The Coagulation System Contributes to Synergistic Liver Injury from Exposure to Monocrotaline and Bacterial Lipopolysaccharide

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Coexposure to a noninjurious dose of bacterial lipopolysaccharide (LPS; 7.4 × 10⁶ EU/kg) and a nontoxic dose of the food-borne toxin monocrotaline (MCT; 100 mg/kg) leads to synergistic hepatotoxicity in Sprague-Dawley rats. Inflammatory factors, such as Kupffer cells (KCs), tumor necrosis factor-α (TNF-α), and neutrophils (polymorphonuclear leukocytes; PMNs), are critical to the pathogenesis. Inasmuch as activation of the coagulation system and sinusoidal endothelial cell (SEC) injury precede hepatic parenchymal cell (HPC) injury, and since fibrin deposition occurs within liver lesions, the coagulation system might be a critical component of injury. In this study, this hypothesis is tested, and the interdependence of the coagulation system and inflammatory factors is explored. Administration of the anticoagulants heparin or warfarin to MCT/LPS-cotreated animals attenuated HPC and SEC injury. Morphometric analysis revealed that anticoagulant treatment significantly reduced the area of centrilobular and midzonal lesions. Heparin treatment also reduced fibrin deposition in these regions. Furthermore, anticoagulant treatment decreased hepatic PMN accumulation but did not affect plasma TNF-α concentration. Neither KC inactivation nor TNF-α depletion prevented activation of the coagulation system. PMN depletion, however, prevented coagulation system activation, suggesting that PMNs are needed for this response. These results provide evidence that the coagulation system and its interplay with PMNs are important in the pathogenesis of MCT/LPS-induced liver injury.

Key Words: liver; inflammation; lipopolysaccharide; monocrotaline; coagulation system; fibrin deposition; heparin; warfarin; MCP-1.

Bacterial lipopolysaccharide (LPS) is an integral constituent of the outer cell wall of Gram-negative bacteria, where it acts as a permeability barrier against hydrophobic molecules and is important for bacterial growth and viability (Rietschel and Brade, 1992; Vaara, 1999). LPS is released from proliferating bacteria or during lysis of their cell walls (Rietschel and Brade, 1992). In mammals, it can elicit a potent inflammatory response (Hewett and Roth, 1993). Under conditions of systemic exposure to large doses of LPS, detrimental pathophysiological changes can result. These life-threatening changes include systemic hypotension, circulatory shock, disseminated intravascular coagulation, and multiple organ failure (Hewett and Roth, 1993; Holst et al., 1996). In rats, an acutely toxic dose of LPS results in liver injury that consists of midzonal (MZ) hepatocellular degeneration and coagulative necrosis with neutrophilic infiltrate and minimal hemorrhage (Hewett et al., 1992; Yee et al., 2000). The mechanism for this injury is complex and involves the interaction of numerous inflammatory cells and soluble mediators (Arthur et al., 1988; Brouwer et al., 1995; Brown et al., 1997; Hewett et al., 1992, 1993; Luster et al., 1994; Moulin et al., 1996).

Mild exposure to LPS is commonplace and episodic, varying with the lifestyle and health of an individual. Systemic LPS concentration can be enhanced during bacterial infection and by increased translocation of LPS from indigenous Gram-negative bacteria in the gastrointestinal tract into the portal circulation (Fink and Mythen, 1999; Ganey and Roth, 2001). A variety of conditions, including disease, dietary alterations, trauma to the gastrointestinal tract, and alcohol consumption, can increase this translocation (reviewed in Roth et al., 1997). Although such episodic exposure to smaller amounts of LPS is insufficient to cause tissue injury, a modest inflammatory response may result. This response includes the accumulation of neutrophils (polymorphonuclear leukocytes; PMNs) and the production of tumor necrosis factor-α (TNF-α) and other inflammatory mediators that have the potential to alter hepatocellular homeostasis (Michie et al., 1988; Spitzer and Mayer, 1993). This may cause tissues to become more susceptible to chemically induced injury. Indeed, LPS exposure augments the toxicity of a number of hepatotoxins (reviewed in Ganey and Roth, 2001).

Monocrotaline (MCT) is a pyrrolizidine alkaloid phytotoxin found in numerous plants of the Crotalaria genus worldwide (Mattocks, 1986; Stegelmeier et al., 1999). It is a well-known hepatotoxicant (Mattocks, 1986). Humans are exposed to this toxin through the accidental consumption of contaminated foodstuffs and the intentional ingestion of MCT and related
induced liver injury and to explore the interdependence between the coagulation system and inflammatory factors that are critical for the toxic response.

