ROLE OF INFLAMMATION IN THE SYNERGISTIC HEPATOTOXICITY OF MONOCROTALINE AND BACTERIAL LIPOPOLYSACCHARIDE

VOLUME I

By

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ABSTRACT

ROLE OF INFLAMMATION IN THE SYNERGISTIC HEPATOTOXICITY OF MONOCROTALINE AND BACTERIAL LIPOPOLYSACCHARIDE

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Inasmuch as noninjurious doses of bacterial lipopolysaccharide (LPS) augment the hepatotoxicity of a variety of xenobiotic agents, exposure to small amounts of LPS can be an important determinant of susceptibility to chemical intoxication. This dissertation tests the hypothesis that inflammatory events participate causally in the synergistic hepatotoxicity from coexposure to the food-borne hepatototoxin monocrotaline (MCT) and LPS. Administration of a small, noninjurious dose of LPS (7.4×10^6 EU/kg; i.v.) 4 hours after a small, nontoxic dose of MCT (100 mg/kg; i.p.) leads to synergistic liver injury in Sprague-Dawley rats. Hepatic parenchymal cell (HPC) injury develops between 6 and 9 hours after MCT administration and is maximal by 18 hours. Both centrilobular (CL) and midzonal (MZ) liver lesions occur and exhibit characteristics similar to lesions associated, respectively, with a larger, toxic dose of MCT or LPS given separately. The nature of the MCT-like, CL and LPS-like, MZ lesion suggests that each agent enhances the injury of the other. Loss of the central vein intima in CL lesions and disruption of sinusoidal architecture and hemorrhage in both lesions suggests vascular injury. This was confirmed by a plasma biomarker for sinusoidal endothelial cell (SEC) injury, immunohistochemistry and electron

microscopy. Since SEC activation and injury can promote hemostasis, activation of the coagulation system was evaluated. A biomarker for coagulation system activation and increased hepatic fibrin deposition confirmed the activation of the coagulation system. Accordingly, both SEC injury and coagulation system activation are characteristics of MCT/LPS-induced liver injury. Interestingly, a study in isolated HPCs cotreated with MCT and LPS failed to reproduce the synergistic injury. This indicates that the enhanced toxicity did not result from a direct interaction of MCT and LPS with HPCs but from an indirect mechanism. Hence, other factors not present in the cell culture system, such as inflammatory cells and/or mediators, may play a role in the synergistic injury *in vivo*. Indeed, inactivation/depletion of inflammatory events, such as Kupffer cells (KCs), tumor necrosis factor (TNF)-α, neutrophils (polymorphonuclear leukocytes; PMNs) and the coagulation system, attenuated HPC and SEC injury. Although the CL and MZ lesions were qualitatively unchanged, the area of both liver lesions decreased with inactivation/depletion of these inflammatory events. Thus, KCs, PMNs, TNF-α and the coagulation system are critical components of this liver injury model. In conclusion, coexposure to small, noninjurious doses of MCT and LPS results in synergistic hepatotoxicity. A complex inflammatory mechanism involving KCs, PMNs, TNF-α and the coagulation system is important to the pathogenesis of MCT/LPS-induced liver injury. This work provides additional evidence that exposure to small amounts of LPS may be a determinant of susceptibility to food-borne hepatotoxins.

Dedications

To my parents, Bruce and Elizabelle Yee, who always believed in me, and to Aunt Pauline for all of her support and encouragement.

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at a moments notice. He, along with Fredo, provided some much needed cheer and fun during all those late night studies.

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LIST OF ABBREVIATIONS

AG	aminoguanidine
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP-1	activating protein-1
ARE	antioxidant response element
AST	aspartate aminotransferase
ATS	anti-rat TNF-α serum
BPI	bactericidal/permeability-increasing protein
$CaCl_2$	calcium chloride
CD	cluster of designation
CINC-1	cytokine induced neutrophil chemoattractant-1
CL	centrilobular
cNOS	constitutive nitric oxide synthase
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CS	control serum
CV	central vein
CVEC	central vein endothelial cell
CYP	cytochrome P-450
DIC	disseminated intravascular coagulation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dUDP	deoxyuridine triphosphate
ECL	enhanced chemiluminescence
EGM-2	endothelial cell medium
ELISA	enzyme linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
EU	endotoxin unit
$FeCl_3$	ferric chloride
g	gram
$GdCl_3$	gadolinium chloride
GGT	gamma-glutamyl transferase
GI	gastrointestinal
GRO	growth related oncogene
GSH	reduced glutathione
hr	hour
HA	hyaluronic acid
HBSS	Hank's balanced salt solution
HCl	hydrochloric acid
HDL	high density lipoprotein
HEP	heparin

HPC	hepatic parenchymal cell
HPF	high power field
HRP	horseradish peroxidase
HVOD	hepatic veno-occlusive disease
ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
IL	interleukin
IL-1	interleukin-1
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
ip	intraperitoneal
IRAK	IL-1 receptor associated kinase
iv	intravenous
JNK	c-Jun Kinase
KC	Kupffer cell
KDO	2-keto-3-deoxy-D-manno-octonic acid
kg	kilogram
L	liter
LBP	LPS binding protein
LDH	lactate dehydrogenase
LPF	low power field
LPS	lipopolysaccharide
M	molar
MAP	mitogen activated protein
MCP-1	monocyte chemoattractant protein-1
MCT	monocrotaline
MCTP	monocrotaline pyrrole
µg	microgram
mg	milligram
$MgSO_4$	magnesium sulfate
µl	microliter
ml	milliliter
µm	micrometer
µM	micromolar
mM	millimolar
MOF/MODS	multiple organ failure/dysfunction syndrome
MPO	myeloperoxidase
MZ	midzonal
N	number
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NAS	neutrophil anti-serum
NF-κB	nuclear transcription factor-kappaB

NF-IL6	nuclear transcription factor-interleukin-6
nm	nanometer
NO	nitric oxide
NO_x	nitrate and nitrite
NOAEL	no observed adverse effect level
NP	nonparenchymal
NS-398	N-(2-cyclohexyloxy-4-nitro-phenyl)methane sulfonamide
PA	pyrrolizidine alkaloid
PAF	platelet activating factor
PAR	protease activated receptor
PAR-1	protease activated receptor-1
PBS	phosphate buffered saline
pg	picogram
PG	prostaglandin
PGD_2	prostaglandin D2
PGE_1	prostaglandin E1
PGE_2	prostaglandin E2
PGI_2	prostacyclin
pH	power of hydrogen
PMN	polymorphonuclear leukocyte; neutrophil
PP	periportal
PT	portal triad
PTX	pentoxifylline
PV	portal vein
RBC	red blood cell
RECA-1	rat endothelial cell antigen-1
RNA	ribonucleic acid
ROS	reactive oxygen species
sCD14	soluble CD14
SEC	sinusoidal endothelial cell
SEM	standard error of the mean
TdT	terminal deoxynucleotidyl transferase
TEM	transmission electron microscope
TF	tissue factor
TLR	toll-like receptor
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling
U	unit
VCAM-1	vascular cell adhesion molecule-1
VII	proconvertin
WARF	warfarin
X	Stuart-Prower factor
XII	Hageman Factor

CHAPTER 1

General Introduction

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1.A. Overview

Some individuals are more susceptible to chemical intoxication than others. There are a variety of determinants that can contribute to this "hypersusceptibility". These include age, sex, diet, disease state, drug interaction, genetics and xenobiotic absorption and metabolism (Grandjean, 1995; Roth *et al.*, 1997; Ganey and Roth, 2001). Another determinant of susceptibility, albeit an understudied one, is inflammation. Although exposure to a modest amount of an inflammagen such as bacterial lipopolysaccharide (LPS) may be insufficient to result in overt tissue injury, the accompanying release of inflammatory mediators has the potential to alter cellular homeostasis (Michie *et al.*, 1988; Hewett *et al.*, 1993; Spitzer and Mayer, 1993; Roth *et al.*, 1997). This may result in tissues that are hypersusceptible to chemical-induced injury (Hewett *et al.*, 1993; Hewett and Roth, 1993; Roth *et al.*, 1997). Accordingly, exposure to LPS may be an important determinant of susceptibility to chemical intoxication (Roth *et al.*, 1997; Ganey and Roth, 2001).

Hepatotoxic chemicals can initiate injury through a variety of mechanisms that can include, but are not limited to, oxidant damage, lipid peroxidation and covalent binding to cellular macromolecules and DNA. Exposure to large doses of these hepatotoxicants leads to overt tissue injury, whereas exposure to smaller doses may alter cellular homeostasis by the same mechanism but not lead to overt tissue injury. Coexposure to LPS-induced inflammatory mediators, that in and of itself would be noninjurious, might result in substantive injury in a

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tissue already predisposed by homeostatic alterations from a subtoxic dose of an hepatotoxicant (Roth *et al.*, 1997; Ganey and Roth, 2001).

The overall objective of this dissertation is to characterize the development of liver injury resulting from coexposure to small, noninjurious doses of monocrotaline (MCT) and LPS, and to explore the mechanisms behind this liver injury model. **In particular, this dissertation tests the hypothesis that inflammatory events participate causally in the synergistic hepatotoxicity from coexposure to MCT and LPS.** Consequently, this dissertation can be divided into two sections. The first section, which consists of chapters 2, 3 and 4, characterizes the development of the MCT/LPS-cotreatment model of liver injury. Following a brief overview (Chapter 1) concerning LPS and MCT toxicity, the MCT/LPS-cotreatment model is developed and characterized in Chapter 2. The effect of the temporal relationship between MCT and LPS coexposure on toxicity is explored in Chapter 3. Next, coagulation system activation and endothelial cell injury are shown to be characteristics of MCT/LPS-induced liver injury (Chapter 4). The second section, consisting of chapters 5, 6 and 7, investigates the inflammatory events involved in the pathogenesis of MCT/LPS-induced liver injury. Evidence that Kupffer cells (KCs) and tumor necrosis factor (TNF)-α are both critical mediators in this synergistic hepatotoxicity is presented in Chapter 5. Neutrophils (polymorphonuclear leukocytes; PMNs) are also shown to be critical to MCT/LPS-induced liver injury. The coagulation system is demonstrated to contribute to the pathogenesis of this model in Chapter 7. Finally, in Chapter 8, the results of these studies are

summarized and a proposal for a mechanism of injury for MCT/LPS-induced hepatotoxicity is discussed.

1.B. Liver Structure and Function

The liver serves as a filter between blood from the portal venous system (which drains the stomach and intestines) and the systemic circulation. Consequently, the liver is exposed to substances (e.g., nutrients, food additives and contaminants, xenobiotics, etc) originating from the gastrointestinal (GI) tract, some of which may be potentially toxic to the liver. The liver is extensively vascularized and receives blood from two different sources. The largest proportion of blood is supplied by the hepatic portal vein. This blood is full of nutrients but is poorly oxygenated. The hepatic artery also delivers blood, albeit a smaller proportion but highly oxygenated, to the liver. Blood drains from the liver via the central vein and enters the inferior vena cava to go on to the systemic circulation. Accordingly, due to its position, structure and function, the liver may be a potential target for injury from toxic substances.

The liver has a variety of important physiological functions besides xenobiotic metabolism. These functions include vitamin storage and metabolism, heme synthesis and degradation, conversion of ammonia (generated from protein and nucleic acid catabolism) to urea (i.e., urea cycle), carbohydrate metabolism, bile synthesis and secretion, lipid and cholesterol metabolism, plasma protein synthesis and the filtration of particulate matter (e.g., cell debris,

bacteria, foreign substances, etc.). The majority of these functions are performed by cuboidal epithelial cells, which are also known as hepatic parenchymal cells (HPCs). HPCs make up approximately 70% of the cell number and 90% of the mass of the liver. The remaining liver cells (i.e., nonparenchymal (NP) cells) consist of sinusoidal endothelial cells (SECs; which make up 15% of the cells in the liver and about 3% of the total liver volume), bile duct epithelial cells, resident macrophages (Kupffer cells; KCs), fat storing stellate cells (Ito cells) and pit cells (natural killer cells; Laskin, 1996; Vandenberghe, 1996; Popp and Catley, 1998).

The liver lobule is the basic structural unit of the liver and consists of cords of HPCs radiating outward from a central vein. The lobule is bordered by several portal triads, each consisting of branches of the portal vein, hepatic artery and bile duct. HPCs in the immediate vicinity (perhaps as far as 4 to 6 HPCs away) of the central vein and the portal triad regions of the liver lobule are referred to as centrilobular (CL) and periportal (PP), respectively. HPCs located between the CL and PP regions are referred to as midzonal (MZ; Vandenberghe, 1996; Popp and Catley, 1998).

Blood entering the liver via the hepatic artery and the portal vein percolates down the space between the cords of HPCs and collects into a branch of the central vein. The space between the cords of HPCs is known as the sinusoid. Sinusoids are considered specialized capillaries with discontinuous basement membranes and are lined with fenestrated SECs. These cells act as a sieve for fluids and particles, which are exchanged between the sinusoidal lumen

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and the space of Disse, wherein lie HPCs (Wisse *et al.*, 1996). Thus, SECs serve as a leaky barrier to HPCs and are responsible for exchange and transport of substances between blood and HPCs. SECs also have a high endocytotic capacity. Consequently, they may assist KCs in the clearance of LPS (Praaning-van Dalen *et al.*, 1981; Nakao *et al.*, 1994; Wisse *et al.*, 1996) and are primarily responsible for the elimination of hyaluronic acid (HA), a glycosaminoglycan component of the extracellular matrix (Laurent and Frazier, 1992). Another NP cell type found in the sinusoid is the KC. These macrophages are responsible for the removal from the circulation of senescent red blood cells (RBCs) as well as cell debris and foreign substances, such as LPS. Also, scattered throughout the sinusoids are Ito and pit cells. Finally, it should be noted that HPCs synthesize bile and secrete it into bile canaliculi, which are formed from tight junctions between adjacent HPCs. Bile flows down these canaliculi and empties into bile ducts located in the portal triads. For more detailed reviews on liver structure and physiology, see Vandenberghe (1996) and Popp and Catley (1998).

1.C. Bacterial Lipopolysaccharide

1.C.1. Structure

LPS is an integral constituent of the outer cell membrane of Gram-negative bacteria, such as *Escherichia coli, Salmonella, Psuedomonas, Shigella,* and others (Rietschel and Brase, 1992; Hewett and Roth, 1993; Holst *et al.*, 1996; Mayeux, 1997; Vaara, 1999). It is an amphiphilic molecule that varies in

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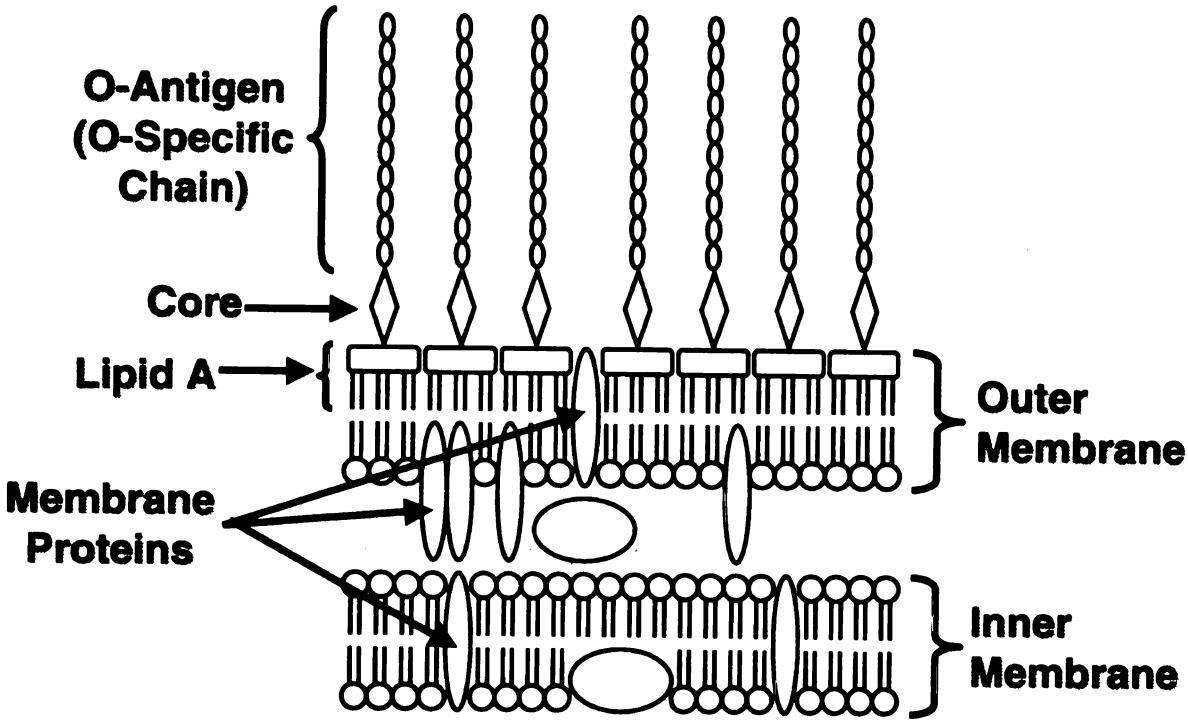
chemical composition among Gram-negative bacterial species. The surface of one bacterium has been estimated to contain approximately 3.5 X 10^6 LPS molecules (Nikaido, 1996). From a biological perspective, mammalian hosts use LPS as an indicator for the specific recognition and elimination of invading Gram-negative bacteria.

