Role of Neutrophils in the Synergistic Liver Injury from Monocrotaline and Bacterial Lipopolysaccharide Exposure

Steven B. Yee,* Umesh M. Hanumegowda,* Jon A. Hotchkiss,†‡1 Patricia E. Ganey,* and Robert A. Roth*†‡2

*Department of Pharmacology and Toxicology; †Department of Veterinary Pathology, National Food Safety and Toxicology Center; and ‡Institute for Environmental Toxicology, Michigan State University, East Lansing, Michigan 48824

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Synergistic liver injury develops in Sprague-Dawley rats from administration of a small, noninjurious dose (7.4 × 10^6 EU/kg) of bacterial lipopolysaccharide (LPS) given 4 h after a nontoxic dose (100 mg/kg) of the pyrrolizidine alkaloid, monocrotaline (MCT). Previous studies demonstrated that liver injury is mediated through inflammatory factors, such as Kupffer cells and tumor necrosis factor-α (TNF-α), rather than through simple interaction between MCT and LPS. In the present study, the hypothesis that neutrophils (polymorphonuclear leukocytes or PMNs) are causally involved in this injury model is tested, and the interdependence between PMNs and other inflammatory components is explored. Hepatic PMN accumulation and the appearance of cytokine-induced neutrophil chemoattractant-1 in plasma preceded the onset of liver injury, suggesting that PMNs contribute to toxicity. Hepatic PMN accumulation was partially dependent on TNF-α. Prior depletion of PMNs in MCT/LPS-cotreated animals resulted in attenuation of both hepatic parenchymal cell (HPC) and sinusoidal endothelial cell (SEC) injury at 18 h. PMN depletion did not, however, protect against early SEC injury that occurred before the onset of HPC injury at 6 h. This observation suggests that SEC injury is not entirely dependent on PMNs in this model. **In vitro**, MCT caused PMNs to degranulate in a concentration-dependent manner. These results provide evidence that PMNs are critical to the HPC injury caused by MCT/LPS cotreatment and contribute to the progression of SEC injury.

**Key Words:** liver; inflammation; lipopolysaccharide; monocrotaline; neutrophil; sinusoidal endothelial cells; cytokine-induced neutrophil chemoattractant.

Bacterial lipopolysaccharide (LPS) is a constituent of the outer cell wall of gram-negative bacteria and is released from proliferating bacteria or during the lysis of their cell walls (Reitschel and Brase, 1992). LPS can elicit a potent inflammatory response in mammals (Hewett and Roth, 1993). Under conditions of systemic exposure to large doses of LPS, a wide spectrum of life-threatening changes can result. These include systemic hypotension, circulatory shock, disseminated intravascular coagulation, and multiple organ failure (Hewett and Roth, 1993; Rietschel and Brase, 1992). In rats, liver injury from large doses of LPS is characterized by neutrophil infiltration associated with hepatocellular degeneration and coagulative necrosis in midzonal regions of liver lobules (Hewett et al., 1992; Yee et al., 2000b). The mechanism for this injury is complex and involves the interaction of numerous soluble mediators and inflammatory cells, including neutrophils (polymorphonuclear leukocytes or PMNs) (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; Pearson et al., 1995).

Exposure to modest levels of LPS is commonplace and episodic, varying with time and the condition of the individual (Ganey and Roth, 2001; Roth et al., 1997). Enhanced systemic exposure occurs during bacterial infection and from increased translocation of LPS into the systemic circulation from indigenous gram-negative bacteria residing in the intestinal lumen (Ganey and Roth, 2001). A variety of conditions, including disease, dietary alterations, surgery, and alcohol consumption, can increase this translocation (Roth et al., 1997). Although such episodes of modest exposure to LPS may be insufficient to cause overt injury, a noninjurious inflammatory response can nevertheless result. This response can include PMN accumulation in tissues and the release of tumor necrosis factor-α (TNF-α) and other inflammatory mediators that have the potential to alter cellular homeostasis (Michie et al., 1988; Spitzer and Mayer, 1993). These events may cause tissues to become more susceptible to chemical-induced injury. Indeed, LPS exposure augments the toxicity of a number of hepatotoxins (reviewed in Roth et al., 1997), including monocrotaline (MCT) (Yee et al., 2000b).

MCT is a pyrrolizidine alkaloid (PA) hepatotoxin found worldwide in numerous plants of the *Crotalaria* genus (Mattocks, 1986; Stegelmeier et al., 1999). Human and animal exposures to PAs occur through the ingestion of contaminated foodstuffs and grains (Huxtable, 1989). Additionally, people may be exposed through the consumption of PA-containing alternative medicines such as *nong ji li*, *zi xiao rong*, and others...
(Roeder, 2000; Stegelmeier et al., 1999). Consequently, MCT was chosen as a toxicant of interest because of the role of PAs as potential food-borne toxins and their occurrence in alternative medicines.

For liver injury to occur, MCT must be bioactivated to monocular pyrrole (MCTP) (Stegalmeier et al., 1999; White and Mattocks, 1972). MCT-induced liver lesions at acutely toxic doses are characterized by centrilobular hepatocellular necrosis, dilated and congested sinusoids, hemorrhage, and injured central venous and sinusoidal endothelial cells (SECs) (Copple et al., 2002; Deleve et al., 1999; Schoental and Head, 1955; Yee et al., 2000b).

