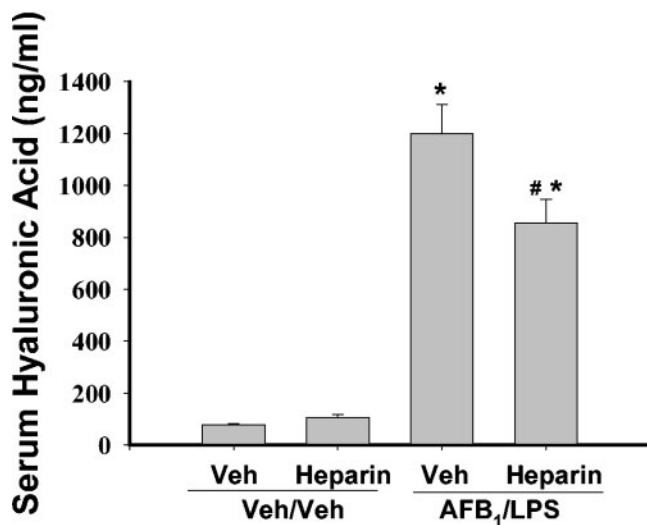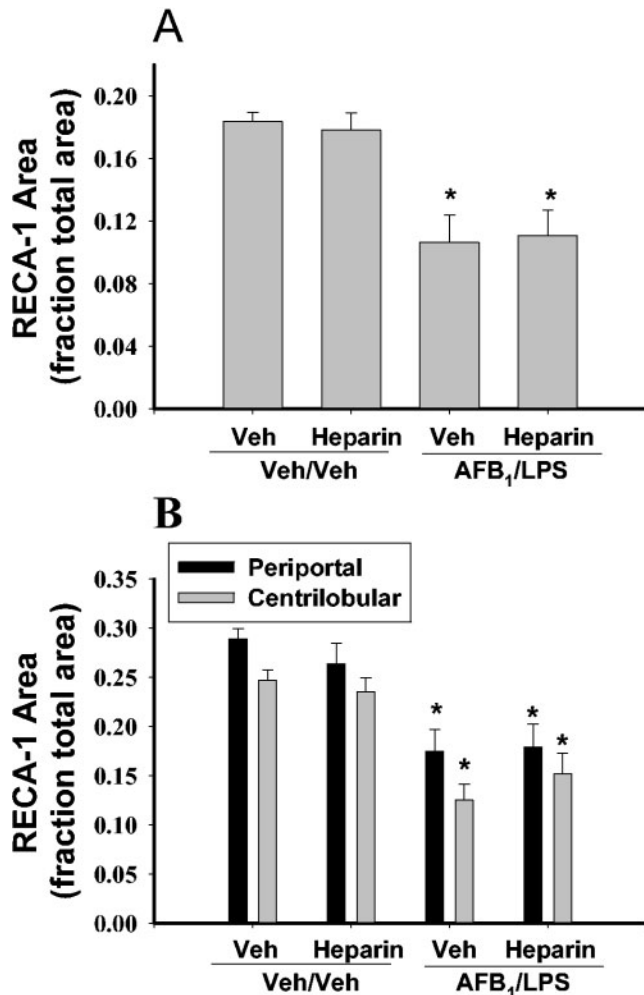
FIG. 12. Effect of heparin on RECA-1 staining after AFB_1/LPS cotreatment. Rats were treated with 1 mg/kg AFB_1 (ip) or its vehicle, then 4 h later with 7.4×10^6 EU/kg LPS or its vehicle (iv). Heparin (2000 U/kg, iv) or its vehicle was administered 1.5 h after LPS. Livers were removed 24 h after AFB_1 administration and processed for RECA-1 immunohistochemistry; $n =$ 5 rats per group. For (A), the total area of RECA-1 staining in 10 randomly chosen fields (magnification $\times100$) per tissue was analyzed morphometrically, as described in Materials and Methods. *Significantly different from respective veh/veh-treated rats. For (B), the areas of RECA-1 staining in 5 randomly chosen fields that contained a centrilobular region and 5 randomly chosen fields that contained a periportal region were analyzed. *Significantly different from the same region (centrilobular or periportal) of respective veh/veh-treated rats.