The general architecture of LPS consists of three regions: lipid A, a core polysaccharide and a somatic (O) antigen or O-specific chain (Figure 1.1; Rietschel and Brase, 1992; Holst *et al.*, 1996; Mayeux, 1997). Lipid A is a phosphoglycolipid component of LPS that anchors the molecule to the outer cell membrane (Holst *et al.*, 1996; Mayeux, 1997). This component is responsible for most of the biologic effects of LPS, both adverse and beneficial (Rietschel and Brase, 1992; Hewett and Roth, 1993; Holst *et al.*, 1996). Lipid A is linked to the O-antigen via a core polysaccharide. This core polysaccharide consists of a short chain of sugars that contains two unusual sugars, heptopyranose and 2-keto-3-deoxy-D-manno-octonic acid (KDO). KDO is present in all endotoxins and directly links the polysaccharide to lipid A (Rietschel and Brase, 1992; Holst *et al.*, 1996). Lastly, the O-antigen or O-specific chain is the variable region of LPS responsible for both diversity of LPS effects among different strains of Gram-negative bacteria and the antigenic component for a particular strain (Rietschel and Brase, 1992; Holst *et al.*, 1996). The O-antigen is the outermost portion of LPS (i.e., in contact with the external environment) and consists of up to 50 oligosaccharides, with each oligosaccharide consisting of between two to eight sugar monomers (Holst *et al.*, 1996).

Figure 1.1. Structure of LPS. LPS resides in the outer membrane of Gram-negative bacteria. LPS can be divided into 3 regions. Lipid A is the innermost portion of this molecule and attaches the molecule onto the outer cell membrane. The core polysaccharide region consists of two unusual sugars, a heptopyranose and KDO. This core region links lipid A and the O-antigen (or O-specific chain). The outermost region is the O-antigen and is made up of a variable number of oligosaccharides.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The *Limulus* amebocyte lysate assay is often used to quantify LPS in plasma. In this assay, LPS activates a proenzyme in the lysate of amebocytes isolated from horseshoe crab (*Limulus polyphemus)* blood. The resulting activated enzyme then cleaves a chromogenic peptide substrate and the absorbance is read. The amount of LPS is compared against a standard curve consisting of U.S. standard endotoxin. Results are expressed in endotoxin units (EUs) per ml of plasma. One EU is equivalent to the activity of 100 pg of U.S. standard endotoxin (Obayashi *et al.*, 1985; Mayeux, 1997). The LPS dose used in subsequent chapters of this dissertation is 7.4×10^6 EU/kg. Hence, the dose is equivalent to 7.4×10^8 pg/kg or 0.74 mg/kg of the U.S. standard endotoxin.

LPS is essential for the growth and survival of Gram-negative bacteria. In conjunction with the outer membrane, it acts as a permeability barrier to exclude hydrophobic molecules and hydrophilic molecules greater than 600 Daltons (Nikaido, 1996; Vaara, 1999). As a consequence, LPS affords protection from a variety of toxic mediators from the environment and the host (e.g., intestinal phospholipases and bile acids). Overall, LPS is required for Gram-negative bacterial growth, viability and effective reproduction (Rietschel and Brase, 1992; Holst *et al.*, 1996; Nikaido, 1996; Vaara, 1999).

In terms of nomenclature, it should be noted that LPS is sometimes used interchangeably with the term "endotoxin." However, there is a difference between the two. Endotoxin consists of LPS in conjunction with outer cell membrane macromolecules (proteins) that may or may not have additional

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biologic activities (Hewett and Roth, 1993; Vaara, 1999). This dissertation will use the term LPS whenever possible.

1.C.2. Sources of LPS in the Host

Systemic LPS exposure can occur through a variety of events, including Gram-negative bacterial infection, respiratory tract exposure to Gram-negative bacteria or LPS-contaminated organic particles and LPS translocation from endogenous Gram-negative bacterial flora in the GI tract (Ditter *et al.*, 1983; Roth *et al.*, 1997). LPS can be released either from proliferating bacteria or from the lysis of bacterial cell walls (e.g., via autolysis, complement, phagocytotic cells, or antibiotics; Rietschel and Brase, 1992; Hewett and Roth, 1993; Mayeux, 1997; Roth *et al.*, 1997). Ironically, though antibiotics abrogate bacterial infection, LPS may greatly increase as a result of bacterial cell death (Jackson and Kropp, 1999). It has been estimated that bactericidal antibiotic therapy during sepsis can increase LPS concentration as much as 2000 fold (Shenep and Morgan, 1984; Shenep *et al.*, 1988).

Exogenous LPS exposure can arise from loci of Gram-negative bacterial infections (which include such bacterial growth from biofilms on implanted medical devices) and through inhalation of Gram-negative bacteria or LPS-contaminated particles (Potera, 1996; Roth *et al.*, 1997). Indeed, inhalation exposure to LPS is relatively common. Gram-negative bacterial pneumonias account for a large number of hospital-related deaths (Leu *et al.* 1989; Roth *et al.*, 1997). Moreover, increased exposure of the respiratory tract to LPS can

occur during occupational conditions (such as in the poultry and swine industry, waste treatment plants, grain and cotton processing and others) and from air from offices or households that contain LPS-laden dust particles and bioaerosols (as reviewed in Roth *et al.*, 1997). Accordingly, exposure to exogenous sources of LPS is ubiquitous, appearing in many media (e.g., dust, air, water and so forth), not just loci of bacterial infection.

Endogenous exposure to LPS can occur from the translocation of LPS through the intestinal mucosa into the circulation (Deitch, 1990; Hewett and Roth, 1993; Roth *et al.*, 1997; Fink and Mythen, 1999). Large, endogenous populations of microbes, which include Gram-negative bacteria, exist in the ileum and colon of the mammalian GI tract and function to aid in host digestion (Fink and Mythen, 1999). Under normal conditions, it is believed that the mucosal lining of the GI tract is a good but not impermeable barrier to translocation. Consequently, a small amount of LPS is known to cross the GI tract and enter the circulation regularly (Jacob *et al.*, 1977; Roth *et al.*, 1997). Under a variety of pathologic conditions, however, increased bacterial and LPS translocation and intestinal mucosal permeability are observed. These conditions include hemorrhage, trauma, bowel ischemia and reperfusion, alterations in diet, alcohol consumption, lifestyle conditions, exposure to cytotoxic chemotherapy and endotoxemia (Hewett and Roth, 1993; Roth *et al.*, 1997; Fink and Mythen, 1999).

Once LPS translocates across the GI tract, it must pass through the liver before entering the systemic circulation. The liver has a vital role in LPS clearance via KCs and, to a lesser extent SECs and PMNs, to prevent systemic

endotoxemia (McCuskey *et al.*, 1984; Munford and Hall, 1986; Hewett and Roth, 1993). Additionally, it is known that LPS binds to certain plasma constituents (e.g., lipoproteins) as well as to natural anti-LPS antibodies, which may act to neutralize it, possibly serving as an additional protective mechanism against LPS toxicity (Ulevitch, 1981). Therefore, a variety of cells and mediators are responsible for clearing LPS and preventing its toxicity.

In summary, modest LPS exposure (i.e., mild endotoxemia) is commonplace and episodic, tending to vary with the clinical condition of the individual, lifestyle, disease, and other factors (Hewett and Roth, 1993; Roth *et al.*, 1997). In extreme conditions (e.g. sepsis), LPS or other inflammagens cause overt tissue injury to organs. More modest LPS exposure results in a mild inflammatory response but without tissue injury, and this could contribute to the toxic response to other stresses (Ganey and Roth, 2001).

1.C.3. Host Response to LPS

LPS exposure, a signal of bacterial infection, can result in a potent inflammatory response in mammals (Raetz *et al.*, 1988; Rietschel and Brase, 1992; Hewett and Roth, 1993; Roth *et al.*, 1997; Mayeux, 1997). Normally, inflammation is part of a controlled, natural process elicited for survival, but an excessive inflammatory response to LPS can lead to deleterious pathophysiological events. These events, however, are highly dependent on the quantity, duration and route of LPS exposure, as well as the bioactivity of LPS (Raetz *et al.*, 1988; Rietschel and Brase, 1992; Jackson and Kropp, 1999).

Paradoxically, LPS may also enhance the host's immune resistance to bacterial and viral infections and cancer (Rietschel and Brase, 1992; Mayeux, 1997; Zhang and Tracey, 1999). In this regard, it is thought that the minimal level of LPS translocation from the GI tract into the circulation may be part of a normal physiologic process. Consequently, LPS may be employed in the development and maintenance of intestinal and systemic immunity (Deitch, 1995), as well as in regulating tissue homeostasis (Komatsu *et al.*, 2000; Ganey and Roth, 2001). For more information on the beneficial effects of LPS, read Rietschel and Brase (1992) and Deitch (1995).

Of particular interest to medical science, however, is the detrimental pathophysiological responses to LPS that occur under conditions of a severe infection or large accumulation of LPS in the circulation (Rietschel and Brase, 1992). These include chills, fever, headache, nausea, leukopenia, systemic hypotension, circulatory shock, adult respiratory distress syndrome, disseminated intravascular coagulation (DIC), multiple organ failure/dysfunction syndrome (MOF/MODS) and death (Holst *et al.*, 1996; Mayeux, 1997; Suffredini and O'Grady, 1999). Systemic sepsis is defined as the presence of bacteria or its toxin in blood or tissue of a host organism that induces a systemic inflammatory response (Mayeux, 1997, Karima *et al.*, 1999). Septic shock arises as a progression of sepsis toward organ dysfunction with hypotension that cannot be controlled by the administration of fluids (Mayeux, 1997). It has been estimated that between 20 to 50% of the 300,000 to 500,000 patients that develop sepsis in the United States each year will develop septic shock (Mayeux, 1997, Karima *et*

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al., 1999). Out of that number, it is further estimated that 50-70% of these patients will die (Dal Nagore, 1991; Mayeux, 1997, Karima *et al.*, 1999). Although a majority of these deaths result from refractory hypotension (i.e., septic shock), MOF/MODS accounts for a significant contribution to the mortality. In particular, organs with extensive vascular beds, such as the liver, lungs, and kidney, are extremely susceptible to dysfunction and failure (Mayeux, 1997, Karima *et al.*, 1999).

1.C.4. LPS Cell Signalling

LPS binds to high-density lipoproteins (HDL) in the circulation to form an LPS-HDL complex. Removal of LPS from this complex by LPS binding protein (LBP) results in the formation of an LPS-LBP complex (Schumann *et al.*, 1990; Karima *et al.*, 1999). LPS-LBP is known to interact with CD14 expressed on the surface of monocytes/macrophages and PMNs (Wright *et al.*, 1990; Watson *et al.*, 1994; Su *et al.*, 1995; Karima *et al.*, 1999). CD14 is a glycerophosphatidylinositol-linked, plasma membrane glycoprotein that in combination with LBP brings LPS to its putative receptor, Toll-like receptor 4 (TLR4; Poltorak *et al.*, 1998; Means *et al.*, 2000). Interestingly, LPS requires CD14 at small concentrations to elicit cellular responses; at large concentrations, however, LPS can stimulate cells through a CD14-independent mechanism. It is unclear what additional molecule recognizes LPS. Although this molecule is likely the TLR4 receptor, some studies have suggested that the TLR2 receptor may be involved but this remains controversial (Ingalls and Golenbock, 1995;

Yang *et al.,* 1998; Liu *et al.*, 2001b; Takeda and Akira, 2001; Andonegui *et al.*, 2002).

For cells that do not express CD14 (e.g., endothelial and smooth muscle cells), the LPS-LBP complex can interact with soluble CD14 (sCD14) that acts as a signal bridge for this LPS-LBP complex (Su *et al.*, 1995; Karima *et al.*, 1999). sCD14 is thought to be secreted or proteolytically cleaved from monocyte/macrophages. sCD14 facilitates systemic circulation and distribution of LPS, thereby promoting its systemic effects (Mayeux, 1997), and acts through the TLR4 receptor (Backhed *et al.*, 2002).

The binding of LPS to TLR4 initiates signaling mechanisms that result in the stimulation of inflammatory cells, activation of transcription factors and the synthesis and release of proinflammatory cytokines. The TLR-induced inflammatory response is dependent on a common signaling pathway that is mediated by the adaptor molecule MyD88. There are also additional pathways that mediate the TLR-induced inflammatory response. Upon stimulation, MyD88 recruits IL-1R-associated kinase (IRAK). Activated IRAK results in the activation of the mitogen-activated protein (MAP) kinase (via c-Jun kinase (JNK), extracellular-signal-regulated kinase (ERK) and p38) signaling pathway and ultimately the activation of transcription factors, such as nuclear factor κB (NFκB; Mukaida *et al.*, 1996; Nick *et al.*, 1996; Takeda and Akira, 2001; Takeda *et al.*, 2003). Activation of this pathway is important in regulating cell function and gene expression (Hill and Treisman, 1995). Moreover, activation of transcription factors of NFκB, activating protein-1 (AP-1) and nuclear factor-interleukin 6 (NF-

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IL-6) are involved in the transcription of many proinflammatory cytokines, adhesion molecules, tissue factor (TF) and inducible nitric oxide synthase (iNOS; Sweet and Hume, 1996; Meng and Lowell, 1997). A more detailed review of the LPS signaling pathway and TLRs is beyond the scope of this introduction. For more information, see the reviews of Takeda and Akira (2001) and Takeda *et al.* (2003).

1.C.5. LPS Clearance

Following intravenous administration of LPS in rats, LPS is cleared from the circulation in a biphasic manner (Warner *et al.*, 1988; Hewett and Roth, 1993). The vast majority of LPS is cleared within seconds after administration. The remainder of LPS elimination occurs over a period of hours (i.e., between 1 and 30 hours in rats; Freudenberg *et al.*, 1984; Warner *et al.*, 1988). This latter elimination of LPS is dependent on a variety of factors such as dose, type of LPS and interactions with plasma constituents (e.g., HDL; Freudenberg *et al.*, 1980; Warner *et al.*, 1988). LPS is found in the liver, lungs, spleen, kidney and other tissues. Circulating LPS accumulates in the liver of rats, where it is primarily eliminated (Praaning-Van Dalen *et al.*, 1981; Warner *et al.*, 1988; Hewett and Roth, 1993). As previously described, KCs, PMNs, and SECs are ultimately involved in clearing LPS (McCuskey *et al.*, 1984; Munford and Hall, 1986; Hewett and Roth, 1993).

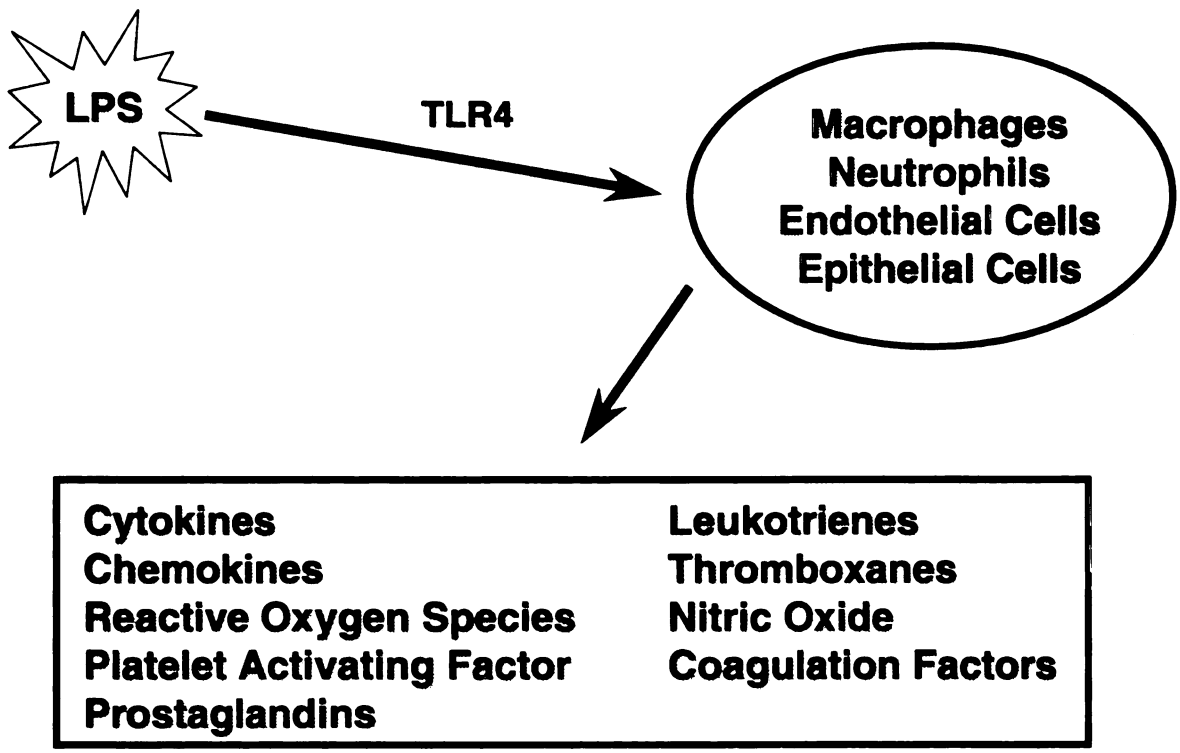
1.C.6. Inflammatory Response to LPS

The innate immune system is responsible for providing a rapid, nonspecific response to invading pathogens that does not require prior exposure to initiate its activity. LPS indirectly or directly activates innate immune cells (i.e., monocytes, macrophages and PMNs as well as other cell types, including platelets and endothelial cells. This results in the release of inflammatory mediators such as interleukins (ILs), TNF-α, platelet activating factor (PAF), arachidonic acid metabolites, reactive oxygen species (ROS), nitric oxide (NO) and lysosomal enzymes (Figure 1.2; Hewett and Roth, 1993; Holst *et al.*, 1996; Karima *et al.*, 1999). LPS can also activate complement. Finally, LPS activation of the coagulation system is important in generating some of the pathophysiologic responses to endotoxemia (Hewett and Roth, 1993; Karima *et al.*, 1999; Copple *et al.*, 2003). It should be noted that this section provides only a brief overview of the cells and mediators stimulated by LPS or its mediators. Whenever possible, priority is given to examples of LPS effects on the liver. For a more comprehensive review of the inflammatory response to LPS, please read Hewett and Roth (1993), Holst *et al.* (1996), and/or Karima *et al.* (1999).