A small, noninjurious dose of LPS given to Sprague-Dawley rats 4 h after a small, nonhepatotoxic dose of MCT causes synergistic, acute liver injury (Yee et al., 2000b). In this model, liver lesions are both centrilobular and midzonal, exhibiting characteristics similar to lesions associated with larger, toxic doses of MCT and LPS given separately. The presence of these MCT-like and LPS-like lesions suggests that each agent enhances the injury of the other. Synergistic liver injury, however, could not be reproduced in isolated hepatic parenchymal cells (HPCs) in vitro, suggesting that the enhanced toxicity does not result from a direct interaction of MCT and LPS in HPCs but rather from an indirect mechanism (Yee et al., 2000b). One such indirect mechanism is an inflammation-induced response to LPS. Recently, Yee et al. (2000a, 2003) demonstrated that Kupffer cells (KCs) and TNF-α, among other mediators, are critical factors in this liver injury model.

PMNs are blood-borne inflammatory cells that are important in the defense against bacterial infection. However, the release of cytotoxic mediators from PMNs, such as proteases and other factors (e.g., reactive oxygen species) can have deleterious effects on host tissues. Indeed, PMNs are causally involved in HPC and SEC injury in several models, both in vitro and in vivo (Ganey et al., 1994; Hewett et al., 1992; Davier et al., 1988; Ohutsuka et al., 2000; Sakamoto et al., 1997).

The present study was designed to investigate the role of PMNs in this model of inflammation-enhanced hepatotoxicity. We examined time courses for appearance of chemoattractant in plasma and in hepatic PMN accumulation and localization. Effects of PMN depletion on HPC and SEC injury were explored in vivo, and the ability of MCT to activate PMNs was studied in vitro.

MATERIALS AND METHODS

Materials. Acetone, ammonium chloride, boron trifluoride in methanol, calcium chloride, o-dianisidine dihydrochloride, p-dimethylaminobenzaldehyde, glycogen, Hanks’ balanced salts, hydrogen peroxide, isopentane, LPS (Escherichia coli, serotype 0128:B12, 1.7 × 10^7 endotoxin units [EU]/mg), magnesium sulfate, mercuric chloride, pentoxyfylline (PTX), phosphate-buffered saline (PBS), pyrrole, sodium citrate and sodium bicarbonate were purchased from Sigma Chemical Company (St. Louis, MO). Gadolinium chloride-6H2O (GdCl3) was purchased from Aldrich Chemical Company (St. Louis, MO). Serum directed against TNF-α (antirat TNF-α serum, ATS) was produced in New Zealand White rabbits (Hewett et al., 1993). Rabbit antirat neutrophil antibody (neutrophil antisera, NAS) and control rabbit serum (CS) were obtained from Inter-Cell Technologies, Inc. (Hopewell, NJ). Mouse antirat endothelial cell antigen (RECA)-1 was purchased from Serotec, Inc. (Raleigh, NC). Goat serum was obtained from Vector Laboratories (Burlingame, CA). Goat antinmouse secondary antibody conjugated to Alexa 594 was purchased from Molecular Probes (Eugene, OR). MCT was acquired from Trans World Chemicals (Rockville, MD). Absolute ethanol was purchased from Quantum Chemical Company (Tuscola, IL). Sterile saline was acquired from Abbott Laboratories (North Chicago, IL). Formalin fixative was obtained from Surgipath Medical Industries, Inc. (Richmond, IL). Diagnostic kits for 58 UV and 59 UV, for the determination of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, respectively, were also purchased from Sigma Chemical Company (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) kit for hyaluronic acid (HA) was purchased from Corgenix, Inc. (Westminster, CO). ELISA kit for rat TNF-α was purchased from Biosource International, Inc. (Camarillo, CA). ELISA kit for rat growth-related oncogene (GRO)/cytokine-induced neutrophil chemoattractant-1 (CINC-1, rat interleuken-8) 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 in vivo studies. Male Sprague-Dawley, retired breeder rats were used for the in vitro PMN 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, Warrensburg, 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, intraperitoneally, followed 4 h later by LPS (7.4 × 10^5 EU/kg) or its saline vehicle via tail vein injection. LPS was administered 4 h after MCT to minimize interference with MCT bioactivation (Allen et al., 1972).

Neutrophil depletion protocol. Rats were pretreated intraperitoneally with NAS or CS (1 ml and 0.5 ml, respectively) 24 and 8 h before LPS administration. This treatment protocol has been shown to deplete PMNs effectively from blood and liver (Barton et al., 2000). Four h after MCT treatment, LPS was administered. Rats were killed and liver injury was assessed at 6 or 18 h after MCT treatment.

Assessment of hepatic injury and plasma TNF-α and CINC-1. At the time indicated in the figure legends, 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, a marker of hepatic SEC injury (Copple et al., 2002; Deaciuc et al., 1993, 1994). Plasma TNF-α activity was determined with a rat TNF-α ELISA kit. Plasma CINC-1 activity was measured using a rat GRO/CINC-1 ELISA kit.

Ehrlich assay. The Ehrlich assay was used to measure the concentration of reactive pyrroles in liver as a marker of MCT bioactivation to its toxic pyrrolic metabolite, MCTP (Mattocks and White, 1970). This assay was used to determine whether NAS interfered with the bioactivation of MCT. It was performed as described previously (Yee et al., 2000b), with the exception that pyrrole was used to generate the standard curve. Vehicle control livers were used to correct for the presence of endogenous pyrroles.

