Augmentation of Aflatoxin B$_1$ Hepatotoxicity by Endotoxin: Involvement of Endothelium and the Coagulation System

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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, 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 x 10$^6$ EU/kg LPS was administered (iv). A time-dependent injury to SECs and parenchymal cells was observed in AFB$_1$/LPS-coated 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-coated 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.

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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 AFB1 on SECs. Kupffer cells and SECs treated in vitro with AFB1 are less sensitive to the damaging effects of AFB1 than are parenchymal cells (Jennings et al., 1994; Lafranconi et al., 1986). In addition, AFB1-treated nonparenchymal cells accumulate fewer AFB1-DNA adducts than parenchymal cells both in vitro and in vivo (Jennings et al., 1992; Schlemer et al., 1991). Whether SEC injury occurs in livers of animals coexposed to AFB1 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 AFB1 and LPS. Furthermore, the hypothesis that activation of the coagulation system is required for LPS to augment AFB1 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 AFB1 hepatotoxicity.

**MATERIALS AND METHODS**

**Materials.** AFB1 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 × 10⁸ 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 (Cr:CD (SD)ICS 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 AFB1, or its vehicle, 8% dimethylsulfoxide (DMSO) in sterile water, by intraperitoneal (ip) injection. Food was returned after AFB1 administration; 4 h later, animals were given 7.4 × 10⁵ EU/kg LPS or sterile saline by tail vein injection. At various times up to 24 h after AFB1 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 (infiniti-ALT, infiniti-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; Corge-nix Medical Corporation, Westminster, CO).

**Immunohistochemistry.** A 1-cm³ 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 antirat 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.
after AFB1/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 AFB1/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 to capture all images. Each digital image encompassed a total area of 1.4 mm² and contained several centrilobular and periportal regions. The circumference of the circle was approximately 4–5 hepatocytes away from the central vein or portal region. This area was measured as described above and divided by the total 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 AFB1/LPS cotreatment.** Development of hepatocellular injury after AFB1/LPS treatment was evaluated by increases in plasma ALT activity. Increases in plasma ALT activity after AFB1/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 AFB1 or LPS alone.

**Sinusoidal endothelial cell injury after AFB1/LPS cotreatment.** To determine if SECs were injured after AFB1/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 function.

**FIG. 1.** Hepatic parenchymal cell injury after AFB1/LPS cotreatment. Rats were treated with 1 mg/kg AFB1 (ip) or its vehicle, then 4 h later were given 7.4 × 10⁶ 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 AFB1 administration; 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 animals.

**FIG. 2.** Effect of AFB1/LPS cotreatment on plasma HA concentration. Rats were treated with 1 mg/kg AFB1 (ip) or its vehicle, then 4 h later were given 7.4 × 10⁶ EU/kg LPS or its vehicle (iv). Plasma HA was measured 6, 12, and 24 h after AFB1 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.
function and therefore have been used as a marker of SEC injury after toxic insult (Deaciuc et al., 1993, 1994). AFB1/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 AFB1 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 AFB1/LPS-cotreated animals (Fig. 3).

Quantitative morphometry confirmed that AFB1 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 AFB1/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 AFB1 or LPS compared to vehicle treatment (Fig. 5). However, in rats cotreated with AFB1/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. 3.** Effect of AFB1/LPS cotreatment on RECA-1 immunostaining. Rats were treated with 1 mg/kg AFB1 (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 AFB1, 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 AFB1/LPS-cotreated rats at (B) 6h, (C) 12 h, and (D) 24 h show a time-dependent decrease in RECA-1 staining intensity.
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 ×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 ×100; Fig. 8A) and zonal analysis (Fig. 8B) showed that heparin markedly attenuated fibrin deposition in livers of AFB1/LPS-treated animals.

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The effect of heparin on the manifestation of hepatic paren-
chymal cell injury was determined by measuring serum activities of ALT and AST twenty-four h after AFB1 administration (Fig. 9). Histopathologic analysis revealed that periportal hepatocellular necrosis and bile duct epithelial cell injury in AFB1/LPS-treated rats were pronounced and similar to lesions described previously (Barton et al., 2000b). Livers from AFB1/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 AFB1/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 AFB1 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 AFB1 administration. Heparin caused a modest (~30%), but statistically significant, attenuation of serum HA concentration after AFB1/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 AFB1/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 AFB1-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 AFB1/LPS cotreatment, as indicated by increases in plasma...
HA and decreased RECA-1 immunostaining (Fig. 2). Livers from animals cotreated with AFB1 and LPS showed a panlobular 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. Alternatively, 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 AFB1 are rendered more sensitive to the action of one or more inflammatory mediators produced in response to LPS exposure. LPS

FIG. 7. Quantitative morphometry of fibrin staining in livers 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 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 ×100) fields per liver, was analyzed morphometrically, as described in Materials and Methods. *Significantly different from veh/veh-treated rats; †significantly different from 12-h AFB1/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 AFB1/LPS-treated rats at the same time.