It is interesting that either anticoagulation with heparin (Fig. 9) or PMN depletion with an anti-PMN antibody (Barton *et al.*, 2000a) protects against parenchymal cell injury, but neither greatly influences bile duct damage caused by AFB_1/LPS. This suggests an interdependence of the coagulation system and PMNs in causing hepatocellular injury. One possibility is that thrombin enhances recruitment and/or activation of PMNs in the liver. Thrombin can modulate adherence of inflammatory cells to endothelium (DeMichele and Minnear, 1992; Zimmerman *et al.*, 1985) and stimulate PMN chemotaxis (Bizios *et al.*, 1986). Consistent with this hypothesis is the observation that

PMN-induced parenchymal cell injury (Barton *et al.*, 2000a) and evidence of augmented thrombin activity (Fig. 7B) occur in periportal regions of livers in AFB_1/LPS-cotreated animals. Conversely, PMNs may promote activation of the coagulation system. Antibody neutralization of PMNs attenuates activation of the coagulation system after administration of a hepatotoxic LPS dose, suggesting that PMNs may aid in coagulation system activation (Hewett and Roth, 1995). In some animal models of sepsis, PMNs express tissue factor in response to stimuli such as LPS (Todoroki *et al.*, 2000). Additionally, PMNs can influence synthesis of tissue factor by other cell types through their release of reactive oxygen species (Cadroy *et al.*, 2000). Thus, PMNs could act to enhance coagulation-dependent injury in the AFB_1/LPS model through upregulation of tissue factor and activation of the extrinsic coagulation pathway.

In summary, coexposure of rats to nontoxic doses of AFB_1 and LPS caused panlobular injury to SECs. This was associated with activation of the coagulation system and sinusoidal fibrin deposition, which persisted in areas of hepatocellular necrosis. Inhibition of coagulation system activation provided protection against the development of parenchymal cell injury but had little effect on bile duct epithelial cell or SEC injury. The data suggest that SEC injury and associated activation of the coagulation system play an important role in parenchymal cell injury after AFB_1/LPS exposure, possibly through ischemia/hypoxia resulting from persistent fibrin clots, or from interaction of coagulation components with inflammatory cells such as PMNs.

REFERENCES

Barton, C. C., Barton, E. X., Ganey, P. E., Kunkel, S. L., and Roth, R. A. (2001). Bacterial lipopolysaccharide enhances aflatoxin B1 hepatotoxicity in rats by a mechanism that depends on tumor necrosis factor-α. *Hepatology* **33,** 66–73.

Barton, C. C., Ganey, P. E., and Roth, R. A. (2000a). Lipopolysaccharide augments aflatoxin B(1)-induced liver injury through neutrophil-dependent and -independent mechanisms. *Toxicol. Sci.* **58,** 208–215.

Barton, C. C., Hill, D. A., Yee, S. B., Barton, E. X., Ganey, P. E., and Roth, R. A. (2000b). Bacterial lipopolysaccharide exposure augments aflatoxin B(1)-induced liver injury. *Toxicol. Sci.* **55,** 444–452.

Bizios, R., Lai, L., Fenton, J. W.,II, and Malik, A. B. (1986). Thrombin-induced chemotaxis and aggregation of neutrophils. *J. Cell Physiol.* **128,** 485–490.

Cadroy, Y., Dupouy, D., Boneu, B., and Plaisancie, H. (2000). Polymorpho-nuclear leukocytes modulate tissue factor production by mononuclear cells: Role of reactive oxygen species. *J. Immunol.* **164,** 3822–3828.

Colman, R. W., Marder, V. J., Salzman, E. W., and Hirsh, J. (1994). Overview of hemostasis. In *Hemostasis and Thrombosis* (R. W. Colman, J. Hirsh, V. J. Marder, and E. W. Salzman, Eds.), p. 3. Lippincott, Philadelphia.

Copple, B. L., Banes, A., Ganey, P. E., and Roth, R. A. (2002a). Endothelial

cell injury and fibrin deposition in rat liver after monocrotaline exposure. *Toxicol. Sci.* **65,** 309–318.

Copple, B. L., Moulin, F., Ganey, P. E., and Roth, R. A. (2000). Endotoxin-induced liver injury requires activation of protease-activated receptor-1. *FASEB J.* **14,** A290.

Copple, B. L., Woolley, B., Banes, A., Ganey, P. E., and Roth, R. A. (2002b). Anticoagulants prevent monocrotaline-induced hepatic parenchymal cell injury but not endothelial cell injury in the rat. *Toxicol. Appl. Pharmacol.* **180,** 186–196.