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 cm^3) of the liver from the middle of the left lateral lobe for immunohistochemical staining was...
frozen in isopentane immersed in liquid nitrogen. 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 a midzonal lesion, (3) the area of midzonal 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 karyorrhexic 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):

\[
\text{area}_{\text{interest}} = \sum \text{points}_{\text{interest}} \times \text{area/point}
\]

\[
\text{area/point} = (\text{distance between points})^2
\]

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, nonoverlapping 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. Eight animals per group were analyzed. Percent lesion area was estimated based on the following formula:

\[
\frac{\text{area}_{\text{lesion of interest}}}{\left(\text{area}_{\text{all lesions}} + \text{area}_{\text{parenchyma}}\right)} \times 100
\]

**Enumeration of hepatic PMNs.** Paraffin-embedded liver tissue (3 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 antirabbit 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, magnification ×400) in each slide (i.e., 10 HPFs per liver section). The analyzed fields represented between 5 to 10% of the total area of each liver section. Analyzed fields were unbiasedly selected from gross regions to cover the entire liver section. PMNs were identified by positive staining and cell morphology. Hepatic PMNs were enumerated at the times indicated in the figure legends in the time course and PMN depletion studies described above. In addition, they were enumerated in MCT/LPS-cotreated animals that had undergone KC inactivation or TNF-α depletion. In the KC inactivation study, 10 mg GdCl₃/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) were administered 1 h before LPS administration. These treatment regimens were effective in preventing KC activation and the LPS-induced increase in plasma TNF-α activity, respectively. None of these three pharmacological agents interfere with MCT bioactivation (Yee et al., 2003).

**PMN distribution.** Hepatic PMN distribution was assessed in MCT/LPS-treated animals 6 h after MCT administration. PMNs were enumerated in 15 randomly selected, low-power fields (LPFs, magnification ×100) in each slide (i.e., 5 LPFs per liver section). In each field, PMNs were counted from a random liver lobule consisting of a clearly defined centrilobular, periportal, and midzonal region. Centrilobular and periportal liver regions were defined as areas up to 5 HPCs away from the central vein and portal triad, respectively. Midzonal regions were arbitrarily determined to constitute the HPCs centrally located between centrilobular and periportal regions. Approximately 5% of the liver lobules were examined in each liver section.

**Immunohistochemistry.** Sections of frozen tissue (8-μm-thick) were fixed in acetone (4°C) for 5 min. They were incubated for 30 min with PBS containing 10% goat serum (i.e., blocking solution) and then with mouse antirat RECA-1 diluted (1:20) in blocking solution overnight at 4°C. The RECA-1 antibody binds to rat endothelium but not to other cell types (Duijvestijn et al., 1992). In the liver, RECA-1 stains both SECs and large venule endothelial cells. After incubation with RECA-1 antibody, the sections were incubated for 3 h with goat antimouse secondary antibody conjugated to Alexa 504 (1:500) in blocking solution containing 2% rat serum. Sections were washed 3 times for 5 min each with PBS and visualized using a fluorescent microscope. No staining was observed in the controls in which the primary or secondary antibody was omitted from the staining protocol. All treatment groups that were compared morphometrically were stained at the same time and evaluated on the same day.

**Quantification of liver endothelial cells.** Endothelial cells in the liver were quantified morphometrically by analyzing the RECA-1 immunohistochemical staining area for each liver section (Copple et al., 2002). A decrease in the endothelial cell staining suggests a loss of these cells from the liver. Fluorescent staining of liver sections was visualized using an Olympus AX-80T microscope. For morphometric analysis of total endothelial cell area, digital images of 5 randomly chosen, LPFs (100X) per tissue section were captured using a SPOT II camera and SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI). 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 centrilobular, periportal, and midzonal regions. The area of staining within each region was calculated from these digital images.

The area of immunohistochemical staining (number of pixels) was quantified using Scion Image software. An inverted, gray-scale digital image of a liver section from a vehicle-treated rat was used to establish the control levels of endothelial cell staining for subsequent analysis of treatment-related effects. The upper and lower threshold gray-scale pixel values were adjusted so that background staining was eliminated from the analysis and only endothelial staining was visualized (i.e., density slice analysis). The same threshold values were used to analyze digital images from sections from all treatment groups. For quantification of endothelial cells, the area of positive staining was measured and divided by the total area of the image. Analysis of the endothelial cells in the centrilobular, periportal, and midzonal areas was conducted by drawing a 145 μm-diameter circle around the central vein or portal triad. The circumference of the circle was approximately 4–5 hepatocytes away from the central vein or portal triad, and this defined centrilobular and periportal regions, respectively, for the purpose of evaluation. The midzonal region was defined as the center of area between the centrilobular and periportal regions, using the same circle circumference, without overlap, of these arbitrary circles. The total area analyzed was 16,512 μm². The area of endothelial cells in that region was measured as described above and divided by the total area of the image. Results from the fields analyzed for each liver section were averaged and counted as a replicate (i.e., each replicate represented a different rat).

**PMN isolation and treatment protocol.** PMNs were isolated from the peritoneal cavity of retired breeder rats by glycogen elicitation, as described by Ganev et al. (1994). The percent yield of PMNs and their viability was routinely greater than 95%. Isolated PMNs were resuspended in Hanks’ balanced salt solution (HBSS), pH 7.35, with 1.6 mM CaCl₂, 0.68 mM MgSO₄, and 14 mM NaHCO₃. The final concentration of cells in all studies was 2.5 × 10⁶ cells/ml. MCT was prepared in HBSS and added to PMNs at a final
concentration ranging from 0 to 0.8 mM. The cell suspension was incubated for 30 min at 37°C and then centrifuged. Aliquots from the cell-free supernatant were taken for measurement of myeloperoxidase (MPO) activity and lactate dehydrogenase (LDH) release.