MATERIALS AND METHODS

Chemicals. Dimethyl sulfoxide (DMSO), heparin (Type II, disodium salt; HEP), isopentane, LPS (Escherichia coli, serotype 0128:B12, 1.7 × 10^6 endotoxin units [EU]/mg), pentoxifylline (PTX), phosphate buffered saline (PBS), sodium citrate, and warfarin (3-α-acetylbenzyl)-4-hydroxycoumarin; WARF) were purchased from Sigma Chemical Company (St. Louis, MO). Gadolinium chloride-6H_2O (GdCl_3) was purchased from Aldrich Chemical Company (St. Louis, MO). Serum directed against TNF-α (anti-TNF-α serum; ATS) was produced in New Zealand White rabbits (Hewett et al., 1993). Rabbit anti-rat neutrophil antibody (neutrophil anti-serum [NAS]) and control rabbit serum (CS) were obtained from Inter-Cell Technologies, Inc. (Hopewell, NJ). Goat anti-rat fibrinogen was purchased from ICN Pharmaceuticals (Aurora, OH). Horse serum was obtained from Vector Laboratories (Burlingame, CA). Donkey anti-goat secondary antibody conjugated to Alexa 594 was purchased from Molecular Probes (Eugene, OR). MCT was acquired from Trans World Chemicals (Rockville, MD). Sterile saline was acquired from Abbott Laboratories (North Chicago, IL). Formalin fixative was obtained from Surgipath Medical Industries, Inc. (Richmond, IL). Diagnostic kits 58 UV and 59 UV for the determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, respectively, and diagnostic kit 886-A for determination of fibrinogen concentration were also purchased from Sigma Chemical Company (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kit for hyaluronic acid (HA) was acquired from Corgenix, Inc. (Westminster, CO). ELISA kits for rat TNF-α and rat monocyte chemotractant protein-1 (MCP-1) were purchased from Biosource International, Inc. (Camarillo, CA). ELISA kit for rat growth-related oncogene (GRO)/cytokine-induced neutrophil chemotactant-1 (CINC-1) was obtained from Assay Designs, Inc. (Ann Arbor, MI).

Animals. Male Sprague-Dawley rats (Crl:CD (SD)IGS BR, Charles River, Portage, MI) weighing 200–300 g were used for all studies. Animals were allowed food (Rodent Chow/Tek 8640, Harlan Teklad, Madison, WI) and water ad libitum. They were housed no more than three to a cage on Aspen chip bedding (Northeastern Products Company, Warrenburg, NY) and were maintained on a 12-h light/dark cycle in a controlled temperature (18–21°C) and humidity (55 ± 5%) environment for a period of 1 week before use. All procedures on animals followed the guidelines for humane treatment set by the American Association of Laboratory Animal Sciences and the University Laboratory Animal Research Unit at Michigan State University.

Treatment protocol. MCT was dissolved in sterile saline minimally acidified by 0.2 M HCl. The pH was brought to 7 by addition of 2 M NaOH, and the volume was adjusted with sterile saline to the appropriate final concentration. Rats were given MCT (100 mg/kg) or an equivalent volume of sterile saline vehicle (Veh), intraperitoneally, followed 4 h later by LP (7.4 × 10^6 EU/kg) or saline Veh via tail vein injection. LPS was administered 4 h after MCT to minimize interference with MCT bioactivation (Allen et al., 1972).

Treatment with HEP. Rats were given HEP (2000 U/kg) or saline Veh intravenously 1.5 h after LPS administration. This treatment has been shown to inactivate the coagulation system and prevent injury from a hepatotoxic dose of LPS (Moulin et al., 1996). Rats were killed and liver injury was assessed 18 h after MCT treatment.

Treatment with WARF. Rats were pretreated with WARF (7.5 mg/kg) or an equivalent volume of DMSO Veh intraperitoneally 34 and 10 h before MCT administration. This treatment has been shown to inactivate the coagulation system (Copple et al., 2002b). Rats were killed and liver injury was assessed 18 h after MCT treatment.

Assessment of hepatic injury and plasma TNF-α and MCP-1 concentrations. At 6, 12, or 18 h after MCT administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). A midline abdominal incision was made,
blood was collected from the inferior vena cava into a syringe containing sodium citrate (0.38% final concentration), and animals were euthanized by exsanguination. HPC injury was evaluated by increases in the activities of ALT and AST in plasma. An ELISA kit was used to measure plasma HA concentration, a marker of hepatic SEC injury. Plasma TNF-α and MCP-1 concentrations were determined with a rat TNF-α and a rat MCP-1 ELISA kit, respectively.

Assessment of plasma fibrinogen concentration and CINC-1. Plasma fibrinogen concentration was evaluated with a BBL fibrometer (Becton, Dickinson and Company, Hunt Valley, MD) and a fibrinogen diagnostic kit. Plasma fibrinogen concentration was determined in the HEF and WARP studies with MCT/LPS-cotreated animals, as well as in cotreated animals that underwent KC inactivation or TNF-α or PMN depletion. In the KC inactivation study, GdCl3 (10 mg/kg) was administered to rats 24 h before LPS administration. In the TNF-α depletion studies, either PTX (100 mg/kg) or ATS (1 ml/rat) was administered 1 h before LPS administration. In the PMN depletion study, animals were pretreated with NAS 24 (1 ml/rat) and 8 (0.5 ml/rat) hours before LPS administration. These treatment regimens were effective in preventing KC activation, the LPS-induced increase in plasma TNF-α activity and hepatic PMN accumulation, respectively (Yee et al., 2003a,b). Administration of GdCl3, PTX, ATS, or NAS did not interfere with MCT bioactivation (Yee et al., 2003a,b). Plasma CINC-1 was measured using a rat GRO/CINC-1 ELISA kit.

Histopathologic evaluation and morphometry. Livers were fixed by immersion in 10% neutral buffered formalin for at least 3 days before being processed for histologic analysis. In addition, a portion (1 cm²) of the liver from the middle of the left lateral lobe was frozen in isopentane immersed in liquid nitrogen for immunohistochemical staining. Serial transverse sections from the left lateral liver lobe were processed for light microscopy. Paraffin-embedded sections were cut at 5 μm, stained with hematoxylin and eosin, and evaluated for lesion size and severity. Slides were coded, randomized, and evaluated by light microscopy.