Deaciuc, I. V., Bagby, G. J., Neisman, M. R., Skrepnik, N., and Spitzer, J. J. (1994). Modulation of hepatic sinusoidal endothelial cell function by Kupffer cells: An example of intercellular communication in the liver. *Hepatology* **19,** 464–470.

Deaciuc, I. V., McDonough, K. H., Bagby, G. J., and Spitzer, J. J. (1993). Alcohol consumption in rats potentiates the deleterious effect of gram-negative sepsis on hepatic hyaluronan uptake. *Alcohol Clin. Exp. Res.* **17,** 1002–1008.

DeMichele, M. A., and Minnear, F. L. (1992). Modulation of vascular endothelial permeability by thrombin. *Semin. Thromb. Hemostasis* **18,** 287–295.

Duijvestijn, A. M., van Goor, H., Klatter, F., Majoor, G. D., van Bussel, E., and van Breda Vriesman, P. J. (1992). Antibodies defining rat endothelial cells: RECA-1, a pan-endothelial cell-specific monoclonal antibody. *Lab. Invest.* **66,** 459–466.

Essani, N. A., Fisher, M. A., Farhood, A., Manning, A. M., Smith, C. W., and Jaeschke, H. (1995). Cytokine-induced upregulation of hepatic intercellular adhesion molecule-1 messenger RNA expression and its role in the pathophysiology of murine endotoxin shock and acute liver failure. *Hepatology* **21,** 1632–1639.

Farhood, A., McGuire, G. M., Manning, A. M., Miyasaka, M., Smith, C. W., and Jaeschke, H. (1995). Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. *J. Leukoc. Biol.* **57,** 368–374.

Fujiwara, K., Ogata, I., Ohta, Y., Hirata, K., Oka, Y., Yamada, S., Sato, Y., Masaki, N., and Oka, H. (1988). Intravascular coagulation in acute liver failure in rats and its treatment with antithrombin III. *Gut* **29,** 1103–1108.

Ganey, P. E., and Roth, R. A. (2001). Concurrent inflammation as a determinant of susceptibility to toxicity from xenobiotic agents. *Toxicology* **169,** 195–208.

Groopman, J. D., Wild, C. P., Hasler, J., Junshi, C., Wogan, G. N., and Kensler, T. W. (1993). Molecular epidemiology of aflatoxin exposures: Validation of aflatoxin-N7-guanine levels in urine as a biomarker in experimental rat models and humans. *Environ. Health Perspect.* **99,** 107–113.

Hewett, J. A., Jean, P. A., Kunkel, S. L., and Roth, R. A. (1993). Relationship between tumor necrosis factor-α and neutrophils in endotoxin-induced liver injury. *Am. J. Physiol.* **265,** G1011–1015.

Hewett, J. A., and Roth, R. A. (1993). Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol. Rev.* **45,** 382–411.

Hewett, J. A., and Roth, R. A. (1995). The coagulation system, but not circulating fibrinogen, contributes to liver injury in rats exposed to lipopolysaccharide from gram-negative bacteria. *J. Pharmacol. Exp. Ther.* **272,** 53–62.

Hewett, J. A., Schultze, A. E., Van Cise, S., and Roth, R. A. (1992). Neutrophil depletion protects against liver injury from bacterial endotoxin. *Lab. Invest.* **66,** 347–361.

Jaeschke, H., Farhood, A., and Smith, C. W. (1991). Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. *Am. J. Physiol.* **261,** G1051–1056.

Jaeschke, H., and Smith, C. W. (1997). Mechanisms of neutrophil-induced parenchymal cell injury. *J. Leukoc. Biol.* **61,** 647–653.

Jaeschke, H., Smith, C. W., Clemens, M. G., Ganey, P. E., and Roth, R. A. (1996). Mechanisms of inflammatory liver injury: Adhesion molecules and cytotoxicity of neutrophils. *Toxicol. Appl. Pharmacol.* **139,** 213–226.

Jennings, G. S., Heck, R., Oesch, F., and Steinberg, P. (1994). Metabolism and cytotoxicity of aflatoxin B1 in cultured rat hepatocytes and nonparenchymal cells: Implications for tumorigenesis. *Toxicol. Appl. Pharmacol.* **129,** 86–94.