**MPO and LDH assays.** MPO activity was used as a marker of PMN activation. MPO activity in the cell-free supernatant was measured according to the method of Harada et al. (2000) with minor modification. Briefly, 200 ml of phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide was added to 10 ml of supernatant. The change in absorbance at 460 nm over 3 min was measured in a 96-well plate reader. MPO activity was reported as fold-increase of the vehicle control (0 mM MCT).

The release of the cytosolic enzyme, LDH by PMNs into the medium was used as a marker of cytotoxicity, which was measured according to the method of Bergmeyer and Bernt (1974). A separate aliquot of PMNs was lysed by sonication, and total LDH activity was determined in the cell-free supernatant. LDH release was expressed as the percent of total cellular LDH released into the medium.

**Statistical analysis.** Results are expressed as means ± 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 ≤ 0.05 for all comparisons.

### RESULTS

**MCT/LPS time course.** Synergistic liver injury from a small, noninjurious dose of LPS (7.4 × 10^6 EU/kg) administered four h following a small, nontoxic dose of MCT (100 mg/kg) is maximal by 18 h after MCT administration (Yee et al., 2000b). A detailed time course study was initiated to define more precisely early and late events in this model of liver injury. Increases in plasma ALT and AST activities occurred between 6 and 9 h and continued to be significant until 72 h after MCT administration (Figs. 1A and 1B). By 96 h, values had returned to control levels. Centrilobular MCT-like and midzonal LPS-like lesions were observed in livers from MCT/LPS-treated animals starting at 9 h and were still apparent by 72 h. During this time, coagulative necrotic subserosal lesions in these livers were also observed. Lesion characteristics were the same as described by Yee et al. (2000b). Centrilobular lesions consisted of moderate to marked hepatocellular apoptotic and oncocytic necrosis, degeneration, hemorrhage, and vascular injury. Midzonal lesions comprised well defined areas of marked hepatocellular coagulative necrosis accompanied by PMN infiltration with congestion and hemorrhage. Histopathologically, no lesions were observed at 6 h, although some disruption of central vein intima was observed. PMN accumulation was apparent at this time in livers of animals that received LPS, irrespective of MCT-treatment. No mortality occurred in MCT/LPS-cotreated animals within 12 h after MCT administration, but approximately 20% of rats died by 24 h, with no deaths thereafter. No animals that received saline vehicles, MCT alone, or LPS alone died.

**Plasma CINC-1 concentration in MCT/LPS-treated animals.** CINC-1, the rat equivalent to human interleukin-8, is a PMN chemoattractant (Luster, 1998; Zhang et al., 1995). Plasma CINC-1 concentration was assessed at various times after administration of MCT or its vehicle (Veh) (Fig. 1C). No increase was observed in Veh/Veh or MCT/Veh cotreatments at any of the times evaluated, but plasma CINC-1 concentration was significantly elevated 2 h after LPS administration, irrespective of MCT-treatment, and declined thereafter. The decline was significantly more rapid in Veh/LPS-treated rats than in those cotreated with MCT/LPS, so that by 18 h plasma CINC-1 concentration was five times as great in MCT/LPS-treated rats (note log scale in Fig. 1C).

**Hepatic PMN accumulation in MCT/LPS-cotreated animals.** Quantitative analysis of liver sections immunostained for PMNs revealed that accumulation of these inflammatory cells occurred by 6 h in livers from animals treated with either LPS alone or MCT/LPS (Fig. 1D). PMN numbers in the livers of animals treated with LPS alone had begun to decline by 12 h and returned to baseline by 48 h. By contrast, PMN accumulation remained significantly elevated in livers from MCT/LPS-treated animals until the final analysis time of 72 h.

Hepatic neutrophil distribution at 6 h was panlobular in livers from animals treated with either LPS alone or MCT/LPS (Fig. 2). Whereas, PMN numbers were elevated at 6 h throughout all the regions of the liver lobules in MCT/LPS-treated animals; by 9 h most PMNs were localized within lesioned areas (i.e., centrilobular and midzonal).

**Effect of PMN depletion on MCT/LPS-induced hepatocellular injury.** To test the hypothesis that PMNs are important in the pathogenesis of MCT/LPS-induced liver injury, rats were pretreated with NAS to deplete PMNs. NAS pretreatment of MCT/LPS-treated animals markedly reduced (by 82%) hepatic PMN accumulation (Fig. 3B) and practically eliminated the increases in plasma ALT and AST activities caused by MCT/LPS exposure 18 h after MCT administration (Figs. 4A and 4B). Mortality was 24% in MCT/LPS-cotreated control animals and 17% in MCT/LPS-cotreated animals that received NAS pretreatment. No animals treated with CS or NAS alone died. NAS pretreatment did not significantly affect survival in MCT/LPS-treated animals.

MCT/LPS-cotreated control animals exhibited centrilobular and midzonal liver lesions as described above. Livers from MCT/LPS-coexposed animals pretreated with NAS demonstrated qualitatively similar centrilobular and midzonal lesions; however, these lesions in both regions were smaller in size and considerably less frequent. Midzonal lesions appeared to have a greater reduction in size and frequency. Morphometric analysis confirmed the observed decrease in the area of the centrilobular (83% decrease) and midzonal (88% decrease) lesions in livers of MCT/LPS-cotreated animals that were pretreated with NAS (Table 1). Histologically, no injury was observed in
animals given CS or NAS alone. Finally, an Ehrlich assay revealed that NAS pretreatment did not alter pyrrolic bioactivation products of MCT in liver tissue (Table 2).