Digitized color images of hematoxylin and eosin-stained liver sections were visualized with an Olympus AX-80T light microscope (Olympus Corp., Lake Success, NY) interfaced with a high-resolution CCD color camera (OLY-750, Olympus-America, Inc., Melville, NY) to quantify treatment-induced changes in liver morphology. Images were evaluated with Scion Image software (Scion Corporation, Frederick, MD) employing a 64-point lattice grid to determine (1) the total area of liver analyzed, (2) the area of CL lesion, (3) the area of MZ lesion, (4) the area of normal parenchyma, and (5) the area of nonparenchymal space. A lesion was defined as hepatic parenchymal cells with either swollen, eosinophilic cytoplasm and karyolytic or pyknotic nuclei (i.e., oncosis), or cells with shrunken cytoplasm and karyorrhexis nuclei or apoptotic bodies (i.e., apoptosis; Levin et al., 1999; Majno and Joris, 1995). Nonparenchymal space was defined as nonparenchymal tissue, vessel lumen, and regions outside the perimeter of the liver section. The area of each object (category) of interest (i.e., lesion) was calculated from the following expression (Cruz-Orive, 1982):

\[
\text{Area}_{\text{Interest}} = \sum \text{Points}_{\text{Interest}} \times \frac{\text{Area}_{\text{Point}}}{(\text{Distance between Points})^2}
\]

Distance between points was 55 μm. Accordingly, the area represented by each point was 3025 μm². One section from the liver of each animal in a treatment group was systematically scanned using adjacent, non-overlapping microscopic fields. The first image field analyzed in each section was chosen using a random number table (i.e., any image field between 1 and 10). Thereafter, every 10th field containing hepatic parenchymal cells was evaluated (minimum of 20 fields measured/section). The measured fields represented approximately 10% of the total area of each liver section. Percent lesion area was estimated based on the following formula:

\[
\left(\frac{\text{Area}_{\text{lesion of Interest}} \times \text{Area}_{\text{All Lesions}} + \text{Area}_{\text{Parenchyma}}}{\text{Area}_{\text{Point}}}ight) \times 100.
\]

Enumeration of hepatic neutrophils. Paraffin-embedded liver tissue (three serial liver sections per slide) was cut into 6 μm-thick slices. Paraffin was removed from the liver tissues with xylene before staining. PMNs within liver sections were stained with a rabbit anti-PMN Ig isolated from serum of rabbits immunized with rat PMNs as described by Hewett et al. (1992). After incubation with the primary antibody, tissue sections were incubated with biotinylated goat anti-rabbit IgG, avidin-conjugated alkaline phosphatase, and Vector Red substrate to stain PMNs. Hepatic PMN accumulation was assessed by averaging the numbers of PMNs enumerated in 30 randomly selected, high power fields (HPFs; ×400) in each slide (i.e., 10 HPFs per liver section). The analyzed fields represented between 5 and 10% of the total area of each liver section. Analyzed fields were selected in an unbiased manner to cover the entire liver section. PMNs were identified by positive staining and cell morphology.

Immunohistochemistry. Sections of frozen tissue (8 μm-thick) were fixed in 10% buffered formalin containing 2% acetic acid for 30 min at room temperature. This fixation protocol solubilizes all fibrinogen and fibrin species except for cross-linked fibrin. Hence, only cross-linked fibrin is stained in liver sections (Schmitt et al., 1993). Sections were incubated for 30 minutes with PBS containing 10% horse serum (i.e., blocking solution) and then with goat anti-rat fibrinogen (diluted 1:1000) in blocking solution overnight at 4°C. Next, the sections were incubated for 3 h in blocking solution with donkey anti-goat secondary antibody conjugated to Alexa 594 (1:1000). Sections were washed three times for 5 min each with PBS and visualized using fluorescence microscopy. No staining was observed in the controls in which the primary or secondary antibody was omitted from the staining protocol. All treatment groups compared morphologically were stained at the same time and evaluated on the same day.

Quantification of hepatic fibrin deposition. Fibrin deposition in the liver was quantified by morphometrically analyzing the area of immunohistochemical staining for each liver section (Coppel et al., 2002a). Fluorescent staining of liver sections was visualized using an Olympus AX-80T microscope (Olympus, Lake Success, NY). For morphometric analysis of fibrin deposition in a liver section, digital images of 5 randomly chosen ×100 fields per tissue section were captured using a SPOT II camera and SPOT Advanced Software (Diagnostic Instruments, Sterling Heights, MD). Samples were coded so that the evaluator was not aware of the treatment, and the same exposure time was used for all captured images. Each digital image encompassed a total area of 1.4 mm² and contained several CL, MZ, and periportal (PP) regions. The area of immunohistochemical staining (number of pixels) within the CL, MZ, and PP regions was quantified using Scion Image software (Scion Corporation, Frederick, MD). For fibrin deposition quantification, a density slice from an inverted, gray-scale digital image of a liver section was used for analysis. A density slice allows analysis of pixels in a defined range of gray values (i.e., densities). The threshold was selected so that little positive staining was present in Veh/Veh/Veh-treated controls. The same threshold value was used to analyze digital images from all treatment groups. The area of positive staining was measured and divided by the total area of the image. Analysis of fibrin deposition in the CL and PP areas was conducted by drawing a 145 μm-diameter circle around the central vein or portal region. The circumference of the circle is about 4~6 hepatocytes away from the central vein or vessels of the portal triad, and this area was arbitrarily defined as the CL and PP regions, respectively. The MZ region was defined as the center of area between the CL and PP regions using the same circle circumference, without having overlap of these arbitrary circles. The area of the circle was 16,512 μm². The area of fibrin staining in each region was measured as described above and divided by the total area of the image. Results from the random fields analyzed for each liver section were averaged and counted as a replicate (i.e., each replicate representing a different rat).