1.C.6.1. Monocytes/Macrophages and Kupffer Cells

Monocytes/macrophages are part of the nonspecific or innate immune system and function by phagocytosing and killing invading bacteria (Hewett and Roth, 1993). Macrophages are produced in the bone marrow and enter the circulation as blood monocytes. These monocytes then migrate to various tissues and differentiate into tissue macrophages. LPS-activated macrophages

Figure 1.2. Inflammatory Response to LPS. LPS can interact with an LBP to combine with CD14. The LPS-LBP-(s)CD14 complex (not shown) interacts with TLR4 in cells to generate the release of a plethora of inflammatory mediators from stimulated cells. LPS is also responsible for the direct (e.g., activation of Factor XII) and indirect (e.g., tissue factor) activation of the coagulation system.


LPS
TLR4
Macrophages
Neutrophils
Endothelial Cells
Epithelial Cells
Cytokines
Chemokines
Reactive Oxygen Species
Platelet Activating Factor
Prostaglandins
Leukotrienes
Thromboxanes
Nitric Oxide
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are responsible for the release of a great many inflammatory mediators (Michie *et al.*, 1988; Rietschel and Brase, 1992). Amongst the numerous endogenous mediators released are the proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-8 (or, its equivalent in rats: cytokine-induced neutrophil chemoattractant (CINC)-1), and interferon-γ (Rietschel and Brase, 1992; Hewett and Roth, 1993; Hack *et al.*, 1997; Roth *et al.*, 1997, Karima *et al.*, 1999). NO, along with various ROS, is also released (Rietschel and Brase, 1992; Karima *et al.*, 1999). Lipid mediators, such as PAF, prostaglandins (PGs), thromboxanes, and leukotrienes, are released as well (Rietschel and Brase, 1992; Karima *et al.*, 1999). Hence, macrophages play a key role in LPS responses since many of the mediators released by these cells activate or modulate the effect on other cells involved in the inflammatory process (Marshall *et al.*, 1987; Hewett *et al.*, 1993; Holst *et al.*, 1996).

1.C.6.1.1. Monocytes

Monocytes originate in the bone marrow from promyoblast stem cells and differentiate into macrophages. Following an inflammatory event in a tissue (e.g., liver, lung, etc.), the number of monocytes increases gradually over time (significantly slower than PMN accumulation), reaching maximal accumulation within approximately 24 hours (O'Grady *et al.*, 2001). Activated monocytes release TNF-α and monocyte chemoattractant protein (MCP)-1 and express TF, which can cause coagulation system activation (Osterud, 1995; Polack *et al.*, 1997). TF is a transmembrane glycoprotein that acts as a cell receptor for

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activated factor VII. The activated factor VII and TF form a complex that in turn is responsible for the activation of factor X, resulting in activated factor X. Activated factor X cleaves prothrombin to form active thrombin. Hence, TF induction results in activation of the coagulation system, thrombin generation and fibrin deposition (de Prost, 1995; Shebuski and Kilgore, 2001). Interestingly, TF induction in monocytes may be dependent on superoxide anion radical (i.e., ROS) and NO generation (Polack *et al.*, 1997). TF has a critical role in DIC (Karima *et al.*, 1999).

1.C.6.1.1.1 Monocyte Chemoattractant Protein-1

MCP-1 is expressed in monocytes, macrophages, stellate cells, HPCs and endothelial cells (Dambach *et al.*, 2002). Expression is induced by oxidative stress, LPS, thrombin and proinflammatory cytokines such as TNF-α and IL-1β. This chemokine is involved in the attraction and activation of monocytes and in the induction of TF expression on the surface of monocytes and macrophages (Luster, 1998; Gu *et al.*, 1999; Dambach *et al.*, 2002). As noted above, TF can lead to activation of the coagulation system (Osterud, 1995; Polack *et al.*, 1997). Furthermore, MCP-1 has been implicated in the expression of intercellular adhesion molecule (ICAM)-1 on rat endothelial cells *in vitro* (Yamaguchi *et al.*, 1998). This adhesion molecule, which is present in SECs (Essani *et al.*, 1995), can interact with PMNs and prime them to release toxic products (Jaeschke *et al.*, 1996).

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1.C.6.1.2. Kupffer Cells

KCs are the resident macrophage of the liver. They reside in hepatic sinusoids where they have a major role in clearing the portal blood of particulate matter, including LPS. These cells remove the polysaccharide moieties of LPS and send the modified LPS to HPCs to be metabolized further. Additionally, KCs help to maintain liver homeostasis by regulating protein synthesis, protein phosphorylation and glycogenolysis in neighboring HPCs (West *et al.*, 1986, 1987, 1988; Castelijn *et al.*, 1988a, 1988b).

KCs are critical to the development of liver injury for a number of different xenobiotics, including carbon tetrachloride (Badger *et al.*, 1996; Wueweera *et al.*, 1996), vinylidene chloride (Wueweera *et al.*, 1996), acetaminophen (Michael *et al.*, 1999), D-galactosamine (Stachlewitz *et al.*, 1999), LPS (Iimuro *et al.*, 1994; Fujita *et al.*, 1995; Sarphie *et al.*, 1996; Brown *et al.*, 1997), and ethanol (Adachi *et al.*, 1994). Although the mechanism by which KCs contribute to injury in these models has not been fully defined, it appears that both oxidative stress and TNF-α release may be important (Iimuro *et al.,* 1994; Ishiyama *et al.*, 1995; Hoglen *et al.*, 1998; Michael *et al.*, 1999; Stachlewitz *et al.*, 1999).

LPS-stimulated KCs become swollen and contain increased numbers of cytoplasmic lysomal granules and phagocytic vacuoles after LPS stimulation. In this active state, KCs produce and release numerous mediators, including TNF-α, IL-1β, IL-6, IL-8 (i.e., CINC-1 in rats), PGE_2, PGD_2, PAF, ROS and NO (Michie *et al.*, 1988; Holst *et al.*, 1996; Decker, 1997).

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1.C.6.1.2.1. Tumor Necrosis Factor-α

LPS exposure stimulates the expression of various cytokines, including TNF-α. It is the first cytokine detected in the host after exposure to Gram-negative bacteria or LPS. A transient increase in circulating TNF-α concentration occurs within 1 – 2 hours after LPS exposure (Hewett *et al.*, 1993).

A number of studies have suggested that TNF-α may contribute to the pathophysiological events (e.g., hypotension and lethality) observed with toxic LPS exposures (Hewett and Roth, 1993). To varying degrees, TNF-α is produced by many cell types including but not limited to, KCs, PMNs, monocytes, endothelial cells and epithelial cells (Vasselli, 1992; Lo *et al*, 1997; Bradham *et al.*, 1998; Zhang and Tracey, 1998). KCs are the major source of this cytokine in the liver (Vasselli, 1992; Zhang and Tracey, 1998). TNF-α can prime and/or activate a number of cells in the liver such as HPCs, liver endothelial cells (e.g., SECs), PMNs and KCs. Activities of TNF-α are mediated through two receptor types found on nearly all cells, TNF receptor types I and II. The type I receptor is responsible for many of the biological activities, as well as activating a pathway to apoptosis (Zhang and Tracey, 1998).

TNF-α can stimulate endothelial cells (including SECs) to increase their permeability, promote coagulation (i.e., via TF expression), and release or express plasminogen activator inhibitor-1, NO, IL-1, ICAM-1, vascular cell adhesion molecule (VCAM)-1, prostacyclin, PAF, and NO (Zhang and Tracey, 1998). Many of these mediators in high quantities are capable of creating deleterious effects in nearby cells and tissues. Additionally, TNF-α has been

shown to have a cytotoxic effect on vascular endothelial cells *in vitro* (Sato *et al.*, 1986; Pohlman and Harlan, 1992) as well as SECs both *in vitro* and *in vivo* (Mochida *et al.*, 1995; Knolle *et al.*, 1996).

In general, TNF-α can exert a variety of effects on cells, ranging from mitochondrial damage and oncotic or apoptotic necrosis to cell proliferation (Bradham *et al.*, 1998; Jaeschke *et al.*, 1998). Indeed, TNF-α can render tissue more sensitive to toxicity and/or can promote tissue injury. For example, *in vitro* TNF-α can render HPCs more susceptible to toxicity (Adamson and Billings, 1992; ElSisi *et al.*, 1993; Hoek and Pastorino, 2002). Alternatively, HPCs altered homeostatically by the actions of hepatotoxicants may be sensitive to TNF-α-induced cell killing (Lawson *et al.*, 1998; Jaeschke *et al.*, 1998). In addition, TNF-α may prompt the accumulation of PMNs by activating endothelial cells (Vasselli, 1992; Bradham *et al.*, 1998) and can indirectly promote toxicity by priming PMNs and KCs to release ROS and proteases that damage nearby cells (Vadas and Gamble, 1990; Nagaki *et al.*, 1991; Vasselli, 1992; Kushimoto *et al.*, 1996, Johnson *et al.*, 1998). Accordingly, because of its role in activating and/or priming inflammatory cells and modulating the effects of other cells, it is considered to be critical in the host response to LPS.

1.C.6.1.2.2. Interleukin-1

IL-1 is a potent proinflammatory cytokine produced by a variety of cell types including macrophages, epithelial and endothelial cells (Dinarello, 1988). This cytokine has effects similar to TNF-α and exists in two forms, IL-1α and IL-

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1β. The biologic activity of either form is nearly indistinguishable from the other. IL-1 enhances the growth of T-lymphocytes, induces the expression of adhesion molecules on endothelium, stimulates rat hepatocytes to release CINC-1, enhances the response of PMNs to TNF-α and elicits the release of inflammatory mediators such as PGs, IL-6 and TNF-α (Kunkel *et al.*, 1986; Movat *et al.*, 1987; Durum and Oppenheim, 1989; Mawet *et al.*, 1996).

1.C.6.1.2.3. Interleukin-6

IL-6 is produced by a variety of cells such as macrophages, endothelial cells and fibroblasts (Durum and Oppenheim, 1989). This cytokine enhances the synthesis of acute phase proteins (Kispert, 1992; Decker, 1997), and its production is greatly influenced by TNF-α and IL-1 (Durum and Oppenheim, 1989; Brouckaert *et al.*, 1993). IL-6 causes PMNs to produce PAF and enhances PMN phagocytic and oxidant production capabilities (Mullen *et al.*, 1995; Biffl *et al.*, 1996). IL-6 has also been identified as an activator of the coagulation system (Van der Poll *et al.*, 1994; Stouthard *et al.*, 1996). Indeed, IL-6 can induce monocytes and endothelial cells to express TF on their surfaces *in vitro* (Shebuski and Kilgore, 2001). Some recent reports suggest that IL-6 may have anti-inflammatory properties as well (Marchant *et al.*, 1999).

1.C.6.1.2.4. Interleukin-8/CINC-1

IL-8 is produced by a great many human cell types including monocyte/macrophages, PMNs, endothelial cells, fibroblasts and hepatocytes

(Van Damme, 1994). This chemokine is a powerful PMN chemoattractant and PMN activator. It enhances numerous functions such as degranulation, chemotaxis, endothelial cell adherence, increased surface expression of key receptors and increased lipoxygenase pathway products (e.g., leukotrienes; Oppenheim *et al.*, 1991). IL-8 is apparent in human volunteers within 90 minutes of administering LPS and becomes maximal by 120 minutes (van Deventer *et al.*, 1993).

The rat equivalent to human IL-8 is CINC-1. This chemokine is produced by SECs, KCs and hepatocytes (Ohkubo *et al.*, 1998). TNF-α and IL-1 induce CINC-1 production by hepatocytes *in vitro* (Thorton *et al.*, 1991; Mawet *et al.*, 1996; Ohkubo *et al.*, 1998). 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 overexpression of CINC-1 in rat liver results in PMN accumulation. Hence, CINC-1 is important in several models in recruiting PMNs into the rat liver (Zhang *et al.,* 1995; Luster, 1998).

1.C.6.1.2.5. Arachidonic Acid Metabolites

Activated macrophages and PMNs can produce a variety of arachidonic acid metabolites consisting of thromboxanes, leukotrienes, and PGs. These metabolites are involved in the production of pain and fever, induction of blood clotting, and inflammation. Thromboxanes mediate many of the vascular effects associated with endotoxic and septic shock. Leukotrienes (i.e., mainly leukotriene B_4) are highly chemotactic for PMNs (Doi *et al.*, 1993), though their

exact role in LPS-induced injury remains controversial (Keppler *et al.*, 1987; Matsuchak *et al.,* 1990; Pearson *et al.*, 1997). Lastly, PGs have many physiologic and regulatory functions. Following LPS exposure, PGs assist in regulating the production of proinflammatory cytokines (e.g., TNF-α, IL-1 and IL-6) and iNOS (Karck *et al.*, 1988; Callery *et al.*, 1990; Gaillard *et al.*, 1991). LPS activated-KCs produce PGE_1, PGE_2, PGD_2, PGI_2, and other PGs (Bowers *et al.,* 1985; Okumura *et al.*, 1987; Brouwer *et al.*, 1988).

Cyclooxygenases (COXs) are responsible for PG synthesis. COX has two isoforms. COX-1 is constitutively expressed as a housekeeping enzyme in most tissues and mediates physiological responses. COX-2 is expressed by inflammatory cells (e.g., monocytes and macrophages, such as KCs) and can be upregulated by various proinflammatory agents (i.e., LPS and cytokines) to enhance synthesis of PGs (Brouwer *et al.*, 1995; Dieter *et al.*, 1999). These mediators activate and/or modulate the function of HPCs or the effects of nearby inflammatory cells (e.g., PMNs) to contribute to pathophysiological alterations in tissues (Casteleijn, *et al.*, 1988c; Decker, 1990, 1997; Ganey *et al.*, 2001) Moreover, COX-2 products have been found to mediate liver injury in some models (Ganey *et al.*, 2001).

1.C.6.1.2.6. Platelet Activating Factor

LPS-activated KCs release PAF. PAF can stimulate KCs to release cytokines and form ROS. PAF can also stimulate peripheral blood monocytes to produce TNF-α (Lo *et al.*, 1997; Kuijpers and Van der Poll, 1999). PAF may be

involved in initial PMN infiltration of the liver, chemotaxis, adhesion to endothelial cells, degranulation and oxidative burst (Lorant *et al.*, 1991; Coughlan *et al.*, 1994; Zimmerman *et al.*, 1994). PAF is also involved in platelet stimulation, vasoconstriction and in the early stages of endotoxemia (Qi and Jones, 1990; Terashita *et al.*, 1992; Balsa *et al.*, 1997). Its role, however, in LPS-induced liver injury is controversial (Imura *et al.*, 1986; Pearson *et al.*, 1997).

1.C.6.1.2.7. Reactive Oxygen Species

Macrophages, including KCs, and PMNs produce oxygen free radicals to destroy invading pathogens. Molecular oxygen is converted to the superoxide anion radical, hydrogen peroxide, and ultimately to the hydroxyl radical. Both LPS activated macrophages and PMNs release a burst of ROS to directly injure/damage tissues (Arthur *et al.*, 1986; Arthur *et al.,* 1988; Bautista *et al.*, 1990; Mayer and Spitzer, 1991). Liver injury induced by ROS occurs in a number of models, and when ROS production is blocked liver injury is likewise prevented (Arthur *et al.*, 1985; Shiratori *et al.*, 1988). ROS can damage cells and tissues through the initiation of lipid peroxidation and oxidant damage to macromolecules such as proteins and DNA.

Although ROS release from macrophages and PMNs can lead to tissue injury directly, intracellular ROS or oxidants may have other functions. They can induce a variety of signal pathways from cell death (i.e., apoptotic and oncotic necrosis) to cell proliferation. In general, activation of a pathway involves the direct modification of a protein or other element of the signaling pathway by ROS.

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ROS can interact with a number of specific molecular targets including protein tyrosine kinase, ERK, MAPK, antioxidant response element (ARE), NF-κB and several caspases (Whitmarsh and Davis, 1996; Maher and Schubert, 2000; Li and Jackson, 2002). MAPK activation (namely, JNK and p38) has been implicated in inflammatory responses and cell cycle arrest, and, with some controversy, apoptosis. ARE is found in the promoter region of genes which encode antioxidant and detoxification proteins and is responsible for the coordinated transcription of such genes. Finally, NF-κB activation can lead to a number of proinflammatory responses including increased TNF-α transcription and release and neutrophil adhesion to vascular endothelium *in vivo* (Kim *et al.*, 2000; Maher and Schubert, 2000; Liu *et al.*, 2001a). There is some controversy, however, about whether ROS activation of NF-κB is required to generate proinflammatory mediators in LPS-activated macrophages (Chandel *et al.*, 2000). In summary, although the release of ROS by macrophages and PMNs can result in cytotoxic effects (i.e., oxidant injury) in nearby cells, ROS can have a role in signal transduction and transcription factor activation that may result in a number of outcomes from apoptotic cell death to antioxidant activation to the release of proinflammatory mediators (Schreck *et al.*, 1991; Canty *et al.*, 1999)

1.C.6.1.2.8. Nitric Oxide

NO is produced by nitric oxide synthase (NOS), which exists in two forms, constitutive (cNOS) and inducible (iNOS). cNOS is found in many different cells including endothelial cells and neurons, where NO is constitutively synthesized.

Macrophages (e.g., KCs) and PMNs have iNOS, which can be induced by cytokines and other agents. Following LPS exposure, iNOS is upregulated in macrophages resulting in NO production (Gaillard *et al.*, 1991; Laskin *et al.*, 1994). NO, a soluble free radical gas, has many different cellular effects including serving as an intracellular messenger, inducing vasodilation, inhibiting platelet aggregation and modulating leukocyte adhesion. NO has also been reported to relax contracted hepatic stellate cells and to potentially upregulate the catalytic activity of COX, though this latter ability is debated (Salvemini *et al.*, 1993; Karima *et al.*, 1999). Excessive formation of NO can lead to LPS-induced hypotensive shock (Ruetten and Thiemermann, 1996; Mayeux, 1997; Wolkow, 1998; Karima *et al.*, 1999) and this, although controversial, has been linked to lethality in experimental animals treated with a toxic dose of LPS (Takano *et al.*, 1997; Wolkow, 1998; Karima *et al.,* 1999). Additionally, NO in the presence of superoxide anion radical forms the cytotoxic peroxynitrite free radical, which can cause cellular injury (Decker, 1997).