Effect of PMN depletion on SEC injury. Hepatic SEC injury has been quantified through increased plasma HA concentration and immunohistochemically as decreased RECA-1 staining. Using these methods, SEC injury has been observed after large, hepatotoxic doses of MCT (Copple et al., 2002) or LPS (Deacuic et al., 1994; Spapen et al., 1999). In MCT/LPS-treated animals SEC injury occurs before the onset of HPC injury (Yee et al., 2002).

To define the importance of PMNs in SEC injury in MCT/LPS-coexposed animals, rats were pretreated with NAS, and injury was assessed 6 h after MCT administration (i.e., before the onset of hepatocellular injury). NAS pretreatment eliminated hepatic PMN accumulation in MCT/LPS-cotreated animals (Fig. 3A). By 6 h, plasma HA concentration was increased in MCT/LPS cotreated animals; however, NAS pretreatment failed to affect this increase (Fig. 5A). No mortality occurred in control or cotreated animals at this time. At 18 h, the increase in plasma HA concentration persisted in MCT/LPS-cotreated animals, and the increase remained in NAS pretreated rats but was less pronounced (Fig. 5C).

To confirm the effect of PMN depletion on SEC injury, endothelial cells were stained immunohistochemically with RECA-1 antibody, which selectively binds to rat endothelial cells (Duijvestijn et al., 1992). RECA-1 staining of liver sections has been used to visualize and quantify the loss of SEC.}

FIG. 1. Time course of events after MCT/LPS exposure. LPS (7.4 × 10^9 EU/kg) or saline vehicle (Veh) was administered iv to rats 4 h after ip administration of MCT (100 mg/kg) or saline vehicle. (A) and (B) present the time course of HPC injury as estimated by changes in plasma ALT and AST activities, respectively. No significant effect of MCT or LPS relative to Veh/Veh animals was observed. (C) and (D) depict time course changes in the plasma concentration of the PMN chemoattractant CINC-1 and in hepatic PMN accumulation, respectively. In (C), both groups that received LPS were significantly different from those groups that did not, at all times evaluated. In (D), the group receiving Veh/LPS was significantly different from both groups not receiving LPS at times encompassing 6 to 24 h, whereas the group receiving MCT/LPS was significantly different from these two groups at all times; n = 4–24 animals. *Significantly different from all other groups; †significant difference between Veh/LPS and MCT/LPS groups.
endothelial cells in the liver (Copple, et al., 2002). In liver sections of rats treated with CS or NAS alone, intense RECA-1 staining was present along the sinusoids and lined the major vessels of the liver. Total RECA-1 staining intensity did not differ from control values in livers from animals that received MCT/LPS cotreatment at 6 h (Fig. 5B); however, it decreased significantly by 18 h (Fig. 5D). NAS pretreatment did not result in a change in total RECA-1 staining intensity in comparison to livers from MCT/LPS control animals at either 6 or 18 h. No effect of NAS pretreatment on liver zonal distribution (i.e., centrilocubular, midzonal, or periportal) of RECA-1 staining intensity was observed in MCT/LPS-cotreated animals at 6 or 18 h (data not shown).

Relationship between TNF-α and PMNs in MCT/LPS-treated rats. LPS can activate KCs to release inflammatory cytokines such as TNF-α (Hewett and Roth, 1993; Michie et al., 1988). In an earlier study (Yee et al., 2003), KC depletion by GdCl3, or attenuation of the TNF-α response by either PTX or ATS was found to protect against HPC injury in MCT/LPS-cotreated rats. As seen in Table 3, each of these treatments also reduced hepatic PMN accumulation. By contrast, PMN depletion was without significant effect on the increase in plasma TNF-α concentration that accompanied MCT/LPS treatment at 6 or 18 h (Figs. 6A and 6B).

Effect of MCT on PMNs in vitro. To determine if MCT caused PMN activation in vitro, isolated PMNs were exposed to various concentrations of MCT (0 to 0.8 mM) for 30 min. MCT treatment resulted in a concentration-dependent increase in MPO release (Fig. 7A) from PMNs without a concurrent increase in LDH release (Fig. 7B).

DISCUSSION

Previously, Yee et al. (2000b) reported that synergistic liver injury develops within 18 h after MCT administration in a model in which a small, noninjurious dose of LPS (7.4 × 10^6 EU/kg) is administered 4 h after a normally nontoxic dose of MCT (100 mg/kg). This dose of LPS evokes modest hepatic inflammation but little or no hepatocellular injury. A more detailed time course study has revealed that after MCT/LPS cotreatment, liver injury develops between 6 and 9 h and continues to be significant until 72 h after MCT administration.
Hepatocellular injury was maximal by 18 h, as marked by increases in plasma ALT and AST activities. No HPC injury was observed in MCT/LPS-treated animals at 6 h. Hepatic PMN accumulation and increases in plasma CINC-1 and TNF-α concentrations in LPS-treated animals confirmed that the animals were undergoing an inflammatory response at this time (Figs. 1C, 1D, and 6A, respectively). In animals given LPS alone, the rise in hepatic PMN numbers gradually decreased to vehicle control levels by 48 h, and liver injury did not develop. In contrast, in MCT/LPS-cotreated animals, the increased number of hepatic PMNs was sustained through 72 h, and liver injury occurred. The significant elevation in hepatic PMN numbers in the cotreated animals before the onset of injury suggested that these inflammatory cells might contribute to the pathogenesis. To test this hypothesis, NAS was used in a treatment regimen known to reduce the number of PMNs in blood and liver (Barton et al., 2000). NAS effectiveness was confirmed by the marked reduction of hepatic PMN accumulation in MCT/LPS-cotreated animals 18 h after MCT administration (Fig. 3B). NAS pretreatment resulted in pronounced attenuation of the increase in plasma ALT and AST activities (Figs. 4A and 4B), suggesting that PMNs play a causal role in the hepatocellular injury.