Statistical analysis. Results are expressed as mean ± SEM. When variances were not homogeneous, data were log-transformed before analysis. Data expressed as percentages were transformed by arc sine square root prior to analysis. Data for single comparisons were analyzed by Student’s t-test or, when appropriate, Fisher’s exact test (Steele et al., 1997). Multiple comparisons of homogeneous data were analyzed by one-way or two-way analysis of variance (ANOVA), as appropriate, and group means were compared using
Tukey’s omega post hoc test (Steele et al., 1997). The criterion for significance was \( p \leq 0.05 \) for all comparisons.

**RESULTS**

**Anticoagulant Treatment of MCT/LPS-Coexposed Animals**

Two anticoagulants, HEP and WARF, were used to investigate the role of the coagulation system in MCT/LPS-induced liver injury. These anticoagulants inhibit the thrombin-catalyzed conversion of circulating fibrinogen to fibrin by different mechanisms (Hewett and Roth, 1995). HEP enhances the binding of antithrombin III to thrombin to inhibit thrombin activity. In contrast, WARF inhibits the formation of vitamin K-dependent coagulation factors (i.e., thrombin precursors; Majerus et al., 1996). During activation of the coagulation system, plasma fibrinogen is converted to fibrin resulting in a decrease in plasma fibrinogen concentration (Copple et al., 2002a), and this decrease was used as a biomarker for activation of the coagulation system and as a monitor of anticoagulant effectiveness.

Plasma fibrinogen concentration was significantly decreased in rats treated with MCT/LPS. Both HEP and WARF prevented the activation of the coagulation system in MCT/LPS-coexposed animals (Figs. 1A and 1B, respectively), confirming their effectiveness.

**Effect of HEP on MCT/LPS-Induced Liver Injury**

Plasma ALT and AST activities, two biomarkers of HPC injury, were elevated in MCT/LPS-cotreated animals 18 h after MCT administration (Figs. 2A and 2B, respectively). HEP treatment attenuated the increase in both of these by approximately 80%. Hepatic SECs remove HA from the circulation, and impairment of this function results in enhanced HA concentration in the plasma. Accordingly, plasma HA concentration has been used as a biomarker for hepatic SEC injury (Copple et al., 2002a; Deaciuc et al., 1993, 1994). In MCT/LPS-cotreated animals, plasma HA concentration was elevated (Fig. 2C), and HEP treatment modestly reduced the elevation (48% decrease).

**Effect of WARF on MCT/LPS-Induced Liver Injury**

WARF treatment significantly attenuated the increase in plasma ALT and AST activities caused by MCT/LPS coadministration by 89% and 75%, respectively (Figs. 3A,B). The increase in plasma HA concentration was also partially attenuated (42% decrease) with WARF treatment (Fig. 3C).

**Mortality**

Mortality was 13% in animals given MCT/LPS/Veh and 7% in MCT/LPS-cotreated animals that received HEP treatment. Mortality was 11% in Veh/MCT/LPS-cotreated animals and 27% in animals given WARF/MCT/LPS coadministration. No animals treated with Veh or anticoagulant alone died. Anticoagulant administration did not significantly affect survival in MCT/LPS-cotreated animals.

**Effect of Anticoagulants on MCT/LPS-Induced Liver Lesions**

MCT/LPS-cotreated control animals exhibited MCT-like, CL and LPS-like, MZ liver lesions as previously described by Yee et al. (2000). CL lesions consisted of moderate hepatoacellular degeneration and apoptotic and oncocytic necrosis, hemorrhage, and loss of central vein intima. CL lesions also exhibited a moderate accumulation of PMNs and monocytes. MZ lesions comprised marked and more frequent, well-
defined areas of hepatocellular coagulative necrosis accompanied by PMN and mononuclear cell infiltration. Pronounced congestion and hemorrhage were also present in these lesions. Livers from MCT/LPS-coexposed animals given either anticoagulant exhibited qualitatively similar CL and MZ lesions; however, both lesion types were smaller and considerably less frequent. The MZ lesions had a slightly greater reduction in size and frequency than the CL lesions (Table 1). Significant decreases in the areas of CL (77% decrease) and MZ (87% decrease) lesions were found in the livers of MCT/LPS-co-treated animals that were treated with HEP. Similar decreases were found in CL (62%) and MZ (85%) lesions in livers from MCT/LPS-coexposed animals given WARF. No histologic evidence of injury was observed in animals given Veh or anticoagulant alone.

**Fibrin Staining in Livers from MCT/LPS-Coexposed Animals**

To demonstrate that HEP treatment of MCT/LPS-coexposed animals decreased insoluble fibrin clots in the liver, hepatic fibrin deposition was examined immunohistochemically (Copple *et al.*, 2002a). No fibrin immunostaining was observed in the sinusoids of liver sections from Veh/Veh/Veh-treated rats, although some staining occurred in the intima of larger vessels. This staining may have been the result of fibrin deposition that occurred after animal sacrifice (Copple *et al.*, 2002a). By 18 h, significant fibrin staining was observed in the livers from the MCT/LPS/Veh-cotreated animals (Fig. 4A). HEP treatment of MCT/LPS-coexposed animals returned hepatic fibrin staining to control (Veh/Veh/Veh-treated) levels. Zonal analysis revealed prevention of fibrin immunostaining in the CL and MZ regions of MCT/LPS-coexposed animals treated with HEP (Fig. 4B).