1.C.6.2. Neutrophils.

PMNs are another important component of the innate immune system and are involved in LPS-induced pathophysiologic responses. PMNs originate from and largely mature in the bone marrow before being released into the general circulation. They are critical in the defense against invading pathogens and are the first cells to migrate to the site of inflammation and phagocytose bacteria and bacterial fragments (Hewett and Roth, 1993; Holst *et al.*, 1996). Moreover,

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PMNs can neutralize LPS through the release of lysosomal enzymes (e.g., acyloxacyl hydrolase; Luchi and Munford, 1993; Holst *et al.*, 1996). They can also release bactericidal/permeability-increasing protein (BPI), which is structurally similar to LBP and binds to neutralize LPS (Marra *et al.*, 1990). Hence, PMNs have a variety of mechanisms to neutralize invading pathogens and limit LPS toxicity.

Although PMNs have largely beneficial activities to protect the host from invading pathogens, they can cause HPC injury and necrosis in the liver. Following chemotaxis to the site of inflammation, adherence to endothelium and diapedesis into nearby tissues, activated PMNs can release proteases and other mediators (e.g., ROS) to facilitate injury (Gallin, 1989; Hewett and Roth, 1993; Holst *et al.*, 1996). Indeed, PMNs contribute to HPC and SEC injury in several models both *in vitro* and *in* vivo (Mavier *et al.,* 1988; Hewett *et al.*, 1992; Ganey *et al.,* 1994; Sakamoto *et al.*, 1997; Ohtsuka *et al.*, 2000).

ROS production in PMNs is dependent on NADPH oxidase activation. The end product of this enzyme is the superoxide anion radical, which can be converted to other cytotoxic free radicals such as the hydroxyl radical and peroxynitrites. With the additional release of myeloperoxidase, the oxidant hypochlorous acid can be formed. As previously noted, ROS can damage cells by initiating lipid peroxidation and causing oxidant damage to macromolecules (Bautista *et al.*, 1990; Spitzer and Mayer, 1993). Activated PMNs also release proteases (Mavier *et al.*, 1988; Harbrecht *et al.*, 1993; Ganey *et al.*, 1994) such as cathepsin G and elastase, which are cytotoxic to HPCs (Ho *et al.*, 1996).

Moreover, ROS can increase the cytotoxicity of these proteases by inhibiting the activity of plasma antiproteases (Jaeschke, 1997). Finally, ROS can activate the NF-κB pathways and consequently contribute to HPC injury by enhancing the inflammatory process (Schreck *et al.*, 1991; Canty *et al.*, 1999). Accordingly, similar to KCs, PMNs have a critical role in LPS-induced injury.

1.C.6.3. Platelets, Endothelial Cells, and Epithelial Cells.

Platelets, endothelial cells, smooth muscle cells and epithelial cells are known to contribute to the inflammatory response (Libby *et al.*, 1991; Pearson *et al.*, 1995; Shibayama *et al.*, 1995; Holst *et al.*, 1996;). Aside from participation in coagulation, platelets can release lipid mediators, such as PAF and thromboxane A_2, that contribute to the activation of other cells and to the development of hepatic injury (Shibayama *et al.*, 1995). Platelets can also release proteases, such as elastase and cathepsins, and ROS, which can promote tissue injury (Holmsen and Day, 1970; James *et al.*, 1985; Gorog and Kovacs, 1995). Following activation by LPS or cytokines such as IL-1 and TNFα, endothelial cells produce IL-1, IL-6, IL-8/CINC-1 and TF. Moreover, they express adhesion molecules and produce PGE_2, PGI_2, ROS, NO, PAF, and interferons (Loppnow and Libby, 1989; Camussi *et al.*, 1995; Holst *et al.*, 1996; Ohkubo *et al.*, 1998). Interestingly, LPS has a direct cytotoxic effect *in vitro* on endothelial cells at concentrations greater than 1 μg/ml (Harlan *et al.*, 1983; Rietschel and Brase, 1992); injury may be due to ROS generation and subsequent lipid peroxidation (Bringham *et al.*, 1987). LPS-activated human endothelial cells *in vitro* can

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trigger PMN granular secretion by unknown factors (Gill *et al.*, 1998). It is possible that if such an event occurred *in vivo*, it could initiate vascular injury. Hence, it is tempting to speculate that SECs potentially could have a role in initiating PMN-induced injury. Finally, after LPS stimulation, epithelial cells can induce IL-6, IL-8/CINC-1, and TNF-α (Schumann *et al.*, 1994; Khair *et al.*, 1996; Ohkubo *et al.*, 1998). Therefore, LPS can affect many different cells, resulting in the release of proinflammatory mediators that modulate effects on these and neighboring cells.

1.C.6.3.1. Sinusoidal Endothelial Cells.

As previously described, SECs line the sinusoids of the liver and are characterized morphologically by an absence of tight junction between cells, a lack of basal lamina and the presence of fenestrations. They perform a variety of functions including exchange and transport of substances between blood and HPCs, and assisting KCs in the clearance of LPS (Praaning-van Dalen *et al.*, 1981; Nakao *et al.*, 1994; Wisse *et al.*, 1996). LPS or LPS-induced inflammatory mediators (e.g., IL-1, TNF-α, IL-8/CINC-1 and thrombin) can activate endothelial cells to promote inflammation, initiate coagulation and affect vascular tone (Pohlman and Harlan, 1992; Holst *et al.*, 1996). Accordingly, endothelial cell activation may alter the function of neighboring parenchymal cells and possibly affect nearby inflammatory cells leading to secondary inflammatory events to further alter or damage parenchymal cells (Pohlman and Harlan, 1992; Holst *et al.*, 1996).

LPS is known to induce changes/injury to SECs *in vivo* and *in vitro* (Deaciuc *et al.*, 1994; Seto *et al.*, 1998; Spapen *et al.*, 1999; Hasegawa *et al.*, 2001). After administration of an acutely toxic dose of LPS, structural alterations (e.g., cell swelling and decreased fenestrae) and functional changes (e.g., increased ICAM-1 expression) are observed before the onset of HPC injury. This progresses to SEC necrosis. LPS-induced structural alteration of SECs is accompanied, but not necessarily correlated with, functional impairment of the cell. HA uptake, a marker of SEC dysfunction/injury, is decreased both before and after the onset of HPC injury (Deaciuc and Spitzer, 1996; Yachida *et al.*, 1998).

Endothelial cells activated by LPS and/or LPS-induced mediators produce a myriad of inflammatory factors. *In vitro,* endothelial cell activation induces the expression of TF and plasminogen activator inhibitor-1. *In vivo,* activation by TNF-α and IL-1 induces procoagulant activity (e.g., via TF expression). Leukocyte adhesion molecule (e.g., ICAM-1 and VCAM-1) expression also increases following endothelial cell activation (Pohlman and Harlan, 1992). These molecules play a critical role in the accumulation and adherence of PMNs at the site of inflammation. Indeed, LPS, TNF-α and/or IL-1 can dramatically induce ICAM-1 and VCAM-1 expression on SECs. ICAM-1 and VCAM-1 can also be expressed on HPCs, though at a level substantially below that observed in endothelial cells (Essani *et al.*, 1995; Farhood *et al.*, 1995; Lalor *et al.*, 2002). For example, Komatsu *et al.* (1994) demonstrated in the galactosamine/LPS liver injury model that the accumulation and activation of PMNs, as well as the

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enhanced expression of adhesion molecules on hepatic endothelial cells, are critically important for injury and could be induced by TNF-α. Furthermore, other inflammatory mediators released by endothelium include IL-1, IL-6, IL-8/CINC-1, and PAF (Pohlman and Harlan, 1992; Ohkubo *et al.*, 1998). Additionally, Jaeschke (1992) found that LPS resulted in an extracellular oxidant stress *in vivo* that caused increased SEC efflux of reduced glutathione (GSH) possibly to counter the release of potentially deleterious ROS generated by KCs. Finally, a number of vasoactivators are produced *in vitro*, including vasoconstrictors, like PAF and endothelin-1, and vasodilators, like prostacyclin and NO (Pohlman and Harlan, 1992). With such a variety of factors released from activated endothelial cells, it is possible that endothelial cell activation/injury could modulate surrounding cells in conditions of endotoxemia (Pohlman and Harlan, 1992; Laskin, 1996).

As mentioned previously, the endothelium is normally antithrombogenic, but in the presence of LPS or LPS-induced mediators, such as TNF-α and IL-1, it can become procoagulant. This procoagulant activity, in part, is due to the induction of TF activity on the surface of endothelial cells, which leads ultimately to thrombin formation (Stern *et al.*, 1985; reviewed in Schultze and Roth, 1998). Interestingly, thrombin also activates endothelial cells (de Groot *et al.*, 1987). Hence, endothelial cells can act as a bridge or link between inflammation and coagulation system activation. Endothelium damaged during inflammation provides a surface whereby proteins involved in both coagulation and inflammation development are expressed (Cicala and Cirino, 1998).

Accordingly, injury to endothelial cells in the liver microvasculature can result in activation of the coagulation system (Machovich, 1985; Ryan, 1986, Hirata *et al.*, 1989). Coagulation system activation is critical for the development of HPC injury in many liver injury models (Perry, 1984; Fujiwara *et al.* 1988; Yamada *et al.* 1989; Hewett and Roth, 1995; Arai *et al.*, 1996). Hence, microcirculatory disturbances (i.e., sinusoidal hypoperfusion) from hemorrhage and/or intrasinusoidal fibrin deposition have been postulated to contribute to HPC injury via ischemic/hypoxic injury (Shibayama, 1987; DeLeve *et al.*, 1996; Ba *et al.*, 2000; Saetre *et al.*, 2000; Copple *et al.*, 2002a, 2002b; Hasegawa *et al.*, 2001).

Taken together, the release of LPS-induced proinflammatory mediators and procoagulant activity from SECs not only have the potential to contribute to SEC injury but also may contribute to injury in surrounding parenchymal cells (Laskin, 1996). Hence, SECs injury may be more than an epiphenomenon in some hepatotoxicity models.

1.C.6.4. Coagulation System and Thrombin

LPS can activate the coagulation system either intrinsically though surface mediated reactions (i.e., activation of factor XII) or extrinsically through the expression of TFs on endothelial cells, monocytes and PMNs (Bone *et al.*, 1992; Polack *et al.*, 1997; Todoroki *et al.*, 2000). With each step in the coagulation system, a previous enzyme activates the next one. This activity requires a cofactor such as calcium and an organizing surface such as platelets or PMNs.

At the distal end of this series of reactions, activated factor X catalytically cleaves prothrombin into active thrombin. Thrombin, a protease, converts circulating fibrinogen into insoluble fibrin clots (Bloom, 1990; Schultze and Roth, 1998).

However, it can have other roles in addition to procoagulant activity. Thrombin has been implicated in the release of proinflammatory cytokines from monocytes, macrophages, endothelial cells and PMNs. It is also considered a weak PMN chemoattractant (Bizios *et al.*, 1986; Hoffman and Cooper, 1995; Holland *et al.*, 1998) and has a role in PMN activation/priming through enhancing ROS production, adherence to endothelium and thromboxane release (Bizios *et al.*, 1987; Chan *et al.*, 1988; Carney, 1992). Interestingly, Moulin *et al.* (2001) recently found *in vitro* that thrombin does not directly prime or activate rat PMNs but instead relies on indirect mechanisms. Further, thrombin is involved in monocyte chemotaxis (Bar-Shavit *et al.*,1983a; Malik, 1986; Bizios *et al.*, 1987) and can promote the release of inflammatory components (i.e., TNF-α and IL-1) from monocytes (Bar-Shavit *et al.*, 1983a; Hoffman and Cooper, 1995). Moreover, thrombin is a potent chemotaxin for macrophages and can alter the production of cytokines and arachidonic acid metabolites (Bar-Shavit *et al.*, 1983b). Overall, this demonstrates that thrombin participates in a larger role than coagulation system activation and further suggests that coagulation system activation may contribute to the upregulation of proinflammatory mediators (Johnson *et al.*, 1998; Karima *et al.*, 1999).

As previously mentioned, thrombin can activate endothelial cells. At physiological levels, it can have a direct permeablizing effect on endothelium

(i.e., by causing endothelial cell retraction; Malik, 1986). Furthermore, thrombin can cause endothelial cells to release prostacyclin, plasminogen activator inhibitor, PAF, chemokines (e.g., MCP-1 and IL-8/CINC-1), ROS and NO and to increase the expression of adhesion glycoproteins on endothelium (Venturini and Kaplan, 1992; Colotta *et al.*, 1994; Ueno *et al.*, 1996).

It is likely that most, if not all, of the effects of thrombin on endothelial cells (e.g., SECs) and on KCs is due to thrombin activation of the G-coupled receptor, protease-activated receptor (PAR)-1 on these cells. Indeed, activated PAR-1 initiates intracellular signal transduction pathways that can contribute to proinflammatory effects by stimulating vasodilation, increasing vascular permeability to plasma proteins, increasing expression of adhesion molecules on endothelium (e.g., ICAM-1 and VCAM-1) and causing release of cytokines such as IL-6 and chemokines (e.g., IL-8; Vergnolle *et al.*, 2001; Derian *et al.*, 2002). Taken together, thrombin has roles other than its procoagulant activity and may be important for chemotaxis, adhesion molecule expression and the release of proinflammatory mediators in endotoxemia.

1.C.6.5. Summary

A wide range of inflammatory mediators is released upon activation of inflammatory cells by LPS and/or LPS-derived mediators. Combination of these mediators, along with coagulation system activation, can result in various pathophysiologic responses to endotoxemia (e.g., shock and lethality). Moreover, some mediators – like TNFα, PAF, and thrombin – could contribute to

enhancing concurrent injury via disruption of blood flow and accumulation and activation of PMNs and macrophages. Accordingly, an underlying inflammatory state could contribute or enhance injury to tissues already predisposed by chemical-induced homeostatic alterations.

1.C.7. LPS and the Liver

Numerous studies have demonstrated that LPS has an hepatotoxic effect on the liver (Levy *et al.*, 1967; Utili *et al.*, 1977; Hirata *et al.*, 1980). Large, acutely toxic doses of LPS produce multifocal MZ coagulative necrosis with neutrophilic infiltrate, minimal hemorrhage and SEC injury (Shibayama, 1987; Hewett and Roth, 1993; Seto *et al.*, 1998). Liver injury is both time- and dose-dependent (Hewett and Roth, 1993; Pearson *et al.*, 1995). For a more detailed characterization of an LPS-induced liver lesion, refer to Chapter 2.

Progressive functional and morphological changes occur in HPCs and SECs after exposure *in vivo* to an acutely toxic dose of LPS (Levy *et al.*, 1967; Hirata *et al.*, 1980; Hewett and Roth, 1993, Roth *et al.*, 1997; Seto *et al.*, 1998). In HPCs, after LPS exposure there is a general decrease in protein synthesis *in vitro* (Keller *et al.*, 1985), and cytochrome P450 expression (Morgan, 1993) and metabolism *in vivo* (Shedlofsky, 1994). Likewise, alterations in protein, DNA and RNA synthesis, and functional impairment (i.e., HA uptake) are observed in SECs *in vivo* (Pober and Coltran, 1990; Deaciuc and Spitzer, 1996; Yachida *et al.*, 1998). Initial morphological changes to HPCs *in vivo* include swelling of microvilli on the sinusoidal border, moderate dilation of organelles and dilation of

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bile canaliculi. Initial morphological changes to SECs, before the onset of HPC injury, include cell swelling and decreased fenestrae *in vivo.* Initial functional changes to SECs include increased ICAM-1 expression. These morphologic changes later progress to SEC necrosis (Deaciuc and Spitzer, 1996; Yachida *et al.*, 1998). Subsequent changes in HPCs become progressively more apparent, culminating in hepatocellular degeneration and coagulative necrosis (Hewett and Roth, 1993).

Several host inflammatory cells and endogenous mediators have key roles in the pathogenesis of LPS-induced liver injury. KCs (Arthur *et al.*, 1985, Arthur *et al.*, 1986; Brown *et al.*, 1997), PMNs (Jaeschke *et al.*, 1991; Hewett *et al.*, 1992; Jaeschke *et al.*, 1993) and platelets (Pearson *et al.*, 1995) appear to be important to this injury. Inactivation or elimination of these cells prior to administration of hepatotoxic doses of LPS results in marked attenuation of liver injury (Hewett *et al.*, 1992; Pearson *et al.*, 1995; Brown *et al.*, 1997). This suggests that these cells are critical to the pathogenesis. Moreover, several cytokines including TNF-α, IL-1 and IL-6 have been implicated in LPS-induced liver injury (Chesnue et al., 1991; Hewett *et al.*, 1993). Lipid mediators (Wise *et al.*, 1980) and coagulation factors, especially thrombin (Hewett and Roth, 1995; Moulin *et al.*, 1996; Pearson *et al.*, 1996b), also play a role in this pathogenesis.

The role of thrombin in LPS-induced liver injury is independent of clot formation (Hewett and Roth, 1995; Moulin *et al.*, 1996). Heparin, warfarin, or hirudin reduced thrombin activity and liver injury *in vivo* during endotoxemia (Hewett and Roth, 1995; Pearson *et al.*, 1996b). Conversely, treatment with

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ancrod, a thrombin-like anticoagulant enzyme, depleted circulating fibrinogen presumably preventing the formation of clots but did not protect against liver injury (Hewett and Roth, 1995; Moulin *et al.*, 1996). These studies, in conjunction with results of isolated perfused liver studies, indicate that thrombin acts on PAR-1 (Moulin *et al*, 1996, 2001; Copple *et al.*, 2003). This may lead to enhancement of proinflammatory mediators (e.g., increasing expression of adhesion molecules on endothelium and causing the release of cytokines and chemokines such as IL-6 and IL-8/CINC-1) that contribute to HPC injury (Vergnolle *et al.*, 2001; Derian *et al.*, 2002). Consequently, in LPS-induced liver injury, the importance of coagulation system activation may not be in fibrin clot formation but in thrombin activation of PAR-1 (Hewett and Roth, 1995; Moulin *et al.*, 2001; Copple *et al.*, 2003).