FIG. 8. Effect of heparin on liver fibrin staining after AFB1/LPS cotreatment. 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 (iv). Heparin (2000 U/kg, iv) or its vehicle was administered 1.5 h after LPS. Livers were removed 24 h after AFB1 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 ×100) per tissue. *Significantly different from respective veh/veh-treated rats. †Significantly different from AFB1/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 AFB1/LPS/veh-treated rats.
causes activation of inflammatory cells implicated in tissue injury. For example, Kupffer cells are important for manifestation of SEC injury after exposure to large, hepatotoxic doses of LPS (Deaciuc et al., 1994; Sarphie et al., 1996). Additionally, 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 AFB1 used did not result in significant changes in either plasma HA concentration or liver RECA-1 immunostaining. Nevertheless, nonparenchymal cells isolated from rats treated with a similar dose of AFB1 have been shown to contain AFB1-DNA adducts, albeit at a markedly lower level compared to parenchymal cells (Jennings et al., 1992). Thus, SECS may be primed by AFB1 to become more sensitive to insult from inflammatory products released from activated PMNs or from Kupffer cells stimulated by LPS.

PMNs accumulate early after AFB1/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 AFB1 (Barton et al., 2000a). This suggests that a second insult is required for PMN-dependent tissue injury in AFB1-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 sinuses, 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 cytotoxicity to parenchymal cells depends on cell-surface adhesion 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 directed against some of these adhesion molecules, including ICAM-1, have proven to be less effective in preventing hepatocellular necrosis (Farhood et al., 1995). This suggests that if the SEC barrier is severely damaged, the requirement for transmigration may be circumvented, allowing direct hepatocyte-PMN interaction and thereby facilitating PMN-induced hepatocellular injury (Jaeschke et al., 1996). Thus, SEC injury in AFB1/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 AFB1/LPS-cotreatment, coagulation 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 quantification confirmed significant fibrin staining in perportal and centrilobular zones of liver lobules, where SEC injury occurred. However, SEC injury by itself is not sufficient to cause parenchymal cell injury, since the latter was only observed in perportal regions, whereas SEC injury occurred in both perportal and centrilobular areas. Interestingly, centrilobular fibrin deposition was transient, clearing by 24 h, whereas fibrin deposits persisted in perportal regions. The persistence of fibrin deposits only in the perportal regions where hepatocellular injury localized suggests the possibility that persistent fibrin plays an important role in the progression to necrosis of hepatocytes homeostatically altered by AFB1 exposure. Alternatively, fibrin may persist in perportal regions as a result of parenchymal cell death.

**FIG. 9.** Effect of heparin on hepatic parenchymal cell injury after AFB1/LPS cotreatment. Rats were treated with 1 mg/kg AFB1 (ip) or its vehicle, then 4 h later with 7.4 × 10⁴ 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 AFB1 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 AFB1/LPS/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., 1996). Inasmuch as SEC injury and coagulation occurred after AFB<sub>1</sub>/LPS cotreatment, we examined whether coagulation system activation contributed to AFB<sub>1</sub>/LPS hepatotoxicity. Anticoagulation with heparin markedly attenuated hepatic parenchymal cell injury but had little effect on bile duct or SEC injury after AFB<sub>1</sub>/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<sub>1</sub>/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 perportal regions must occur in order to maintain flow and oxygen delivery to uninjured, downstream areas. Homeostatic alterations in perportal parenchymal cells by AFB<sub>1</sub> may render them more sensitive to hypoxic injury, whereas hepatocytes unaffected by AFB<sub>1</sub> 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<sub>1</sub> hepatotoxicity.

**FIG. 10.** Effect of heparin on biliary injury after AFB<sub>1</sub>/LPS cotreatment. Rats were treated with 1 mg/kg AFB<sub>1</sub> (ip) or its vehicle, then 4 h later with 7.4 × 10<sup>6</sup> 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<sub>1</sub>, 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.

**FIG. 11.** Effect of heparin on serum HA concentration after AFB<sub>1</sub>/LPS cotreatment. Rats were treated with 1 mg/kg AFB<sub>1</sub> (ip) or its vehicle, then 4 h later with 7.4 × 10<sup>6</sup> 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<sub>1</sub>, administration, using a commercially available ELISA. *Significantly different from Veh/Veh/Veh-treated animals. #Significantly different from AFB<sub>1</sub>/LPS/Veh-treated animals.
PMN-induced parenchymal cell injury (Barton et al., 2000a) and evidence of augmented thrombin activity (Fig. 7B) occur in periportal regions of livers in AFB₁/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 AFB₁/LPS model through upregulation of tissue factor and activation of the extrinsic coagulation pathway.

In summary, coexposure of rats to nontoxic doses of AFB₁ 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 AFB₁/LPS exposure, possibly through ischemia/hypoxia resulting from persistent fibrin clots, or from interaction of coagulation components with inflammatory cells such as PMNs.

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