**Plasma TNF-α Concentration after Anticoagulant Administration in MCT/LPS-Coexposed Animals**

The proinflammatory cytokine TNF-α is important in the pathogenesis of MCT/LPS-induced liver injury (Yee *et al.*, 2003a). To explore the relationship between plasma TNF-α and the coagulation system in this model, plasma TNF-α concentration was assessed after administration of anticoagulant to MCT/LPS-cotreated animals. Neither HEP (Fig. 5A) nor WARF treatment (Fig. 5B) had an effect on plasma TNF-α concentration.

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**FIG. 2.** Effect of HEP on MCT/LPS-induced liver injury. MCT/LPS-coexposed rats were treated with HEP anticoagulant as described in Figure 1 legend. ALT (A) and AST (B) activities, plasma biomarkers for HPC injury, and plasma HA concentration (C), a biomarker for SEC injury, were evaluated 18 h after MCT or its Veh. *n* = 5–14 animals. aSignificantly different from respective value in the absence of MCT/LPS. bSignificantly different from MCT/LPS/Veh group.
PMNs are an important component in MCT/LPS-induced liver injury (Yee et al., 2003b). To examine the relationship between PMNs and the coagulation system in this model, hepatic PMN accumulation was assessed in MCT/LPS-co-treated animals given anticoagulants. Treatment with either HEP (Fig. 6A) or WARF (Fig. 6B) attenuated hepatic PMN accumulation by approximately 60%.

Plasma MCP-1 Concentration in MCT/LPS-Cotreated Animals

The chemokine MCP-1 causes monocyte attraction and activation (Gu et al., 1999; Luster, 1998), and evidence in vitro suggests that this chemokine may be involved in increasing expression of intercellular adhesion molecule-1 on rat endothelial cells (Yamaguchi et al., 1998). Activated monocytes express TF, which can cause coagulation system activation (Osterud, 1995; Polack et al., 1997). Plasma MCP-1 concentration was assessed at various times after MCT/LPS-cotreatment (Table 2). MCT administration caused no change at any of the times evaluated. By contrast, an elevation in plasma MCP-1 concentration occurred 2 h after LPS administration, irrespective of MCT treatment. Plasma MCP-1 remained elevated in Veh/LPS-cotreated animals at all times examined. By 12 h after MCT administration, plasma MCP-1 was nearly twice as great in MCT/LPS-cotreated animals compared to 18 h after MCT or its Veh. n = 4–8 animals. *Significantly different from respective value in the absence of anticoagulant treatment.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Centrilobular</th>
<th>Midzonal</th>
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<tbody>
<tr>
<td>HEP study</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCT/LPS/Veh</td>
<td>3.9 ± 0.7</td>
<td>7.7 ± 1.6</td>
</tr>
<tr>
<td>MCT/LPS/HEP</td>
<td>0.9 ± 0.2 *</td>
<td>1.0 ± 0.3 *</td>
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<tr>
<td>WARF study</td>
<td></td>
<td></td>
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<tr>
<td>Veh/MCT/LPS</td>
<td>4.2 ± 0.9</td>
<td>7.4 ± 0.8</td>
</tr>
<tr>
<td>WARF/MCT/LPS</td>
<td>1.6 ± 0.6 *</td>
<td>1.1 ± 0.3 *</td>
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*Significantly different from respective value in the absence of anticoagulant treatment.

*WARF or Vehicle (Veh) was administered 10 and 34 h before MCT administration, whereas, in a separate study, HEP or Veh was administered 1.5 h after LPS administration. LPS (7.4 × 10^6 EU/kg) or Veh was administered i.v. to rats 4 h after ip administration of MCT (100 mg/kg) or Veh. Livers were taken 18 h after MCT administration and processed for morphometric analysis. n = 6–7 animals.
animals that received LPS alone, and this relationship remained through 18 h.

Coagulation System Activation in Animals Depleted of KCs, TNF-α, or PMNs

Yee et al. (2003a,b) demonstrated that KC depletion by GdCl₃, attenuation of the TNF-α response by either PTX or ATS, or PMN depletion by NAS diminished HPC and SEC injury in MCT/LPS-cotreated rats. To explore the relationship between these inflammatory components and the coagulation system, plasma fibrinogen concentration was assessed (Table 3). Neither KC inactivation nor attenuation of TNF-α response affected activation of the coagulation system. However, PMN depletion prevented the decrease in plasma fibrinogen in MCT/LPS-cotreated animals at 6 (data not shown) and 18 h after MCT administration.

Plasma Chemokine Concentration in Animals after Inactivation of KCs, TNF-α, PMNs, or the Coagulation System

The changes observed in this study with MCP-1 (Table 2) were similar to those of the PMN chemoattractant CINC-1, which was elevated in plasma at 6 h in animals that received LPS treatment, irrespective of MCT coadministration (Yee et
Plasma CINC-1 concentration declined thereafter, but it did so at a much slower rate in MCT/LPS-cotreated animals than in Veh/LPS animals, such that by 18 h plasma CINC-1 concentration was five times greater in MCT/LPS-cotreated animals than in animals treated with LPS alone. To examine the interplay between inflammatory factors and chemokines, plasma concentrations of CINC-1 and MCP-1 were evaluated in MCT/LPS-cotreated animals after treatment with various anti-inflammatory pharmacologic agents. Neither GdCl3 nor NAS treatment diminished plasma CINC-1 measured at 18 h after MCT/LPS-cotreatment (data not shown). However, plasma CINC-1 concentration was attenuated by treatment with ATS or WARF (Table 4). A similar decrease was observed with HEP treatment in MCT/LPS-coexposed animals (data not shown). None of these pharmacologic treatments affected MCP-1 concentration at 18 h (data not shown).