Furthermore, thrombin can act as a PMN chemoattractant (Bizios *et al.*, 1987). This role, however, is controversial in LPS-induced hepatotoxicity (Pearson *et al.*, 1996b; Woodman *et al.*, 2000), though thrombin may be important in PMN extravasation (Jaeschke, 1997). Hence, LPS-induced liver injury is dependent on cells from the innate immune system as well as platelets and other proinflammatory mediators and cytotoxic factors.

Exposure to a modest dose of LPS does not result in overt tissue injury but does generate an inflammatory response. Hepatic PMN accumulation is observed at noninjurious doses of LPS (Spitzer and Mayer, 1993; Spitzer *et al.*, 1994; Shibayama *et al.*, 1995). *In vitro*, KCs and other macrophages are activated, resulting in the release of TNF-α, PGs and other soluble mediators

(Michie *et al.*, 1988; Hewett and Roth, 1993; Hewett and Roth, 1995; Roth *et al.*, 1997). It has been demonstrated that these mediators can alter parenchymal cell homeostasis (reviewed in Roth *et al.*, 1997).

In summary, exposure to an acutely toxic dose of LPS results in the activation of inflammatory cells, release of proinflammatory mediators and coagulation system activation ultimately leading, through a complex interaction of these factors, to liver injury (Hewett and Roth, 1993). Exposure to a smaller, noninjurious dose of LPS results in the presence of inflammatory cells and production of proinflammatory mediators at levels that do not cause overt injury but can alter cellular homeostasis. This mild inflammatory response in conjunction with hepatic cells that already have undergone xenobiotic-induced cellular homeostatic alterations can produce frank injury (Roth *et al.*, 1997; Ganey and Roth, 2001).

1.C.8. LPS Potentiates Hepatotoxicant-Induced Injury

LPS potentiation of hepatotoxicant-induced injury occurs in a number of models (reviewed in Roth *et al.*, 1997; Ganey and Roth, 2001), with hepatotoxicants such as carbon tetrachloride (Formal *et al.*, 1960), cadmium (Cook *et al.*, 1974), galactosamine (Galanos *et al.*, 1979), ethanol (Nolan *et al.*, 1980; Bhagwandeen et al, 1987), halothane (Lind *et al.*, 1984), T-2 toxin (Tai and Pestka, 1988), allyl alcohol (Sneed *et al.*, 1997), aflatoxin B_1 (Barton *et al.*, 2000b), and cocaine (Labib *et al.*, 2002). Other inflammagens (such as vitamin A and *Corynebacterium parvem*) in addition to LPS can also potentiate liver injury

in conjunction with hepatotoxicant coexposure (Raiford and Thigpen, 1994; Wueweera *et al.*, 1996; Ganey and Roth, 2001). Despite these hepatotoxicants being mechanistically diverse and causing injury to different regions of the liver lobule, LPS coexposure augments the liver damage. Futhermore, for each of these the overall mechanism of injury appears to involve aspects of the inflammatory system, including, but not limited to, KCs, TNFα, PMNs, COX-2, PAF and others (reviewed in Ganey and Roth, 2001).

The mechanism by which LPS augments hepatotoxicant-induced injury appears to be complex and not necessarily universal. For example, in the allyl alcohol/LPS-induced liver injury model, KCs, PMNs and COX-2 products are critical factors, but TNF-α is not (Sneed *et al.*, 1997; Kinser *et al.*, 2000; Ganey *et al.*, 2001). Conversely, in the aflatoxin B_1/LPS-induced liver injury model, PMNs and TNF-α are important in the mechanism of injury but COX-2 products are not (Barton *et al.*, 2000a; Barton *et al.*, 2001). The reason for these differences in critical inflammatory mediators with these models is unclear but may have to do with the mechanistic contribution of the particular hepatotoxicant (i.e., allyl alcohol or aflatoxin B_1) to the overall injury mechanism (Ganey and Roth, 2001).

LPS potentiation of hepatotoxicant-induced injury depends on the exposure time between LPS and the hepatotoxicant. LPS pretreatment a day or more before the administration of carbon tetrachloride, acetaminophen or α-naphthylisothiocyanate, for example, protects against liver injury (Calcamuggi *et al.*, 1992; Liu *et al.*, 2000), whereas concurrent LPS exposure enhances carbon tetrachloride-induced liver injury (Formal *et al.*, 1960). This decreased toxicity, or

cross-tolerance, is thought to develop from decreased cytochrome P450 levels or an increased amount of antioxidants that develop in part from the downregulation of proinflammatory mediators (Liu *et al.*, 2000). Consequently, augmented toxicity develops when LPS is administered closer in time (e.g., within a few hours) to administration of the hepatotoxicant (Ganey and Roth, 2001).

Interestingly, for liver injury to develop in the allyl alcohol/LPS cotreatment model it does not matter whether LPS administration immediately (i.e., within a few hours) precedes or occurs shortly after allyl alcohol administration for liver injury to develop. However, in the LPS/cocaine-induced liver injury model, if LPS administration follows (i.e., about 4 hours) cocaine administration, liver injury develops (Labib *et al.*, 2002), whereas when LPS administration precedes cocaine administration by 3 to 6 hours liver injury does not develop (Reid and Bornheim, 2001). Moreover, alterations in the sequence of hepatotoxicant and LPS administration can change the nature of the toxicity (i.e., from hepatotoxicity to extrahepatic toxicity). This change has been observed in several models of LPS augmentation of injury, such as with cadmium (Cook *et al.*, 1974, 1975), lead (Selye *et al.*, 1966; Trejo *et al.*, 1972) and galactosamine (Galanos *et al.*, 1979). In these models, the greatest mortality occurred when rats were treated with hepatotoxicant and LPS simultaneously rather than hours apart. In a primate model, 100% lethality was observed upon the simultaneous administration of nonlethal doses of LPS and lead acetate. Death from this coexposure was likely due to circulatory shock (Holper *et al.*, 1973). Taken together, the temporal relationship between LPS and hepatotoxicant exposure

seems to dictate the target organ (i.e., extrahepatic injury) and whether there will be augmentation in liver injury or a protective effect.

LPS has a potential to decrease the threshold for toxicity for a number of mechanistically diverse hepatotoxicants. Indeed, exposure of animals to an acute, modest, noninjurious dose of LPS may enhance injury in liver cells homeostatically altered by a hepatotoxicant (Roth *et al.*, 1997; Ganey and Roth, 2001). To elucidate further this phenomenon, monocrotaline (MCT), a pyrrolizidine alkaloid (PA), was selected as the chemical of interest.

1.D. Monocrotaline

1.D.1. Pyrrolizidine Alkaloids

PAs encompass a group of structurally related plant compounds consisting of a pyrrolizidine nucleus with various functional groups attached (McLean, 1970). The pyrrolizidine nucleus consists of two fused, five-membered rings connected by a single nitrogen atom at the center of the molecule. Side chains of various lengths and compositions are attached to the 1 and/or 7 position of the pyrrolizidine nucleus (Huxtable, 1979; Prakash *et al.*, 1999). PAs often occur as free alkaloids or alkaloid N-oxides. Nearly four hundred PAs have been identified in over 6,000 plants – chiefly of the Boraginaceae, Compositae, and Leguminosae families – spanning the globe (Huxtable, 1989; Schultze and Roth, 1998). Most of these plants contain various mixtures of PAs at different concentrations and with different toxicities. For plants, these phytotoxins may

play an important defensive role against insect herbivores. In terms of mammalian toxicity, however, PA-containing plants represent an often "understudied, but potentially important source of exposure to plant toxins and carcinogens" for animals and humans (Huxtable, 1989; Schultze and Roth, 1998).

PA-containing plants are responsible for livestock and human morbidity and mortality, especially in third-world countries. They are often considered noxious weeds that can easily invade fields and crops. However, some PA-containing plants have agricultural uses. *Crotalaria spectabilis*, for example, has been employed as a leguminous cover crop to prevent soil erosion and to increase soil nitrogen content (Cheeke, 1989; Schultze and Roth, 1998; Stegelmeier *et al.*, 1999).

Wildlife and livestock grazing on PA-containing plants often find them to be unpalatable and subsequently do not usually forage on them. However, they may be exposed to these plants through accidental grazing or by consuming them outright when no other forage is available. A more common way for livestock to be exposed to PAs is through the consumption of prepared feeds or grain contaminated with PAs (Stegelmeier *et al.*, 1999). In some areas of the U.S., a significant portion of livestock has become critically ill or died as a result of liver disease from grazing on PA-containing plants. In the U.S. alone in the 1970's, losses to cattleman and other livestock owners was estimated to be in the tens of millions of dollars each year (Huxtable, 1979).

Human exposure to PAs occurs through the consumption of contaminated foodstuffs (such as cereal grain, cooking oils, milk, and possibly honey) and through the ingestion of alternative medicines. Accidental contamination of cereal crops in the past has led to epidemic PA poisonings in Tadjikistan, Uzbeckistan, Japan, Nigeria, Afghanistan, India, Sri Lanka, South Africa, Iraq, and the Caribbean. The largest reported incident of PA poisoning occurred in Afghanistan in 1974. Approximately 7,800 individuals were affected out of a population of 35,000 in nearly 100 villages from the consumption of wheat flour heavily contaminated with seeds from the PA-containing *Heliotropium* genus. Many deaths resulted from this exposure, primarily due to hepatic veno-occlusive disease (HVOD; Prakash *et al.*, 1999; Stegelmeier *et al.*, 1999). This is not surprising since the major target organ for humans poisoned with PA is the liver (Kasturi *et al.*, 1979). HVOD is characterized by the occlusion of central veins of the hepatic circulation that leads to cirrhosis and liver failure (Stegelmeier *et al.*, 1999). A more comprehensive characterization of HVOD is provided in the next section. Besides HVOD, hepatomegaly and meglaocytosis also occur (Schoental and Head, 1955; McLean, 1970; Mattocks, 1986). Although epidemic poisonings have become less of a problem in recent years due to the use of herbicides and to grain inspection in more modernized countries, PA-poisoning in humans and livestock may still be an area of concern in less developed regions.

Humans can also be poisoned after consuming PA-containing plants in alternative medicines, such as herbal teas. Previously in Jamaica, HVOD was endemic because of the consumption of teas prepared from leaves of wild scrub

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from the *Senecio* or *Crotalaria* genera (Huxtable, 1979). In the U.S. during the 1970's, PA poisoning also occurred in Arizona due to the consumption of a widely used and commercially marketed herbal tea called gordoloba yerba (Stillman *et al.*, 1977). Within the last decade there has been increasing interest in alternative medicines, especially the use of herbal remedies. PAs have been identified in traditional herbal medicines of South America, Africa and China. Numerous PA-related poisonings have occurred through deliberate ingestion or accidental contamination of herbal preparations. In one case, an individual consuming a powder supposedly containing ground comfrey root (*Symphytum officinale*), for use as a dietary supplement, consumed 85 mg of PA over a six-month period with the result of HVOD. In another case, an 18-month-old boy consumed an herbal tea mixture contaminated with the PA-containing plant *Adenostyles alliariae* (mistaken for coltsfoot) for 15 months to aid in the healthy development of the child. The child consumed approximately 60 µg/kg body weight per day for 15 months with the result of HVOD. Recently, the German Federal Health Department limited the use of over 600 herbal remedies, including several that contained large concentrations of PAs. Similar regulations have been proposed worldwide. Furthermore, due to PA toxicity, several herbal remedies in traditional Chinese medicine are no longer recommended for therapeutic use (Prakash *et al.*, 1999; Stegelmeier *et al.*, 1999; Roeder, 2000).

The toxic effects of PAs tend to vary with species, age, gender, physiological and nutritional state, plant species consumed, duration of exposure, and total dose of alkaloid consumed (McLean, 1970; Huxtable, 1989). Injury

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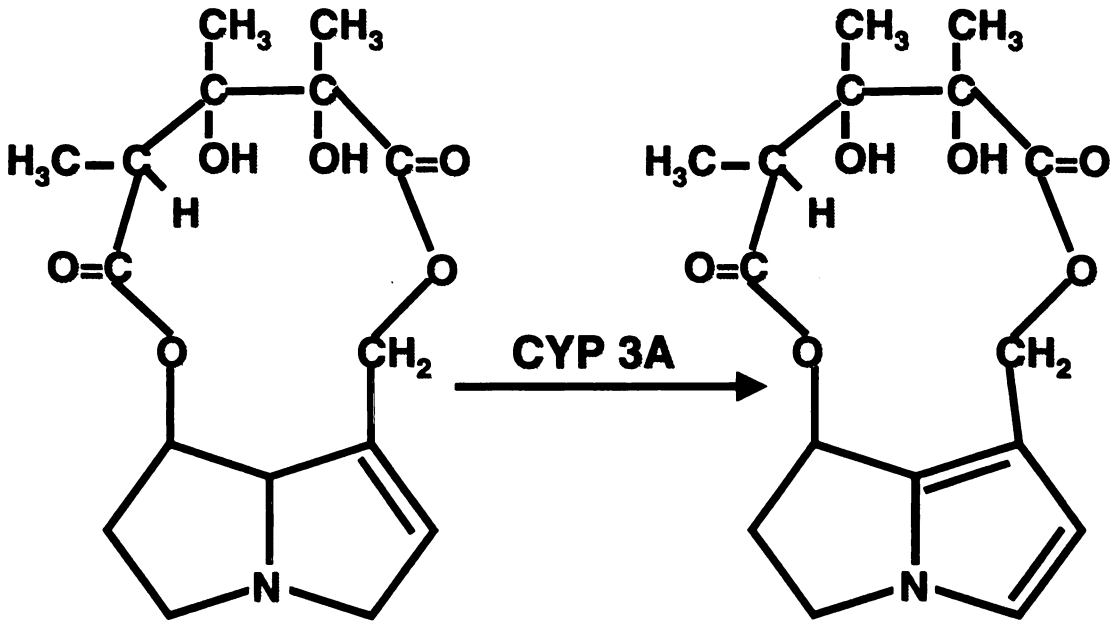
from chronic PA poisoning is not limited to the classic features of HVOD, hepato-splenomegaly and emaciation, but also includes injury to the lungs, heart, GI tract, pancreas, and kidneys. In addition, teratogenesis and carcinogenesis have occurred in some experimental animals as a result of PA exposure. No cases of cancer have been reported from PA exposure in humans or domesticated animals, however (Mattock, 1986; Prakash *et al.*, 1999; Stegelmeier *et al.*, 1999).

1.D.2. Monocrotaline as a Food-borne Hepatotoxicant

MCT is a PA phytotoxin that is well known for hepatic and cardiopulmonary toxicity (Figure 1.3; Schultze and Roth, 1998). MCT occurs in the foliage and seeds of plants from the genus, *Crotalaria* (Huxtable, 1989). Major sources of MCT include *Crotalaria spectabilis* or rattlebox (the most common source of MCT in the U.S.; mostly southern and southeastern states), *Crotalaria retusa*, *Crotalaria recta* and *Crotalaria sericea* (IARC, 1976).

Like other PAs, human exposure to MCT occurs through consumption of cereal grains, cooking oils, and herbal teas and medicines (Huxtable, 1989), whereas animal exposure occurs primarily through grazing on pastures with pyrrolizidine-containing plants (McLean, 1970).

Figure 1.3. Chemical Structure of MCT and MCTP. MCT or 12β,13β-dehydroxy-12α,13α,14α-trimethyl-crotal-1-enine is a PA phytotoxin that is a macrocyclic diester of retronecine to which monocrotalic acid is esterified at the 1 and 7 position. It is normally stable and nontoxic. MCT must be bioactivated by the cytochrome P450 (CYP) 3A subfamily to MCTP to produce toxicity.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MCT causes hepatic injury, which is clinically evident as HVOD (Bras *et al.*, 1954). MCT is often used experimentally to generate a reproducible HVOD model (McLean, 1970). Although HVOD still arises in humans and animals from PA poisoning, most cases of HVOD are the result of high-dose cytoreductive therapy in bone marrow and stem cell transplantation (Bearman, 2000; Carreras, 2000, DeLeve, 2001). Moreover, HVOD is one of the leading causes of mortality in bone marrow transplant patients (DeLeve, 2001). In the rat model for HVOD, early HVOD (i.e., day 3 to 5) is characterized by centrilobular necrosis, severe sinusoidal injury, severe sinusoidal hemorrhage, and severe damage to the central vein endothelium (DeLeve *et al.*, 1999). In late HVOD (i.e., days 6 and 7), livers are characterized by subendothelial and advential fibrosis of the central vein, damage to the central vein endothelium with subendothelial hemorrhage, and some recovery of the sinusoidal wall (DeLeve *et al.*, 1999). Accordingly, while MCT may raise concern as a food-borne hepatotoxin, it is also useful for generating a reproducible HVOD model in rats to study clinical HVOD potentially arising from bone marrow transplantation (DeLeve *et al.*, 1999).

Prior to the 1980's, diagnosis of PA poisoning (and consequently MCT poisoining) was often made on episodic, circumstantial evidence and observation of characteristic liver lesions. Hence, determination of the exact dose of MCT, following chronic exposure, needed to produce HVOD in humans is difficult. For example, Huxtable (1989) reported that grain contamination in central India led to human symptoms of HVOD. In forty-two percent of the 67 cases studied, poisoning resulted in death. Contamination of grain was due to *Crotalaria nana*,

a PA-containing species consisting of MCT and fulvine. It is estimated that villagers consumed on average less than 40 mg of PAs per day (Huxtable, 1989). Additionally, Mattocks (1986) estimated that a cumulative dose (taken within a few weeks) of PAs from *Crotalaria* exceeding 10 mg/kg would result in HVOD. Children appear to be more susceptible to PA-induced toxicity than adults (Mattocks, 1986).