The distribution of PMNs at 6 h was panlobular in livers of LPS-treated animals. No zonal differences in PMN distribution in LPS-treated animals were observed, regardless of MCT administration (Fig. 2). This indicated that the presence of PMNs was not a response to injury, since injury occurred only in centrilobular and midzonal regions, whereas PMN accumulation was panlobular. Moreover, PMN accumulation without liver injury occurred in rats treated with LPS alone. Taken together, these observations suggest that activation of these cells follows their accumulation and is required for hepatocellular injury to occur. As previously described, cotreatment of rats with normally nontoxic doses of MCT and LPS results in liver lesions consisting of a centrilobular, MCT-like lesion and a midzonal, LPS-like lesion (Yee et al., 2000b). NAS pretreatment of these

(Figs. 1A and 1B). Hepatocellular injury was maximal by 18 h, as marked by increases in plasma ALT and AST activities.

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<table>
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<tr>
<th>TABLE 1</th>
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<tr>
<td>PMN Depletion Reduces Liver Lesions in MCT/LPS-Cotreated Rats</td>
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<table>
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<tr>
<th>Treatment</th>
<th>Percent lesion area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Centrilobular</td>
</tr>
<tr>
<td>CS/MCT/LPS</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>NAS/MCT/LPS</td>
<td>0.4 ± 0.1*</td>
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</tbody>
</table>

Note. Neutrophil antiserum (NAS) or control (nonimmune) serum (CS) was given ip to rats at 24 and 8 h before LPS. LPS (7.4 × 10⁶ EU/kg) was administered iv to rats 4 h after ip administration of MCT (100 mg/kg). Livers were taken 18 h after MCT administration and processed for morphometric analysis, n = 8 animals.

*Significantly different from CS/MCT/LPS group.

<table>
<thead>
<tr>
<th>TABLE 2</th>
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<td>NAS Does Not Influence MCT Bioactivation</td>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pyrrole (µg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS/MCT/LPS</td>
<td>6.3 ± 0.4</td>
</tr>
<tr>
<td>NAS/MCT/LPS</td>
<td>6.4 ± 0.2</td>
</tr>
</tbody>
</table>

Note. NAS or CS was given to rats (ip) 24 and 8 h before LPS. Rats were treated with MCT and/or LPS as described in the Table 1 footnote. Liver samples were taken 18 h after MCT administration and analyzed for pyrrole concentration by Ehrlich assay. Resulting values were corrected for endogenous pyrrole by subtracting the average value (i.e., 1.4 µg pyrrole/g liver) from animals given the CS/Veh/Veh combination; n = 5 animals.
animals did not affect the qualitative nature of the lesions. However, both centrilobular and midzonal lesion size and frequency were greatly attenuated (Table 1). Moreover, the amount of hemorrhage and congestion within centrilobular and especially midzonal areas was reduced by NAS pretreatment. Overall, this suggests that NAS pretreatment of MCT/LPS-cotreated animals results in a reduction of both midzonal and centrilobular lesions.

To demonstrate that the attenuation of HPC injury was not the result of NAS pretreatment interfering with MCT bioactivation, an Ehrlich assay was performed on liver homogenates. This assay has been used to monitor hepatic accumulation of pyrrolic bioactivation products of MCT (Mattocks and White, 1970; Yee et al., 2000b). NAS pretreatment of MCT/LPS-cotreated animals did not reduce liver pyrrole concentration in comparison to animals pretreated with CS (Table 2). Thus, NAS pretreatment does not appear to interfere with MCT bioactivation.

Over 90% of circulating HA is removed and degraded by hepatic SECs (Kobiyashi et al., 1999). SEC injury impairs the ability of these cells to clear circulating HA, resulting in an increase in plasma HA concentration. Consequently, such increases have been used as a biomarker of SEC injury in vivo. The increase in plasma HA concentration correlated well with histopathologic evidence of SEC injury and destruction in rats given a large, hepatotoxic dose of either MCT (Copple et al., 2002) or LPS (Deacuic et al., 1994; Spapen et al., 1999). In this and previous studies (Yee et al., 2000b, 2002), coexposure to doses of MCT and LPS that are by themselves nontoxic resulted in an increase in plasma HA concentration, and this increase was consistent with histopathologic evidence of endothelial injury.

FIG. 5. Effect of PMN depletion on MCT/LPS-induced SEC injury. NAS or CS was given to rats 24 and 8 h before LPS. Rats were treated with MCT and/or LPS as described in the Figure 1 legend. (A) and (B) show plasma hyaluronic acid (HA) concentration, a biomarker of SEC injury, and the total area of RECA-1 (an antibody selective for endothelial cells) immunohistochemical staining in liver sections 6 h after MCT administration, respectively. (C) and (D) present the same markers 18 h after MCT administration. Note ordinal scale differences in 5A and 5C; n = 3 to 6 animals. aSignificantly different from respective value in the absence of MCT/LPS; bsignificantly different from CS/MCT/LPS group.
Increased plasma HA concentration was observed by 6 h, well before the onset of HPC injury, and became more pronounced by 18 h (Figs. 5A and 5C, respectively). To determine if PMNs were responsible for this early injury to SECs, MCT/LPS-cotreated animals were examined at 6 h. NAS pretreatment of these animals significantly reduced hepatic PMN accumulation (Fig. 3A) but did not affect the elevation in plasma HA concentration at this time (Fig. 5A). RECA-1 immunohistochemical staining was not able to detect the subtle changes in SECs at 6 h after MCT/LPS cotreatment (Fig. 5B). This lesser sensitivity of RECA-1 as a biomarker of SEC injury compared to plasma HA concentration has been observed previously (Copple et al., 2002). The inability of NAS pretreatment to reduce the increase in plasma HA concentration at 6 h suggests that PMNs are not responsible for the early SEC injury.