**DISCUSSION**

This investigation tested the hypothesis that the coagulation system plays a causal role in the development of MCT/LPS-induced liver injury and explored the interdependence between the coagulation system and critical inflammatory factors. Anticoagulant therapy involved either HEP or WARF administration. Although HEP is well known for anticoagulant activity, it has anti-inflammatory effects as well. These effects include inhibition of PMN adhesion, chemotaxis, and production of superoxide anion and nitric oxide (Beltran et al., 1999; Matzner et al., 1984; Riesenberg et al., 1995; Shin et al., 1997; Silvestro et al., 1994). Because PMNs are critical to the pathogenesis of MCT/LPS-induced liver injury (Yee et al., 2003b), HEP could have effects independent of its anticoagulant activity. Consequently, WARF, a drug that prevents coagulation by a different mechanism, was also used in this investigation.

Either HEP (Fig. 1A) or WARF (Fig. 1B) treatment of MCT/LPS-cotreated animals prevented the activation of the coagulation system, confirming the effectiveness of these agents. Anticoagulant treatment caused a marked decrease in HPC injury but only a modest decrease in SEC injury 18 h after MCT administration (Figs. 2 and 3). This suggests that the

![FIG. 6. Effect of anticoagulants on PMN accumulation in MCT/LPS-cotreated animals. MCT/LPS-coexposed rats were treated with HEP (A) or WARF (B) anticoagulants as described in Figure 1 legend. Hepatic PMN accumulation (i.e., number of PMNs per high powered field (HPF; 400X) was determined in MCT/LPS treated animals 18 h after MCT or its Veh were administered. n = 4–14 animals. *Significantly different from respective value in the absence of MCT/LPS. †Significantly different from respective MCT/LPS value in the absence of anticoagulant treatment.](image)

<table>
<thead>
<tr>
<th>Time after MCT or MCT vehicle</th>
<th>Plasma MCP-1 concentration (pg/ml)</th>
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<tbody>
<tr>
<td>6 h</td>
<td></td>
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<tr>
<td>Veh/Veh</td>
<td>32 ± 7</td>
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<tr>
<td>Veh/LPS</td>
<td>934 ± 108†</td>
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<tr>
<td>MCT/Veh</td>
<td>32 ± 6</td>
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<tr>
<td>MCT/LPS</td>
<td>894 ± 34‡</td>
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<tr>
<td>12 h</td>
<td></td>
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<tr>
<td>Veh/Veh</td>
<td>32 ± 6</td>
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<tr>
<td>Veh/LPS</td>
<td>941 ± 113‡</td>
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<tr>
<td>MCT/Veh</td>
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<tr>
<td>MCT/LPS</td>
<td>1552 ± 80‡</td>
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<tr>
<td>18 h</td>
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<tr>
<td>Veh/Veh</td>
<td>38 ± 15</td>
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<tr>
<td>Veh/LPS</td>
<td>798 ± 150‡</td>
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<tr>
<td>MCT/Veh</td>
<td>40 ± 12</td>
</tr>
<tr>
<td>MCT/LPS</td>
<td>1462 ± 137‡</td>
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*Significantly different from respective values in the absence of LPS at the indicated time.
†Significantly different from all other groups at the indicated time.
‡Rats were treated with MCT and/or LPS as described in Table 1 footnote.

n = 3–6 animals.
coagulation system only partially contributes to SEC injury and that additional factors are likely involved. Zonal analysis of liver sections revealed that anticoagulant treatment significantly reduced the areas of CL and MZ lesions (Table 1). Accordingly, the coagulation system appears to contribute causally to MCT/LPS-induced liver injury.

MCT is nontoxic and must be bioactivated by cytochromes of the P450 3A family to its toxic metabolite, monocrotaline pyrrole (MCTP), in order to produce liver injury (Kasahara et al., 1997; Stegelmeier et al., 1999; White and Mattocks, 1972). This conversion happens rapidly, with peak MCTP production occurring within 2 h after MCT exposure (Allen et al., 1972). Thus, HEP was given to MCT/LPS-cotreated animals after the bioactivation of MCT to MCTP was nearly complete (i.e., 5.5 h after MCT). A recent study by Copple et al. (2002b) demonstrated that WARF does not interfere with MCT bioactivation. Therefore, it is unlikely that either anticoagulant reduced injury by interfering with MCT metabolism.