1.D.3. Chemical, Structure, Properties and Metabolism

MCT or 12β,13β-dehydroxy-12α,13α,14α-trimethyl-crotal-1-enine is a macrocyclic diester of retronecine to which monocrotalic acid is esterified at positions 1 and 7 (Figure 1.3; Schultze and Roth, 1998). The chemical structure of MCT has been confirmed by infrared, nuclear magnetic resonance, and mass spectrometry (Culvenor and Dal Bon, 1964; Prakash *et al.*, 1999). When purified, this bitter-tasting compound has a molecular weight of 325.3 g and a melting point of 202-203 °C (see IARC, 1976 for more chemical and physical data; Schultze and Roth, 1998).

MCT is chemically stable and nontoxic. Within the liver, MCT can undergo N-oxidation, hydroxylation and ester hydrolysis to form nontoxic products. MCT may also undergo dehydrogenation to result in a toxic product (McLean, 1970; Mattocks and White, 1971; Mattocks, 1986; Schultze and Roth, 1998). Indeed, for injury to occur, MCT must be metabolized by cytochrome P450 (CYP) to its toxic metabolite, monocrotaline pyrrole (MCTP or dehydromonocrotaline; Figure 1.3; Mattocks, 1968; McLean, 1970). When inhibitors or inducers of CYPs are

administered *in vivo*, toxicity and tissue concentrations of pyrroles are altered accordingly (i.e., decreased or increased, respectively; Mattocks and White, 1971; Allen *et al.*, 1972; Mattocks, 1972; Schulltze and Roth, 1998). The CYP 3A subfamily is the primary CYP isozyme family responsible for the bioactivation of MCT (Kasahara *et al.*, 1997; Reid *et al.*, 1998). Currently, it is unclear which specific isozyme(s) of the subfamily is involved in MCT bioactivation. For the related PA senecionine, CYP 3A2 (PCN-E) was found to responsible for bioactivation (Williams, *et al.*, 1989). Finally, the Ehrlich assay is often used to estimate the formation of pyrroles in liver, which may serve as a marker of bioactivation of MCT to toxic pyrrole metabolites (such as MCTP; McLean, 1970).

The generation of MCTP is a major metabolic pathway for MCT, and there is a strong correlation between the amount of MCTP found in tissues (e.g., liver, lung, etc.) and tissue injury (Mattocks, 1972; Chesney *et al.*, 1974). Indeed, MCTP has been implicated as a causative agent in HVOD (Huxtable, 1989). It is thought that MCTP, formed in the liver, is responsible for hepatotoxicity as well as extrahepatic toxicity (such as pneumotoxicity; Huxtable, 1990; Lame *et al.*, 1991; Yan and Huxtable, 1995; Schultze and Roth, 1998). Chemically synthesized MCTP, when administered via the tail vein (i.e., bypassing the liver) results in lung lesions in rats, but when MCTP is administered via the mesenteric vein (i.e., which goes directly to the liver) liver injury develops (Butler, 1970; Bruner *et al.*, 1983, 1986). MCTP is a reactive electrophile that is capable of causing tissue injury via covalent binding with nucleophilic macromolecules such as DNA, proteins and GSH (Robertson *et al.*, 1977; Seawright, 1995;

Stegelmeier *et al.*, 1999). Additionally, MCTP can act as a bifunctional crosslinking agent that binds macromolecules including DNA and proteins (Petry *et al.*, 1984; Hincks *et al.*, 1991; Hoorn *et al.*, 1993). Hence, bioactivation of MCT to MCTP is responsible for liver injury as well as injury in other tissues.

1.D.4. Toxicokinetics

Metabolic bioactivation of MCT to MCTP begins early after MCT administration and is evident as increased pyrrole concentration in liver for more than 24 hours (Allen *et al.*, 1972; Lame *et al.*, 1991). The peak level of metabolic pyrrole concentration occurs within 1.5 hours after MCT administration and then tapers off over time. By 4 hours, the concentration of MCTP is approximately 50% of its peak value, decreasing to 25% by 24 hours and continuing to be present at low levels by 48 hours (Allen *et al.*, 1972). Following administration of radiolabeled MCT, 90% of radioactivity appeared in the urine and bile by 7 hours (Estep *et al.*, 1991). Data from the study by Estep *et al.* (1991) suggest that erythrocytes may serve as carriers of MCT metabolites, and this may be involved in pulmonary toxicity. Indeed, Lame *et al.* (1997) determined that hemoglobin and other erythrocyte proteins could serve as carriers for reactive pyrrolic metabolites. Subsequently, these reactive metabolites travel to other tissues (e.g., lung) and induce injury.

Pyrroles formed in the liver from PAs react with a number of cellular nucleophiles, especially those with sulfhydryl groups, such as GSH and proteins (Mattocks, 1986, Mattocks and Jukes, 1990; Seawright, 1995). These thiol

adducts may exist for some time after their formation (Mattocks and Jukes, 1992). A GSH-conjugated pyrrole has been identified in blood and urine of rats treated with radiolabeled MCT (Estep *et al.*, 1990, 1991). As previously noted, some adducts have the potential to further damage to cells when they bind to more significant macromolecules like proteins and DNA.

1.D.5. Biologic Effects of Monocrotaline

MCT causes toxicity in a variety of animal species, including cattle, horses, dogs, cats, poultry, rabbits, mice, rats, non-human primates and humans (reviewed in Schultze and Roth, 1998). MCT affects a number of organs other than the liver, depending on the dose, route of administation and post-treatment time. However, the focus of this dissertation is on the acute liver injury following coexposure to small, nontoxic doses of LPS and MCT. Therefore, a discussion on the biological effects of MCT in kidney, heart, lung and other organs is beyond the scope of this dissertation. The reader is referred to several reviews, including: Huxtable, 1990; Wilson *et al.*, 1992; Schultze and Roth, 1998.

At acutely toxic doses, MCT-induced liver lesions are characterized by centrilobular hepatocellular necrosis, dilated and congested sinusoids, hemorrhage and injured central venous endothelial cells and SECs (Schoental and Head, 1955; DeLeve *et al.*, 1996, 1999; Yee *et al.*, 2000b; Copple *et al.*, 2002a). Twenty-four hours after a toxic dose of MCT, monocytes increase in the liver with a noticeable decrease in the presence of Kupffer cells (DeLeve *et al.*,

1999). For a more detailed characterization of MCT-induced liver lesions, refer to Chapter 2.

MCT can cause endothelial cell injury both *in vivo* (Schoental and Head, 1955; Allen *et al.*, 1972; DeLeve *et al.*, 1999; Copple *et al.*, 2000a) and *in vitro* (Reindel and Roth, 1991; Reindel *et al.*, 1991; Hoorn *et al.*, 1993; DeLeve *et al.*, 1996). *In vitro,* MCTP is capable of inducing endothelial cell dysfunction (Taylor *et al.*, 1996). MCTP binds to endothelial cellular DNA and can induce apoptosis (Thomas *et al.*, 1996, 1998). Moreover, MCT has been reported to be selectively more toxic to SECs than to HPCs *in vitro,* and this SEC toxicity may originate from profound GSH depletion (DeLeve *et al.*, 1996). MCT toxicity in HPCs is largely unaffected by depletion of GSH, but this is not the case with SECs (Pan *et al.*, 1993; DeLeve *et al.*, 1996; Reid *et al.*, 1999). MCT toxicity to SECs is attenuated by the addition of exogenous GSH (DeLeve *et al.*, 1996).

It is tempting to speculate that a small, noninjurious dose of MCT could decrease GSH concentration in SECs without resulting in overt injury. Prooxidant inflammatory mediators evoked by LPS (as discussed in 1.C.6.3.1.) could precipitate overt injury in these SECs wherein GSH has been decreased. Alternatively, as previous mentioned, LPS-induced extracellular oxidant stress might result in an increased efflux of GSH from SECs to counter the release of deleterious ROS from phagocytic cells (Jaeschke, 1992). Hence, LPS exposure could further decrease GSH in SECs that have been exposed to this small, noninjurious dose of MCT. Therefore, it seems possible that the combined loss

of GSH from SECs coexposed to small, noninjurious doses of MCT and LPS could lead to overt SEC injury.

Additionally, MCT can elicit oxidative stress in pulmonary vascular endothelial cells *in vitro,* and subsequent treatment with dimethylthiourea, an oxygen radical scavenger, prevented MCT-induced increase in cellular oxidation (Aziz *et al.*, 1999). Recently, Baybutt and Molteni (1999) showed that treatment with the antioxidant β-carotene *in vivo* resulted in decreased hemorrhage and protection of liver parenchyma after a toxic dose of MCT. This decrease in hemorrhage may suggest that β-carotene offered some protection to SECs. Consequently, SEC injury may be due in part to oxidative stress, though this is controversial (DeLeve *et al.*, 1999).

Copple *et al.* (2000b) have recently found that coagulation system activation is involved in HPC but not in SEC injury in rat livers after an acutely toxic dose of MCT. Fibrin deposition in the microvasculature surrounding and within the centrilobular lesion was postulated to lead to local hypoperfusion (i.e., microcirculatory disturbances) and subsequent hypoxic/ischemic cellular injury (Copple *et al.*, 2000a, 2000b). Taken together, it is tempting to speculate that the initial target for MCT toxicity is SECs and that subsequent injury to these cells results in coagulation system activation and fibrin deposition, leading to local hypoperfusion that contributes to HPC injury (DeLeve *et al.*, 1996; Copple *et al.*, 2000a, 2000b). Hence, both HPC and SEC injury occur in MCT-induced toxicity, and SEC injury can potentially contribute to HPC injury.

In conclusion, liver injury induced by an acutely toxic dose of MCT results from MCTP effects on HPCs and SECs. SEC injury may be an epiphenomenon or could contribute to HPC injury through coagulation system activation and subsequent fibrin deposition resulting in local hypoperfusion leading to hypoxic/ischemic injury. MCT was chosen as the hepatotoxicant of interest for this dissertation because it is known to be poisonous to both animals and humans and because of its potential to damage the liver vasculature, which has not been characterized well in other models of LPS augmentation of hepatotoxicant-induced injury. Hence, investigation of liver injury resulting from MCT/LPS-cotreatment may offer additional perspectives into this interesting phenomenon.

1.E. Summary

Inflammation can decrease the threshold for toxicity, making an individual more susceptible to hepatotoxicants (Roth *et al*, 2001; Ganey and Roth, 2001). To understand this phenomenon better, LPS potentiation of MCT-induced toxicity was explored. The focus of this dissertation is to characterize the development of liver injury resulting from coexposure to small noninjurious doses of MCT and LPS and to explore the mechanisms behind this liver injury model. In particular, the hypothesis is tested that inflammatory events participate causally in the synergistic hepatotoxicity from coexposure to MCT and LPS. "Synergy" is defined as the condition in which the coadministration of two agents causes a

change in a toxic biomarker (e.g., plasma alanine aminotransferase) that is greater than the sum of effects occurring when each agent is given alone. "Synergy" includes the appearance of toxicity from the coadministration of doses of two agents that are nontoxic when given alone.

In the following chapters, development and characterization of a model for acute, synergistic hepatotoxicity from coexposure to small, noninjurious doses of MCT and LPS is described. Further, it is demonstrated that alterations in the sequence of MCT and LPS coexposure changes the nature of toxicity and that SEC injury and activation of the coagulation system are both characteristics of this model. Finally, evidence is presented that KCs, TNF-α, PMNs and the coagulation system are causal to the pathogenesis of MCT/LPS-induced hepatotoxicity.

CHAPTER 2

Synergistic Hepatotoxicity from Coexposure to Bacterial Lipopolysaccharide and the Pyrrolizidine Alkaloid Monocrotaline

2.A. Abstract

Individuals are commonly exposed to bacterial endotoxin (lipopolysaccharide; LPS) through Gram-negative bacterial infection and from its translocation from the gastrointestinal lumen into the circulation. Inasmuch as noninjurious doses of LPS augment the hepatotoxicity of certain xenobiotic agents, exposure to small amounts of LPS may be an important determinant of susceptibility to chemical intoxication. Monocrotaline (MCT) is a pyrrolizidine alkaloid phytotoxin that at large doses produces centrilobular liver lesions in rats. In the present study, the food-borne toxin MCT was coadministered with LPS to determine if LPS would enhance its hepatotoxicity. Doses of MCT (100 mg/kg, i.p.) and LPS (7.4 x 10^6 EU/kg, i.v.), which were nonhepatotoxic when administered separately, produced significant liver injury in male, Sprague-Dawley rats, when given in combination. Within 18 hours after MCT administration, this cotreatment resulted in enhanced plasma alanine aminotransferase and aspartate aminotransferase activities, two markers of liver injury. Histologically, overt hemorrhage and necrosis appeared between 12 and 18 hours. The lesions were centrilobular and midzonal and exhibited characteristics similar to lesions associated with larger doses of MCT and LPS, respectively. In the presence of LPS, the threshold for MCT toxicity was reduced to 13 – 33% of the dose required for toxicity with MCT alone. A study in isolated, hepatic parenchymal cells revealed no interaction between MCT and LPS in producing cytotoxicity. In summary, coexposure of rats to noninjurious doses of MCT and LPS resulted in pronounced liver injury. Results *in vitro* suggest that

the enhanced toxicity does not result from a direct interaction of MCT and LPS with hepatic parenchymal cells. These results provide additional evidence that exposure to small amounts of LPS may be a determinant of susceptibility to food-borne hepatotoxins.

2.B. Introduction

In the present investigation, noninjurious doses of LPS were administered to rats in conjunction with nontoxic doses of MCT to test the hypothesis that LPS exposure augments the hepatotoxicity of MCT. In this study, we developed and characterized an animal model for LPS and MCT interaction. This model included elucidation of dose-response relationships and of development of acute toxicity. Finally, we explored whether the interaction between LPS and MCT could be reproduced in hepatocellular preparations *in vitro*.

2.C. Materials and Methods

2.C.1. Materials

LPS (*Escherichia coli,* serotype 0128:B12), heparin (Type II, disodium salt), mercuric chloride, p-dimethylaminobenzaldehyde, boron trifluoride in methanol, hydrogen peroxide, 3, 3'-diaminobenzidine, proteinase K (EC 3.4.21.64), deoxyribonuclease I (EC 3.1.21.1), hematoxylin solution (Gill No. 3), collagenase (Type II), and diagnostic kits 58 UV, 59 UV, 245, and 419 for the

determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyltransferase (GGT) activities, respectively, were purchased from Sigma Chemical Company (St. Louis, MO). MCT was obtained 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). *In situ* cell death detection kit with horseradish peroxidase was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Williams' Medium E and gentamicin were acquired from GIBCO (Grand Island, NY). Fetal calf serum was purchased from Intergen (Purchase, NY). Formalin fixative was obtained from Surgipath Medical Industries, Inc. (Richmond, IL).

2.C.2. Animals

Male, Sprague-Dawley rats (Crl: CD BR(SD) VAF/Plus, Charles River, Portage, MI) weighing 200 – 300 g were used in 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). The animals were maintained on a 12-hr 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.

2.C.3. Treatment Protocol

Rats were given MCT intraperitoneally at doses indicated in the text and figures or an equivalent volume of sterile saline vehicle (Veh). 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 adjusted with sterile saline to the appropriate final concentration. Four hours after treatment with MCT or its vehicle, LPS diluted in sterile saline or equivalent volume of sterile saline Veh was injected intravenously via tail vein at the doses indicated. Hepatotoxicity was assessed at the times specified in the text and figures. Allen *et al.* (1972) reported that peak metabolic bioactivation of MCT occurred 90 minutes after administration. LPS was given 4 hours following MCT to reduce the possibility of altering MCT bioactivation. In addition, preliminary studies revealed that this dosing protocol resulted in enhanced liver injury without substantial mortality. In contrast, concurrent administration of MCT and LPS resulted in considerable mortality, apparently through extrahepatic mechanisms (Yee *et al.*, 1998).

2.C.4. Determination of Hepatotoxicity

At various times after treatment with MCT or its vehicle (i.e., 12, 18, or 24 hours), rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A midline abdominal incision was made, blood was collected from the inferior vena cava into a syringe containing heparin (final concentration, 50 U/ml), and animals were euthanized by exsanguination. Hepatocellular injury was estimated as

increases in activity of plasma ALT and AST. Biliary injury was estimated as increased plasma ALP and GGT activities. Livers were removed intact and fixed by immersion in 10% neutral buffered formalin for a minimum of 3 days.

2.C.5. Histopathologic Evaluation and Morphometry

Two transverse sections from the middle of the left lateral liver lobe and one from the right lateral 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. A computerized image analysis system was employed to quantify treatment-induced changes in liver morphology. This system consisted of a light microscope (Olympus AX-80T, Olympus Corp., Lake Success, NY) interfaced with a high resolution CCD color camera (OLY-750, Olympus-America, Inc., Melville, NY), a color monitor, and a Power Macintosh 9600/300 computer with the public domain National Institutes of Health image analysis program (developed by the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image). Digitized color images of hematoxylin- and eosin-stained liver sections were evaluated with a 25-point lattice grid (Stereology Toolbox, Morphometrix, Davis, CA) to determine (1) the total area of liver analyzed, (2) the area of lesion, (3) the area of normal parenchyma, and (4) the area of non-parenchymal 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). Non-

parenchymal space was defined as non-parenchymal tissue, vessel lumen, and regions outside the perimeter of the liver section. The area of each object (category) of interest was calculated from the following expression (Cruz-Orive, 1982):

$$Area_{Interest} = \Sigma\ Points_{Interest}\ X\ Area/Point$$

$$Area/Point = (Distance\ Between\ Points)^2$$

Distance between points was 70 μm. Accordingly, the area represented by each point was 4900 μm^2. 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 20). Thereafter, every 20th field containing hepatic parenchymal cells was evaluated (minimum of 10 fields measured/section). The measured fields represented approximately 5% of the total area of each liver section. A minimum of 10 animals per group was analyzed. Percent lesion area was estimated based on the following formula:

$$[Area_{Lesion}/(Area_{Lesion} + Area_{Parenchyma})] \times 100.$$

Following the recommendations of the Society of Toxicologic Pathologists' Committee on the Nomenclature of Cell Death (Levin *et al.*,1999), the term "necrosis" is used to describe cell death, regardless of the particular pathway by which cell death occurred. The terms "apoptosis" and "oncosis" are used to distinguish necrotic cells based on morphological characteristics largely described in Levin *et al.* (1999). Apoptotic necrosis characterizes individual or aggregates of shrunken cells with condensed and marginated nuclear chromatin

or karyorrhexis and cell fragments which were either anuclear or contained condensed nuclear fragments (Majno and Joris, 1995; Levin *et al.*, 1999). Oncotic necrosis is defined by swollen parenchymal cells with intensely eosinophilic cytoplasm, indistinct borders and pyknotic or karyolytic nuclei (Majno and Joris, 1995; Levin *et al.*, 1999).