Jennings, G. S., Oesch, F., and Steinberg, P. (1992). *In vivo* formation of aflatoxin-B1-DNA adducts in parenchymal and non-parenchymal cells of rat liver. *Carcinogenesis* **13**, 831–835.

Kobayashi, H., Horikoshi, K., Yamataka, A., Yamataka, T., Okazaki, T., Lane, G. J., and Miyano, T. (1999). Hyaluronic acid: A specific prognostic indicator of hepatic damage in biliary atresia. *J. Pediatr. Surg.* **34,** 1791–1794.

Lafranconi, W. M., Glatt, H., and Oesch, F. (1986). Xenobiotic metabolizing enzymes of rat liver nonparenchymal cells. *Toxicol. Appl. Pharmacol.* **84,** 500–511.

Luyendyk, J. P., Shores, K. C., Ganey, P. E., and Roth, R. A. (2002). Bacterial lipopolysaccharide exposure alters aflatoxin B1 hepatotoxicity: Benchmark analysis for markers of liver injury. *Toxicol. Sci.* **68,** 220–225.

Moulin, F., Copple, B. L., Ganey, P. E., and Roth, R. A. (2001). Hepatic and extrahepatic factors critical for liver injury during lipopolysaccharide exposure. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281,** G1423–1431.

Neubauer, K., Knittel, T., Armbrust, T., and Ramadori, G. (1995). Accumulation and cellular localization of fibrinogen/fibrin during short-term and long-term rat liver injury. *Gastroenterology* **108,** 1124–1135.

Pearson, J. M., Schultze, A. E., Schwartz, K. A., Scott, M. A., Davis, J. M., and Roth, R. A. (1996). The thrombin inhibitor, hirudin, attenuates lipopolysaccharide-induced liver injury in the rat. *J. Pharmacol. Exp. Ther.* **278,** 378–383.

Roebuck, B. D., and Maxuitenko, Y. Y. (1994). Biochemical mechanisms and biological implications of the toxicity of aflatoxins as related to aflatoxin carcinogenesis. In *The Toxicology of Aflatoxins* (D. L. Eaton and J. D. Groopman, Eds.), pp. 27–43. Academic Press, San Diego.

Roth, R. A., Harkema, J. R., Pestka, J. P., and Ganey, P. E. (1997). Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? *Toxicol. Appl. Pharmacol.* **147,** 300–311.

Sarphie, T. G., D'Souza, N. B., and Deaciuc, I. V. (1996). Kupffer cell inactivation prevents lipopolysaccharide-induced structural changes in the rat liver sinusoid: An electron-microscopy study. *Hepatology* **23,** 788–796.

Schlemper, B., Harrison, J., Garner, R. C., Oesch, F., and Steinberg, P. (1991). DNA binding, adduct characterization, and metabolic activation of aflatoxin B1 catalysed by isolated rat liver parenchymal Kupffer and endothelial cells. *Arch. Toxicol.* **65,** 633–639.

Schnitt, S. J., Stillman, I. E., Owings, D. V., Kishimoto, C., Dvorak, H. F., and Abelmann, W. H. (1993). Myocardial fibrin deposition in experimental viral myocarditis that progresses to dilated cardiomyopathy. *Circ. Res.* **72,** 914–920.

Smedly, L. A., Tonnesen, M. G., Sandhaus, R. A., Haslett, C., Guthrie, L. A., Johnston, R. B., Jr., Henson, P. M., and Worthen, G. S. (1986). Neutrophil-mediated injury to endothelial cells. Enhancement by endotoxin and essential role of neutrophil elastase. *J. Clin. Invest.* **77,** 1233–1243.

Takei, Y., Kawano, S., Nishimura, Y., Goto, M., Nagai, H., Chen, S. S., Omae, A., Fusamoto, H., Kamada, T., Ikeda, K., Kawada, N., and Kaneda, K. (1995). Apoptosis: A new mechanism of endothelial and Kupffer cell killing. *J. Gastroenterol. Hepatol.* **10**(Suppl. 1), S65–67.

Todoroki, H., Nakamura, S., Higure, A., Okamoto, K., Takeda, S., Nagata, N., Itoh, H., and Ohsato, K. (2000). Neutrophils express tissue factor in a monkey model of sepsis. *Surgery* **127,** 209–216.

Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1985). Thrombin stimulates the adherence of neutrophils to human endothelial cells *in vitro*. *J. Clin. Invest.* **76,** 2235–2246.