There are a variety of mechanisms by which the coagulation system might contribute to the liver injury of this model. Fujiwara et al. (1988) and others (Aria et al., 1996; Ba et al., 2000; Copple et al., 2002a; DeLeve et al., 1996; Saetre et al., 2000) have postulated that fibrin clots in the liver cause local hypoperfusion and can thereby result in cellular injury. Consistent with this hypothesis, HEP substantially reduced fibrin deposition in CL and MZ regions of liver lobules (Fig. 4B) from MCT/LPS-cotreated rats. Hence, it is possible that the reduction in hepatic fibrin deposition was responsible for the decrease in liver injury. However, in liver injury caused by a hepatotoxic dose of LPS, thrombin appears to be the critical factor, acting independently of fibrin clot formation (Hewett and Roth, 1995; Moulin et al., 1996, 2001). Thrombin can contribute to liver injury by causing the stimulation and aggregation of platelets (Kito et al., 1985; Shuman, 1986; Sinha et al., 1983; Wise et al., 1980), by inducing PMN chemotaxis (Bizios et al., 1986) and/or by enhancing MCP-1 release from liver stellate cells, monocytes, and endothelial cells (Colotta et al., 1994; Marra et al., 1995). Moreover, thrombin’s action on a protease-activated receptor present on KCs and SECs in liver might contribute to HPC injury, as it does after a large, hepatotoxic dose of LPS (Copple et al., 2003; Moulin et al., 2001). Indeed, activation of protease-activated receptor-1 by thrombin results in the release of various cytokines and growth factors, as well as the expression of adhesion molecules on endothelium that could contribute to inflammatory liver injury (Derian et al., 2002). In MCT/LPS-cotreated animals, further study will be needed to define fully the mechanism by which an activated coagulation system promotes hepatotoxicity.

The relationship between the coagulation system and other inflammatory factors was explored. Although neither KC inactivation nor TNF-α depletion prevented activation of the coagulation system, PMN depletion did (Table 3). This suggests that PMNs promote coagulation system activation in this model. PMNs can affect the coagulation system through a

### TABLE 3
Treatment with NAS but Not with GdCl₃, PTX or ATS Prevents Coagulation System Activation in MCT/LPS-Co-treated Animals

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Plasma fibrinogen concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdCl₃ Study</td>
<td></td>
</tr>
<tr>
<td>Veh/Veh/Veh</td>
<td>257 ± 19</td>
</tr>
<tr>
<td>GdCl₃/Veh/Veh</td>
<td>247 ± 17</td>
</tr>
<tr>
<td>Veh/MCT/LPS</td>
<td>115 ± 12</td>
</tr>
<tr>
<td>GdCl₃/MCT/LPS</td>
<td>129 ± 7*</td>
</tr>
<tr>
<td>PTX Study</td>
<td></td>
</tr>
<tr>
<td>Veh/Veh/Veh</td>
<td>234 ± 7</td>
</tr>
<tr>
<td>Veh/PTX/Veh</td>
<td>227 ± 12</td>
</tr>
<tr>
<td>MCT/Veh/LPS</td>
<td>72 ± 10*</td>
</tr>
<tr>
<td>MCT/PTX/LPS</td>
<td>109 ± 22*</td>
</tr>
<tr>
<td>ATs Study</td>
<td></td>
</tr>
<tr>
<td>Veh/CS/Veh</td>
<td>325 ± 25</td>
</tr>
<tr>
<td>Veh/ATS/Veh</td>
<td>324 ± 26</td>
</tr>
<tr>
<td>MCT/CS/LPS</td>
<td>77 ± 10*</td>
</tr>
<tr>
<td>MCT/ATS/LPS</td>
<td>119 ± 14*</td>
</tr>
<tr>
<td>NAS Study</td>
<td></td>
</tr>
<tr>
<td>CS/Veh/Veh</td>
<td>301 ± 7</td>
</tr>
<tr>
<td>NAS/Veh/Veh</td>
<td>310 ± 28</td>
</tr>
<tr>
<td>CS/MCT/LPS</td>
<td>101 ± 10*</td>
</tr>
<tr>
<td>NAS/MCT/LPS</td>
<td>336 ± 49*</td>
</tr>
</tbody>
</table>

*Significantly different from respective value in the absence of MCT/LPS.  
**Significantly different from all other groups.

*Rats were treated with MCT and/or LPS as described in Table 1 footnote. In addition, rats were given GdCl₃ (Kupffer cell inactivator), PTX (TNF-α synthesis inhibitor), ATS (TNF-α depleter) or NAS (PMN depletor) or their Vehs as described in Materials and Methods. Plasma fibrinogen concentration, a biomarker of coagulation system activation, was determined 18 h after MCT or its Veh administration. n = 3–13 animals.

### TABLE 4
Treatment with ATS or WARF Attenuates Plasma CINC-1 in MCT/LPS-Co-treated Animals

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Plasma CINC-1 concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATS Study</td>
<td></td>
</tr>
<tr>
<td>Veh/CS/Veh</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Veh/ATS/Veh</td>
<td>58 ± 8*</td>
</tr>
<tr>
<td>MCT/CS/LPS</td>
<td>2899 ± 1263*</td>
</tr>
<tr>
<td>MCT/ATS/LPS</td>
<td>329 ± 42*</td>
</tr>
<tr>
<td>WARF Study</td>
<td></td>
</tr>
<tr>
<td>Veh/Veh/Veh</td>
<td>34 ± 15</td>
</tr>
<tr>
<td>WARF/Veh/Veh</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>Veh/MCT/LPS</td>
<td>3296 ± 94*</td>
</tr>
<tr>
<td>WARF/MCT/LPS</td>
<td>2002 ± 800*</td>
</tr>
</tbody>
</table>

*Significantly different from respective value in the absence of MCT/LPS.  
**Significantly different from all other groups.

*Rats were treated with MCT and/or LPS as described in Table 1 footnote. In addition, rats were given ATS (TNF-α neutralizer) or WARF (anticoagulant) or their Vehs as described in Materials and Methods. Plasma CINC-1 concentration, a PMN chemokine, was measured 18 h after administration of MCT or its Veh. n = 3–8 animals.
number of mechanisms, including the expression of TF on their surfaces (Todoroki et al., 2000) and the release of cathepsin G (Bray et al., 1987). TF activates the coagulation system, leading to thrombin formation (Osterud and Rapaport, 1977; Schultz and Roth, 1998). In addition to its cytotoxic protease activity (Ho et al., 1996), cathepsin G activates Factor X, which converts prothrombin to thrombin (Bray et al., 1987; Goel and Diamond, 2001; Plescia and Altieri, 1996). Hence, the critical role of PMNs may derive both from their ability to release cytotoxic mediators and their participation in activating the coagulation system.