[All photomicrographs in this chapter were taken by Jack R. Harkema].

2.C.6. TUNEL Assay

An *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was used to determine if DNA strand breakage was associated with cell death. In brief, liver sections from paraffin-embedded tissue were deparaffinized and rehydrated. After treatment with proteinase K, liver sections were subsequently treated with fluorescein-conjugated dUTP label and TdT (i.e., TUNEL reaction mixture) followed by a horseradish peroxidase conjugated anti-fluorescein antibody from an *in situ* cell death detection kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Slides were treated with the chromagen 3,3'-diaminobenzidine and counterstained with hematoxylin. Liver sections were analyzed by light microscopy. Only TUNEL positive nuclei that also met the morphologic criteria of apoptosis were classified as having undergone apoptotic necrosis (Levin *et al.*, 1999).

2.C.7. Effect of LPS on MCT-induced Cytotoxicity In Vitro

Hepatic parenchymal cells (HPCs) were isolated from rats by collagenase (type II; 0.24 mg/ml) digestion according to the method of Seglen (1973), as modified by Klaunig *et al.* (1981). The cell pellet was washed twice with Williams' Medium E, then the cells were suspended in Williams' Medium E containing 10% fetal calf serum and 1% gentamicin and placed in 6-well Falcon, Primaria plates (Becton Dickinson & Company, Lincoln Park, NJ) at 1 x 10^6 HPCs/well. Greater than 95% of the cells in the final preparation were hepatic parenchymal cells as determined by light microscopy. The viability of the isolated HPCs was greater than 90% (via trypan blue exclusion).

After HPCs were stabilized in culture for 4 hours at 37°C in 92.5% O_2/7.5% CO_2, the wells were washed with medium, and MCT was added to the fresh medium (0, 0.04, 0.4, or 1 mM, final concentration). After 4 hours of incubation, LPS was added (0, 7.4 x 10^3, 7.4 x 10^4, or 7.4 x 10^5 EU/ml), and the cells were incubated for an additional 20 hrs. Concentrations of MCT were based on those used by DeLeve *et al.* (1996). Concentrations of LPS were chosen to span that expected to occur *in vivo* assuming a dose of 7.4 x 10^6 EU/kg distributed initially into plasma volume.

After incubation, the medium was collected, and HPCs were lysed with 2 ml 1% Triton X-100. Percent ALT release was assessed by dividing ALT released into the medium by the ALT measured in the medium plus the cell lysates.

2.C.8. Ehrlich Assay

The Ehrlich assay was used to measure the concentration of pyrroles in liver as a marker of bioactivation of MCT to toxic pyrrolic metabolites (Mattocks and White, 1970). Monocrotaline pyrrole synthesized from MCT was used to generate a standard curve (Mattocks, 1969; Mattocks *et al.*, 1989). A liver sample (0.5 g) from the left and right lateral lobes was homogenized in 5% mercuric chloride and then centrifuged. The resulting pellet was washed with absolute ethanol and centrifuged again. The pellet was resuspended in a 1:1 solution of absolute ethanol and Ehrlich's reagent (3% 4-dimethylaminobenzaldehyde in methanolic 14% boron trifluoride). The solution was heated to 90°C for 1 minute and then allowed to cool to room temperature. Solids were pelleted by centrifugation, and the absorbance of the supernatant at 565 and 625 nm was measured. The λ_{max} of the Ehrlich pyrrole complex is 565 nm. The λ_{max} of Ehrlich reagent-treated blood is 625 nm. Absorbance values were corrected for the presence of residual blood in the liver (Mattocks and White, 1970), and saline/saline control livers were used to correct for presence of endogenous pyrroles unrelated to MCT treatment.

2.C.9. Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). When variances were not homogeneous, data were log-transformed before analysis. Data for single comparisons were analyzed by Student's t-test (Steel *et al.*, 1997). Multiple comparisons were analyzed by one-way, two-way or three-way analysis of variance (ANOVA), as appropriate, and group means were

compared using Tukey's omega post hoc test. Data expressed as percentages were transformed by arc sine square root prior to analysis (Steel *et al.*, 1997). The criterion for statistical significance was $p \leq 0.05$ for all comparisons.

2.D. Results

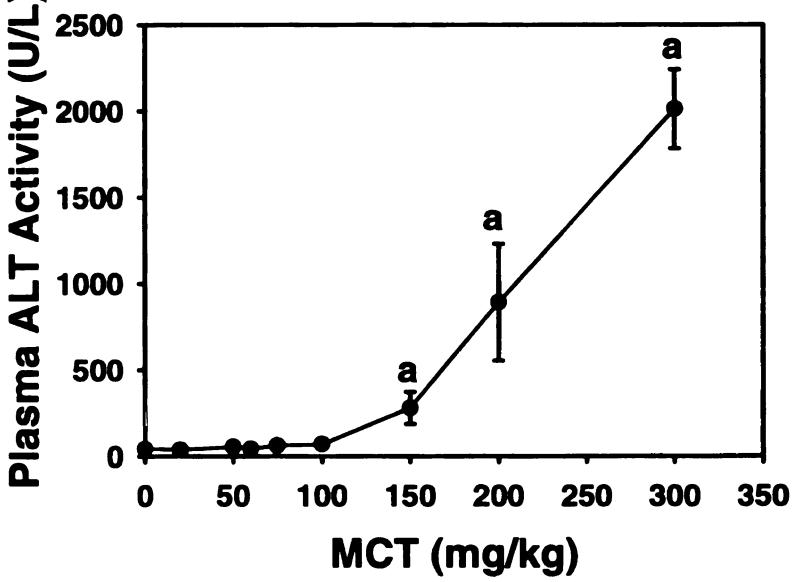
2.D.1. Dose-Ranging Studies: Monocrotaline

In a dose-ranging study to determine a nontoxic MCT dose for further examination, MCT (0 - 300 mg/kg) was administered i.p. to rats and followed 4 hours later by intravenous saline. At 24 hours, liver injury was assessed from increases in plasma ALT and AST activities (Figure 2.1). Hepatotoxicity was dose-dependent, and the threshold (i.e., start) for toxicity was between 100 and 150 mg MCT/kg. A dose of 100 mg/kg was determined to be noninjurious using plasma ALT and AST activities as criteria. Upon histopathologic examination minor congestion was observed infrequently in the sinusoids of centrilobular regions. Within this region, minimal apoptotic necrosis of parenchymal cells was observed at 24 hours. Typically, one or two such individual apoptotic cells or cell fragments were observed around a central vein. A TUNEL assay confirmed the observation of a mild increase in apoptotic necrosis compared to the saline/saline control (data not shown). The periportal and midzonal regions had normal hepatic sinusoidal architecture and parenchymal cells.

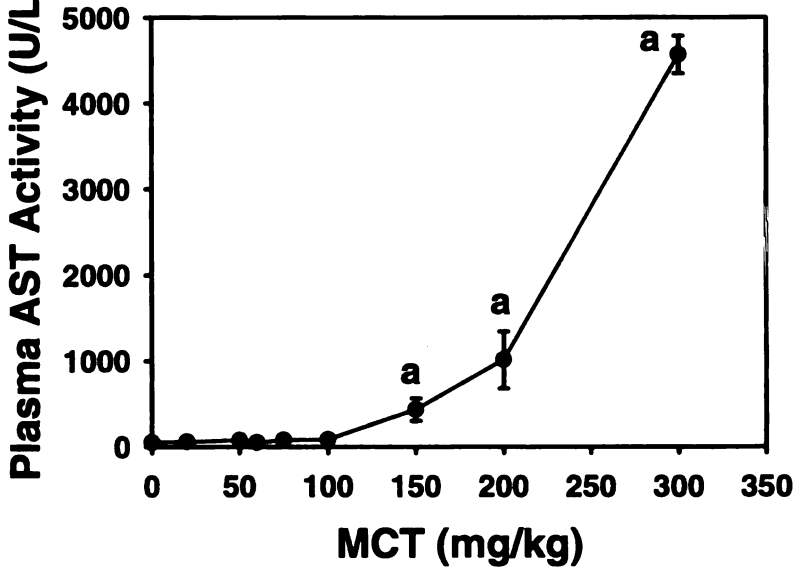
Figure 2.1. Dose-Related Hepatotoxicity of MCT. Rats were given a single administration of MCT (0 – 300 mg/kg) i.p. then an i.v. injection of sterile saline Veh 4 hours later. Hepatotoxicity was evaluated 24 hours after MCT administration by plasma ALT (A) and AST (B) activities. Error bars for SEM that are smaller than symbols are not shown. N = 4 – 30 animals.

[a] Significantly different ($p \leq 0.05$) from controls given only Veh.

A


Plasma ALT Activity (U/L)
2500
2000
1500
1000
500
0
a
a
a
0
50
100
150
200
250
300
350
MCT (mg/kg)

B

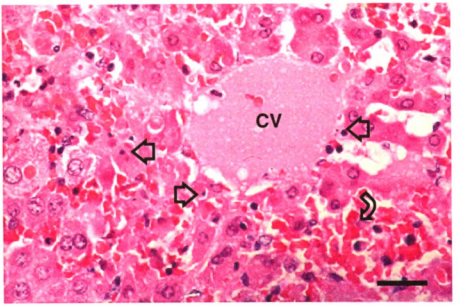

Plasma AST Activity (U/L)
5000
4000
3000
2000
1000
0
a
a
a
0
50
100
150
200
250
300
350
MCT (mg/kg)

At 200 mg MCT/kg (Figure 2.2), marked hepatocellular apoptotic necrosis occurred in centrilobular areas, but hepatocytes with oncotic or degenerative characteristics, such as shrunken or overtly pyknotic nuclei and indistinct cell borders, were also visible. Disruption of the vascular intima typified central venules. This was associated with severe hemorrhage and loss of tissue architecture in surrounding parenchyma. Indeed, some central veins were obscured by marked hemorrhage and centrilobular necrosis. Minimal inflammatory cell accumulation (i.e., neutrophils and monocytes) was observed in sinusoids. These centrilobular lesions were not uniform, however, and sometimes exhibited projections of injury radiating toward the midzonal region, resulting in occasional bridging of lesions. The midzonal and periportal regions remained largely normal, although there was slight congestion in both regions.

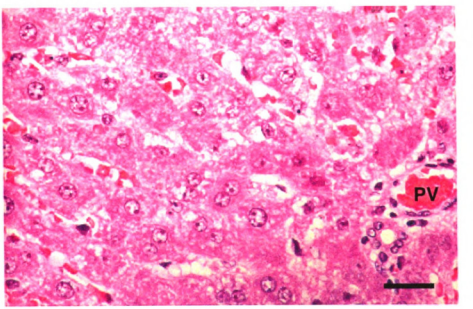
At a larger dose of MCT (300 mg/kg), marked to severe necrosis was observed in the centrilobular regions of liver lobules. Necrosis also extended to the midzonal and, occasionally, periportal regions. Affected hepatic parenchymal cells exhibited predominantly apoptotic morphology, although lesions contained parenchymal cells with oncotic characteristics. Compared to the 200 mg MCT/kg dose there was increased hemorrhage and loss of central venular intima and sinusoidal architecture. An increased presence of neutrophils and monocytes was also observed within the affected centrilobular and midzonal regions. Mortality was 66% in rats treated with 300 mg MCT/kg by 24 hours. No animals died within 24 hours after administration of smaller doses of MCT. Based on

Figure 2.2. Representative Photomicrographs of Liver from a Rat Given an Hepatotoxic Dose of MCT. Centrilobular and midzonal regions of liver lobules from an animal treated with 200 mg MCT/kg and sterile saline Veh 4 hours later. Twenty-four hours after MCT administration marked, centrilobular apoptotic necrosis (wide open arrows) is apparent. This centrilobular lesion is associated with severe hemorrhage (curved open arrow), loss of sinusoidal architecture, and disruption of the vascular intima of the central vein. The remainder of the liver lobule appears normal. CV = Central Vein. PV = Portal Vein. Bar indicates 30 μm. [Images in this dissertation are presented in color].

Centrilobular



Midzonal



plasma enzyme markers and histopathology, 100 mg MCT/kg was chosen as a minimally toxic dose for initial cotreatment studies.

2.D.2. Dose-Ranging Studies: Endotoxin

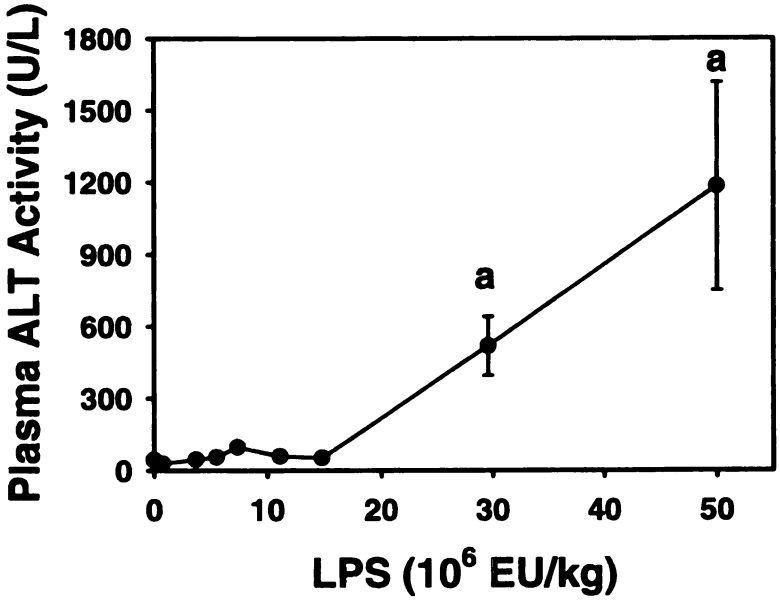
A similar dose-ranging study was conducted for LPS. LPS (0 – 50 x 10^6 EU/kg) was administered by tail vein injection to animals which had received an injection of saline (i.p.) 4 hours earlier. Hepatotoxicity was assessed at 24 hours via plasma ALT and AST activities (Figure 2.3). Increases in these plasma enzyme activities were apparent at doses exceeding 15 x 10^6 EU LPS/kg. After a dose of 7.4 x 10^6 EU LPS/kg, approximately one quarter of the animals treated exhibited occasional, mild to moderate, midzonal coagulative (oncotic) necrosis with an associated influx of neutrophils at 24 hours. At this small dose, the lesions were apparent predominantly in the subserosal areas of the liver sections. However, approximately three-quarters of the animals treated at this dose had normal livers or small, infrequent hepatic lesions restricted to the midzonal regions. A widespread increase in neutrophils and a mild increase in mononuclear cells were apparent in hepatic sinusoids. At doses greater than 7.4 x 10^6 LPS EU/kg, midzonal coagulative necrosis was present. Neutrophilic infiltration was associated with the hepatocellular necrosis.

At 50 x 10^6 EU LPS/kg (Figure 2.4), moderate to marked midzonal coagulative hepatocellular necrosis was observed, with a mixed inflammatory cell infiltrate of mononuclear cells and neutrophils and minimal hemorrhage. Both the centrilobular and periportal regions of the liver lobules remained normal.

Figure 2.3. Dose-Related Hepatotoxicity of LPS. Sterile saline Veh was administered i.p. to rats followed 4 hours later by a single i.v. administration of LPS (0 – 50 x 10^6 EU/kg). Hepatotoxicity was assessed 24 hours after saline Veh administration by plasma ALT (A) and AST (B) activities. Error bars for SEM that are smaller than symbols are not shown. N = 4 – 30 animals.

[a] Significantly different ($p \leq 0.05$) from Veh/Veh control.

A


Plasma ALT Activity (U/L)
1800
1500
1200
900
600
300
0
a
a
0
10
20
30
40
50
LPS (10^6 EU/kg)

B

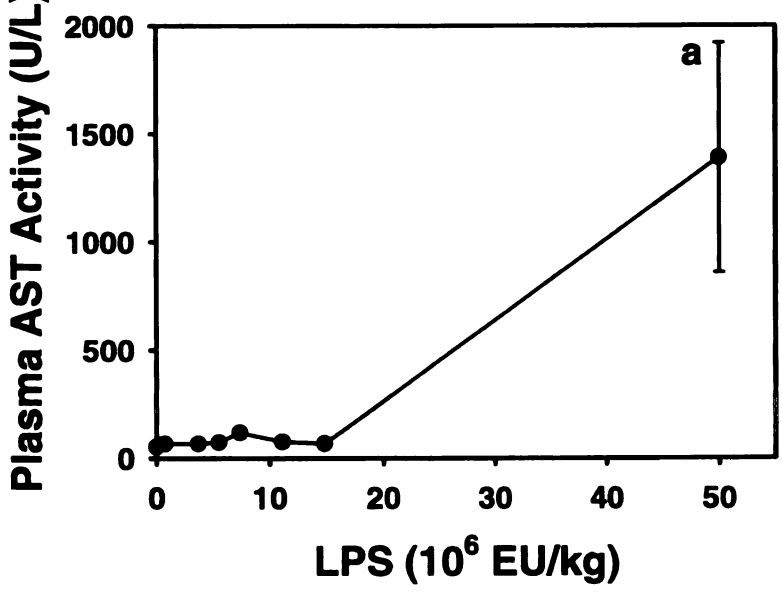
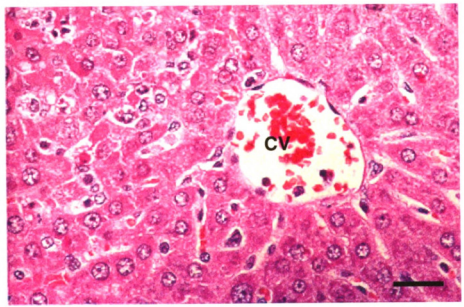
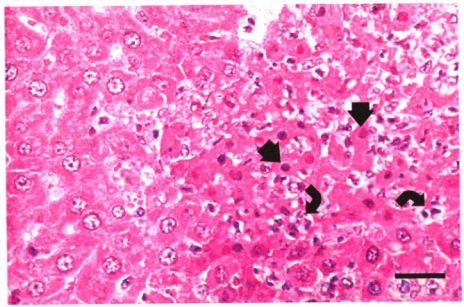

Plasma AST Activity (U/L)
2000
1500
1000
500
0
a
0
10
20
30
40
50
LPS (10^6 EU/kg)

Figure 2.4. Representative Photomicrographs of Liver from a Rat Given an Injurious Dose of LPS. Centrilobular and midzonal regions of liver lobules from an animal treated with 50 x 10^6 EU LPS/kg 4 hours after sterile saline Veh injection. Twenty-four hours after saline administration, moderate to marked midzonal hepatocellular coagulative necrosis (wide closed arrows) is apparent, whereas the centrilobular and periportal (not shown) regions appear normal. Increased numbers of neutrophils (curved closed arrows) are present. CV = Central Vein. Bar indicates 30 μm. [Images in this dissertation are presented in color].