At later stages in the progression of liver injury, PMNs appear to contribute to the injury to SECs. Indeed, other studies have demonstrated that PMNs can cause SEC injury in vivo (Ohtsuka et al., 2000; Sakamoto et al., 1997). NAS pretreatment of MCT/LPS-cotreated animals resulted in a small but significant attenuation of plasma HA concentration at 18 h (Fig. 5C). Immunohistochemical staining confirmed endothelial cell injury in MCT/LPS-cotreated animals as a decrease in RECA-1 staining in NAS-treated animals (Fig. 5D). Regardless of NAS pretreatment, MCT/LPS treatment reduced RECA-1 staining in both centrilobular and midzonal regions of liver lobules (data not shown). However, RECA-1 immunostaining was not sensitive enough to reflect the modest differences in plasma HA concentration that occurred at 18 h in MCT/LPS-cotreated animals that did and did not receive NAS pretreatment. Taken together, these results suggest that PMN depletion in MCT/LPS-coexposed animals partially but incompletely attenuated late-developing SEC injury. Overall, the results suggest that SEC injury early in pathogenesis occurs independently of PMNs, but that PMNs contribute to the progression of SEC injury at later times (Fig. 8).

The cause of the early injury to SECs in MCT/LPS-treated rats is not understood. Although SECs express cytochromes P450 (DeLeve et al., 1997; Lester et al., 1993), it is not known if the particular P450 family (i.e., 3A) responsible for MCT bioactivation is expressed in these cells (Kasahara et al., 1997). DeLeve et al. (1996) observed that MCT is toxic to SECs in vitro and suggested that the toxicity might be due to the production of a toxic metabolite (i.e., MCTP). Indeed, pyrrolic metabolites of MCT are produced by SECs in vitro (unpub-
lished observation), supporting the possibility that MCT may injure SECs directly through the formation of MCTP. It is possible that bioactivation products of MCT, produced either by HPCs or SECs, work in combination with inflammatory factor(s) to cause early SEC injury independently of PMNs (Fig. 8). This possibility remains untested. In this regard, it is interesting that the MCT/LPS cotreatment causes an early and sustained elevation in TNF-α and, like early SEC injury, this was not reduced by PMN depletion. The possibility that early SEC injury in this model may be due to inflammatory factors such as LPS itself or TNF-α acting on MCT-compromised SECs deserves further attention.

To understand better the interdependence between PMNs and other inflammatory factors, we studied the effect of PMN depletion on plasma TNF-α. In rats given an acutely toxic dose of LPS, PMN depletion significantly increased plasma TNF-α concentration (Hewett et al., 1993). However, in this study, plasma TNF-α concentration in MCT/LPS-cotreated animals at 6 and 18 h was unaffected by PMN depletion (Figs. 6A and 6B, respectively). This suggests that PMNs are not needed to provoke or maintain TNF-α release in this model.

Treatment of rats with TNF-α-depleting agents or with GdCl₃, a KC inactivator, decreased hepatic PMN accumulation (Table 3). This result suggests that TNF-α released by activated KCs contributes to PMN accumulation and/or retention in liver (Fig. 8). This finding is different from observations in rats given a larger, hepatotoxic dose of LPS (Hewett et al., 1992) or in a model of LPS potentiation of aflatoxin B₁ hepatotoxicity (Barton et al., 2001), in which no change in hepatic PMN accumulation occurred after TNF-α depletion. However, in a liver/ischemia reperfusion model, GdCl₃ treatment resulted in a decrease in PMN infiltration (Mosher et al., 2001). In the present study, the effects of TNF-α depleters on hepatic PMN accumulation were measured at 18 h (Table 3). It is possible that initial influx of PMNs into livers occurs independently of TNF-α, but that tissue retention of PMNs is mediated by this cytokine. Interestingly, the increase in plasma TNF-α concentration is short lived (about 2 h) in the other models noted above, whereas a prolonged increase in TNF-α occurs in this model (Fig. 6B). It may be that the sustained release of TNF-α underlies the prolonged accumulation of PMNs. Indeed, in animals given only LPS, in which the elevation in plasma TNF-α is transient (Chensue et al., 1991; Iimuro et al., 1994), the accumulation of PMNs in the liver is relatively short-lived (Fig. 1D).

TNF-α might be involved in promoting hepatic PMN accumulation/retention in this model by activating SECs (Bradham et al., 1998; Sakamoto et al., 2002; Vasselli, 1992). This cytokine can increase the expression on endothelial cells of adhesion molecules that promote PMN retention (May and Gnosh, 1998; Sakamoto et al., 2002). Moreover, PMNs can be activated by interaction with adhesion molecules expressed on endothelial cells (Crockett-Torabi, 1998; Lawson et al., 2000), and such activation might contribute to the late SEC injury that appeared to have a PMN-dependent component (Fig. 8). It should be noted, however, that the biologic significance of the prolonged but small increase in plasma TNF-α (Fig. 6B) is unclear. Local tissue TNF-α concentration may be substantially greater than that circulating in plasma. Further study will be required to ascertain if the small concentrations detected have a contributory role in the pathogenesis in this model.