Although PMNs appear necessary for activation of the coagulation system in this model, they may not be sufficient. Another necessary factor may be damage to SECs, which is known to activate the coagulation system in other models (Copple et al., 2002a; Hirata et al., 1989; Seto et al., 1998). SEC injury occurs concurrently with activation of the coagulation system (Yee et al., 2003), but further study will be needed to determine whether both PMN accumulation and SEC injury are required events and, if so, how they interact to promote coagulation system activation.

The involvement of TNF-α in coagulation system activation varies with different models of inflammation. After a large, hepatotoxic dose of LPS, TNF-α depletion prevents activation of the coagulation system (Hewett and Roth, 1995). In that model, TNF-α is important for coagulation system activation through either stimulation of TF activity on endothelial cells (Bevilacqua et al., 1986; Esmon, 2000; Kirchhofer et al., 1994) or through activation of PMNs (Goel and Diamond, 2001; Klebanoff et al., 1986; Todoroki et al., 2000). By contrast, in the MCT/LPS-cotreatment model TNF-α does not appear to be necessary for activation of the coagulation system (Table 3). Likewise, KC inactivation in this model failed to prevent coagulation system activation, a result consistent with the lack of effect of TNF-α depletion, since activated KCs are a major producer of TNF-α in the liver. Although both KCs and TNF-α are critical for the development of MCT/LPS-induced injury (Yee et al., 2003a), their involvement is apparently not through activation of the coagulation system.

In MCT/LPS-cotreated rats, an early, pronounced increase in plasma TNF-α concentration occurs, and this is followed by a more modest but sustained elevation that continues through 18 h (Yee et al., 2003a,b). Anticoagulant administration to MCT/LPS-cotreated animals did not affect this later, sustained phase of increased plasma TNF-α concentration (Figs. 5A,B), suggesting that an activated coagulation system is not needed for sustained TNF-α release. Although it cannot be ruled out that the coagulation system influenced TNF-α formation at an earlier time, these results provide additional evidence that TNF-α generation and coagulation system activation are not interdependent. Previously, ATS treatment was shown to attenuate PMN accumulation at 18 h in livers of MCT/LPS treated rats (Yee et al., 2003b). The observation that it also reduced plasma CINC-1 concentration (Table 4) suggests that PMN influx occurs through a TNF-dependent mechanism involving CINC-1.

Anticoagulant administration reduced hepatic PMN accumulation (Figs. 6A,B). The coagulation system could affect PMN influx into liver tissue through the chemotactic activity of thrombin (Bizios et al., 1986). PMN accumulation, however, was not completely eliminated by administration of anticoagulant, suggesting that thrombin may work in conjunction with other PMN chemoattractants (e.g., CINC-1; Luster et al., 1998; Zhang et al., 1995). In this regard, it is of interest that anticoagulant administration reduced but did not eliminate CINC-1 appearance in plasma. Overall, the results provide evidence of interplay between the coagulation system and PMNs in contributing to HPC injury in this model.

MCP-1 can attract and activate monocytes and induce the expression of TF on the surfaces of these cells (Gu et al., 1999; Luster, 1998). Furthermore, MCP-1 has been implicated in the expression of intercellular adhesion molecule-1 on rat endothelial cells in vitro (Yamaguchi et al., 1998). This adhesion molecule, which is present in hepatic SECs (Essani et al., 1995), can interact with PMNs and prime them to release toxic products (Jaeschke et al., 1996). Accordingly, MCP-1 may be an important chemokine in the development of MCT/LPS-induced liver injury. In this model, elevated plasma MCP-1 was observed early in LPS-treated animals, irrespective of MCT treatment (Table 2). By 18 h, MCP-1 remained elevated in both groups of LPS-treated animals, but the increase was much greater in MCT/LPS-cotreated animals. The prolonged elevation in MCP-1 was unaffected by several pharmacologic manipulations, suggesting that neither KCs, PMNs, TNF-α, nor coagulation system activation are required for increased MCP-1 production after MCT/LPS coadministration.

Since the coagulation system is not activated in Veh/LPS animals (Yee et al., 2003), and since the same level of MCP-1 was seen in MCT/LPS and Veh/LPS-treated animals at 6 h when coagulation activation occurs, it seems unlikely that MCP-1 contributes to the early activation of the coagulation system. However, MCP-1-induced TF expression might contribute to liver injury at a later time by enhancing fibrin deposition (Falati et al., 2002; Orvim et al., 1994; Shebuski and Kilgore, 2001). Further study will be needed to determine the function of MCP-1 in this model. It is tempting to speculate, based on the sustained increase in MCP-1 at 18 h, that it may help prolong PMN adhesion (Yamaguchi et al., 1998) and sustain monocyte accumulation in liver.

In summary, anticoagulant therapy reduced fibrin deposition in CL and MZ regions of liver lobules, prevented HPC injury, and caused a modest attenuation in SEC injury in MCT/LPS-cotreated animals. These results point to a critical role of the coagulation system in MCT/LPS-induced liver injury. Moreover, PMNs and the coagulation system appear to cooperate in inducing HPC injury in this model of synergistic hepatotoxicity.
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