Since the decrease in PMN accumulation caused by TNF-α-depleting agents was not complete, other inflammatory mediators in addition to TNF-α may be involved. CINC-1, a rat
neutrophil chemoattractant (Luster, 1998; Zhang et al., 1995), was significantly elevated in plasma 2 h after LPS administration, regardless of MCT treatment (Fig. 1C). This result suggests that CINC-1 contributes to the recruitment of hepatic PMNs (Fig. 8). Plasma CINC-1 concentration declined after 2 h but remained significantly elevated in the LPS-treated animals; however, the decline in CINC-1 was much slower in rats cotreated with MCT. This maintenance of CINC-1 production may contribute to the greater persistence of PMNs in the livers of MCT/LPS-cotreated rats.

It is tempting to speculate that the prolonged elevation in CINC-1 and in hepatic PMNs in MCT/LPS-treated rats is the result of CINC-1 gene expression maintained by persistently elevated production of cytokines such as TNF-α (Calkins et al., 2002; Deutschman et al., 1996; Ohkubo et al., 1998). TNF-α has been shown to induce CINC-1 production by hepatocytes in vitro (Ohkubo et al., 1998; Thorton et al., 1991). Moreover, Zhang et al. (1995) demonstrated that an anti-CINC antibody attenuates hepatic PMN accumulation in LPS-treated rats, and Maher et al. (1997) found that adenovirus-mediated over expression of CINC-1 in rat liver results in PMN accumulation. Accordingly, CINC-1 is important in several models in recruiting PMNs into the liver.

TNF-α depletion in this model results in reduced liver injury (Yee et al., 2003). In addition to its apparent role in PMN accumulation (Table 3), this cytokine might promote injury by priming PMNs to release toxic products that can damage nearby cells (Nakagi et al., 1991; Vasselli, 1992; Kushimoto et al., 1996; Fig. 8). However, additional inflammatory factors must be required, since LPS alone resulted in elevated plasma TNF-α and hepatic PMN accumulation but did not produce hepatic injury (Fig. 1D). Indeed, one action of TNF-α might be to render the HPCs more susceptible to injury (Adamson and Billings, 1992; Bradham et al., 1998; Hoek and Pastorino, 2002; Jones et al., 2000; Xu et al., 1998).

MCT was able to induce PMN degranulation in vitro at nontoxic concentrations (Figs. 7A and 7B). Treatment of isolated PMNs with 0.4 and 0.8 mM MCT resulted in a significant increase in MPO activity but no change in LDH release. Assuming that MCT is rapidly absorbed and distributed into total body water, a dose of 100 mg/kg could produce a transient plasma MCT concentration approaching 0.5 mM, which is in the range needed to cause PMN activation in vitro. The 100 mg MCT/kg dose did not by itself result in HPC or SEC injury or result in hepatic PMN sequestration. It is possible that in the presence of PMNs recruited by LPS, this otherwise nontoxic dose of MCT promotes PMN activation, resulting in the release of toxic factors that precipitate injury (Fig. 8). However, MCT elimination by metabolism is rapid in vivo (Allen et al., 1972); therefore, whether tissue MCT concentration remains elevated for a period sufficient to contribute to PMN activation is uncertain. It is conceivable that early exposure of circulating PMNs to MCT primes them to respond with heightened sen-
sitivity to other stimuli (Kushimoto et al., 1996; Nakagi et al., 1991; Vassalli, 1992).

Episodic exposure to LPS, and probably other inflammagens, is commonplace, and resulting modest inflammation may be an important factor governing susceptibility of individuals to intoxication by a variety of chemicals (Ganey and Roth, 2001). Moreover, modest inflammation may play a role in interactions among toxicants. For example, alcohol consumption can result in endotoxemia in people and animals (Tarao et al., 1977; Bode et al., 1987), and it is conceivable that this may contribute to ethanol-drug interactions by lowering the threshold for drug toxicity. Results of the present study suggest that people experiencing a mild inflammatory response due to alcohol consumption or other conditions may be at particular risk for illness from foods or alternative medicines that contain PAs or other potentially hepatotoxic compounds.

In summary, PMN depletion in MCT/LPS-cotreated animals protected against HPC injury and caused a modest attenuation of late-onset SEC injury. Accordingly, PMNs appear to be critically important in the pathogenesis of liver injury in this model. Figure 8 depicts a series of events that could explain the role that PMNs and other inflammatory factors play in the synergistic hepatotoxicity resulting from MCT and LPS. Co-exposure to these two agents likely causes early SEC activation and injury by unknown mechanisms. This results in arrest of PMNs in sinusoids. Concurrent activation of KCs and perhaps other cells causes release of inflammatory factors such as TNF-α and CINC-1, which prompt PMN migration and activation in parenchyma. Activation of PMNs may be enhanced by MCT. Activated PMNs release toxic factors that damage HPCs sensitized to injury by MCT exposure and cause progression of SEC injury. Although consistent with available evidence (Yee et al., 2000a, 2003), this scenario represents a hypothesis that requires additional testing. Nevertheless, it is clear that PMNs, along with TNF-α, KCs, and other inflammatory components, are part of an array of factors contributing to the synergistic, hepatotoxic interaction between MCT and LPS.

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