Centrilobular



Midzonal



In this study, LPS doses of 11.1 and 30 x 10^6 EU/kg resulted in 8% and 14% mortality (i.e., one animal death per group), respectively, whereas no animals died within 24 hours at the other doses.

A dose of 7.4 x10^6 EU LPS/kg was chosen for initial MCT/LPS cotreatment studies. This dose was designated "noninjurious" because it did not produce a significant increase in plasma aminotransferase activities and it resulted in a minimal to mild inflammatory response, with an absence of histologic lesions in a majority of animals.

2.D.3. Hepatotoxicity in Animals Cotreated with MCT and LPS

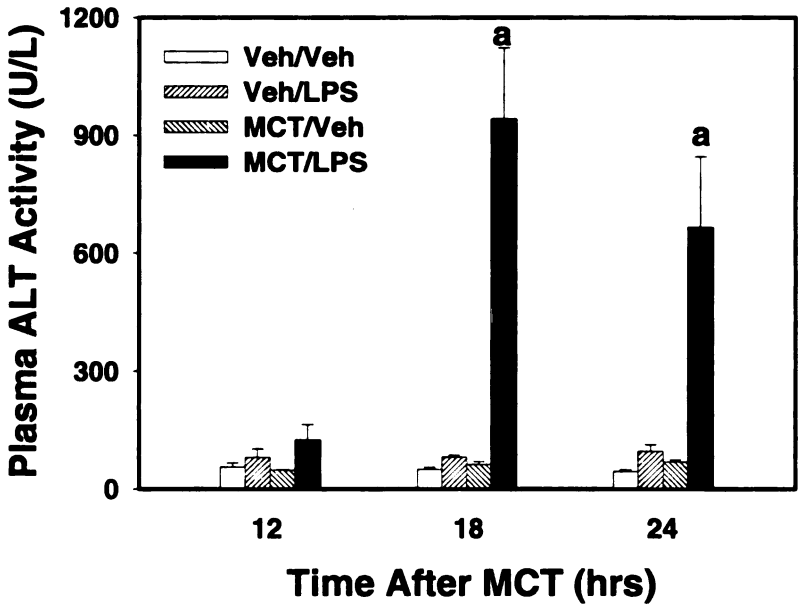
Hepatotoxicity in animals cotreated with noninjurious doses of MCT (100 mg/kg) and LPS (7.4 x 10^6 EU/kg) was assessed 24 hours after MCT treatment (Figure 2.5). Histologically, livers from Veh/Veh-treated rats had no evidence of injury (Figure 2.6A). Livers from the Veh/LPS-treated animals had normal liver architecture as well (Figure 2.6B), although minimal midzonal lesions were apparent in some animals. A mild increase in neutrophils in hepatic sinusoids was apparent in livers from all animals in this treatment group. Animals treated with MCT/Veh had minimal to no centrilobular histopathologic change (Figure 2.6C), as noted in the dose-ranging study above, and midzonal and periportal regions were normal. No significant increases in plasma aminotransferase activities were observed for the either Veh/LPS or MCT/Veh treatment groups compared to the Veh/Veh group (Figure 2.5) or to naïve animals (data not shown).

Figure 2.5. Development of Hepatotoxicity in Rats Cotreated with MCT and LPS. Animals were given 7.4 x 10^6 EU LPS/kg or saline Veh, i.v., 4 hours after i.p. injection of 100 mg MCT/kg or saline Veh. Hepatotoxicity was evaluated at 12, 18 or 24 hours after MCT administration by plasma ALT (A) and AST (B) activities. N = 6 – 30 animals.

[a] Significantly different ($p \leq 0.05$) from all other groups at the same time.

[b] Significantly different ($p \leq 0.05$) from MCT/LPS groups at both of the other times.

A


Plasma ALT Activity (U/L)
1200
900
600
300
0
Veh/Veh
Veh/LPS
MCT/Veh
MCT/LPS
a
a
12
18
24
Time After MCT (hrs)

B

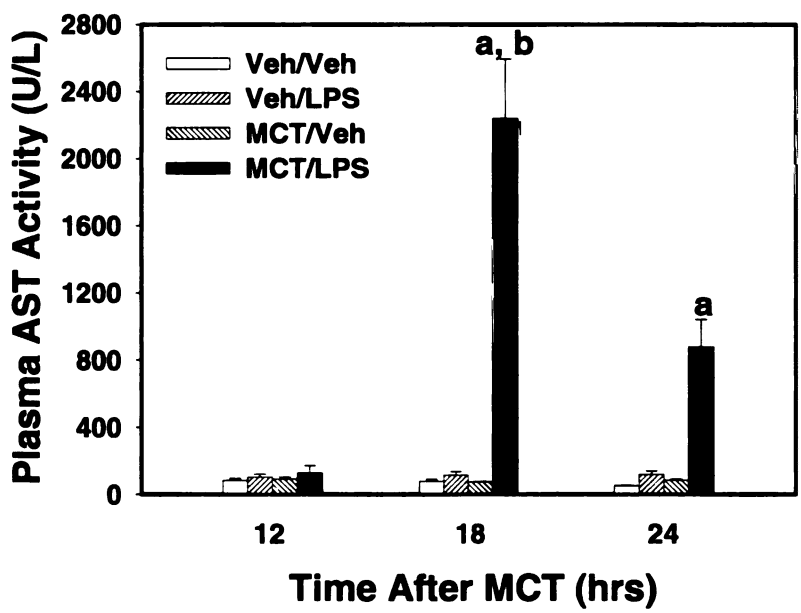
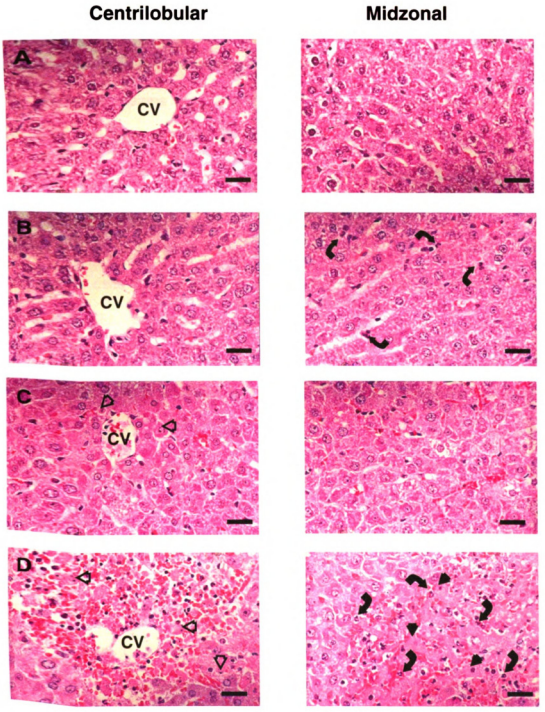

Plasma AST Activity (U/L)
2800
2400
2000
1600
1200
800
400
0
Veh/Veh
Veh/LPS
MCT/Veh
MCT/LPS
a, b
a
12
18
24
Time After MCT (hrs)

Figure 2.6. Representative Photomicrographs of Liver Sections from Rats Treated with LPS and/or MCT. Animals were given 100 mg MCT/kg or Veh and 4 hours later 7.4 x 10^6 EU LPS/kg or Veh. Livers were removed 24 hours after MCT or its Veh administration and processed for histopathology. Photomicrographs of centrilobular (CL) and midzonal (MZ) regions of liver from each group are presented. (A) Liver from an animal treated with Veh/Veh appears normal; (B) Liver from an animal treated with Veh and 7.4 x 10^6 EU LPS/kg. Except for a mild increase in the number of neutrophils in the sinusoids (curved closed arrows), most livers were normal. About one quarter of the livers had minimal MZ changes (i.e., occasional coagulative necrosis); (C) Liver from an animal treated with 100 mg MCT/kg and Veh. The architecture of the liver lobule is normal. Occasionally, single, scattered parenchymal cells around the central vein had characteristics of apoptosis (wide open arrows). No histopathological changes are apparent in the MZ or periportal (not shown) regions; (D) Liver from an animal cotreated with 100 mg MCT/kg and 7.4 x 10^6 EU LPS/kg. "Hybrid" lesion consists of moderate CL lesion and distinctly different, marked MZ lesion. CL lesion exhibits characteristics of apoptotic necrosis (wide open arrows) and hemorrhage. The MZ lesion exhibits coagulative necrosis (wide closed arrows) with a conspicuous influx of neutrophils (curved closed arrows). The periportal region of the liver lobule is normal (not shown). CV = Central vein. Bar indicates 30 μm. [Images in this dissertation are presented in color].


Centrilobular
Midzonal
A
CV
B
CV
C
CV
D
CV

In contrast, MCT/LPS cotreatment resulted in both midzonal and centrilobular injury (Figure 2.6D). Generally, the effect of the cotreatment was more pronounced in the midzonal region of the liver lobule than the centrilobular. The midzonal lesions consisted of moderate to marked coagulative necrosis similar to that produced by large, hepatotoxic doses of LPS. The midzonal lesion in these MCT/LPS cotreated rats corresponded most closely to the lesion produced by 30 x 10^6 EU LPS/kg given alone, except that hemorrhage adjacent to the areas of necrosis was more pronounced. At the lesion periphery, occasional cells with apoptotic morphology (i.e., marginated chromatin and positive TUNEL staining) were observed.

In centrilobular areas of livers from rats treated with the MCT/LPS combination, lesions varied in size from one animal to another. In most livers, there was partial to complete loss of intima in many of the central venules. This was typically associated with pericentral hemorrhage and loss of sinusoidal architecture. In some areas, central veins were obscured by these changes. In other livers, dilated sinusoids and congestion were more typical, with loss of sinusoidal architecture in some centrilobular areas. Centilobular hepatocellular changes included moderate to marked apoptotic necrosis and other changes noted above in livers of animals treated with larger doses of MCT. Hepatocellular degeneration with smaller than normal, faintly staining nuclei were observed within this region of the liver lobule. Moderate accumulation of neutrophils and monocytes was another prominent feature of the lesion. Hepatocytes with apoptotic morphology and the accumulation of inflammatory

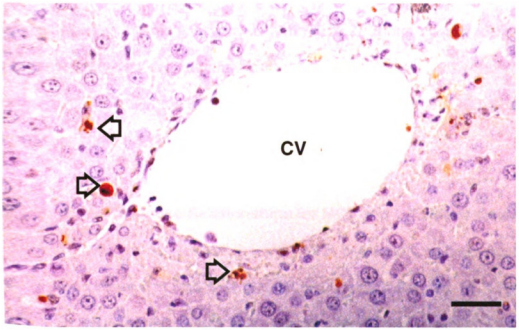
cells in centrilobular areas was more pronounced than in the livers of rats given the same dose of MCT alone. Indeed, the centrilobular lesions in this cotreated group resembled in severity the centrilobular lesions in the livers of rats given doses of MCT alone of 150 – 200 mg/kg (see Figure 2.2). Positive TUNEL staining (Figure 2.7) corroborated the presence of apoptotic necrosis apparent in the H & E sections.

Morphometric analysis supported the pronounced increase in liver injury in MCT/LPS cotreated rats. The minor injury found in rats treated either with MCT/Veh or with Veh/LPS was due predominantly to individual cell necrosis and comprised less than 2% of the area in livers examined (1.7 ± 0.3 and 1.1 ± 0.2, respectively). By contrast, 22 ± 2% of the liver area was injured in animals treated with MCT and LPS. ANOVA revealed a significant interaction between MCT and LPS treatments.

2.D.4. Time Course of Injury in MCT/LPS-Cotreated Rats

MCT/LPS cotreatment produced no change in plasma aminotransferase activities by 12 hours after MCT administration (Figure 2.5). Livers removed from these rats had either no injury or infrequent, mild histopathologic change. In the centrilobular area, there was slight congestion and mild apoptotic necrosis. The intima of the central veins remained intact. Midzonally, mild to moderate, mixed apoptotic and coagulative necrosis was observed infrequently. A slight increase in numbers of neutrophils was observed midzonally. The periportal regions remained normal. MCT/saline and saline/LPS controls at 12 hours revealed

Figure 2.7. Photomicrograph of a TUNEL-Stained Liver from a Rat Given MCT/LPS. TUNEL assay was performed on liver from an animal cotreated with 100 mg MCT/kg and 7.4 x 10^6 EU LPS/kg. TUNEL-positive staining (wide open arrows) in the centrilobular region was associated with bodies having morphological features of apoptosis. An increase in apoptosis was observed in the centrilobular region relative to livers from animals treated with Veh/Veh, MCT/Veh, or Veh/LPS. CV = Central Vein. Bar indicates 30 μm. [Image in this dissertation is presented in color].


CV

normal liver architecture, although the latter group showed a mild increase in neutrophils.

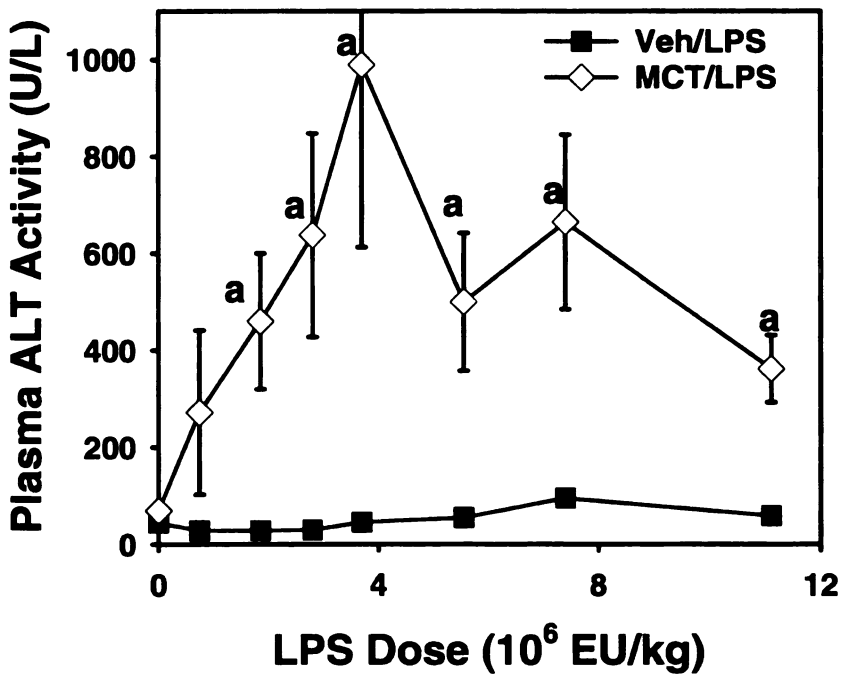
Hepatotoxicity as determined by increases in plasma ALT and AST activities was observed at 18 and 24 hours in rats cotreated with MCT and LPS (Figure 2.5). Pronounced centrilobular and midzonal lesions were present by 18 hours. Lesion characteristics were the same as those described for cotreated animals evaluated at 24 hours. Throughout the 12, 18, and 24-hour timepoints, no significant treatment-related increase in plasma ALP or GGT activities was observed (data not shown).

2.D.5. Dose Response Relationships for MCT and LPS Cotreatment

To characterize further the MCT/LPS interaction, various doses of LPS were administered to rats that had received either 100 mg MCT/kg or saline vehicle 4 hours earlier (Figure 2.8). Hepatotoxicity was assessed 24 hours after MCT administration. Rats receiving only LPS had no significant increase in plasma ALT activity at any of the LPS doses tested. By contrast, a dose-related increase in plasma ALT activity occurred in LPS-treated animals that were cotreated with 100 mg MCT/kg. A significant elevation in plasma ALT activity occurred with 1.85×10^6 EU LPS/kg in cotreated rats. The maximal effect occurred at 3.7×10^6 EU LPS/kg. At each of the LPS doses that resulted in significantly elevated ALT activity (Figure 2.8), midzonal and centrilobular liver lesions qualitatively similar to those described above were observed. Mortality was approximately 20% in cotreated rats given doses of LPS less than 7.4×10^6

Figure 2.8. Hepatotoxicity from MCT/LPS Coadministration: LPS Dose-Response. Animals were given 100 mg MCT/kg or its saline Veh and then 4 hours later LPS (0 – 11.1 x 10^6 EU/kg). Hepatotoxicity was assessed 24 hours after MCT administration from plasma ALT activity. N = 4 – 20 animals.

[a] Significantly different ($p \leq 0.05$) from value at the same dose of LPS.


Plasma ALT Activity (U/L)
1000
800
600
400
200
0
0
4
8
12
LPS Dose (10^6 EU/kg)
Veh/LPS
MCT/LPS
a
a
a
a
a
a

EU/kg. Mortality of 50% occurred in animals receiving the cotreatment of MCT with LPS at a dose of 11.1 x 10^6 EU/kg.

The dose-response relationship for MCT was also elucidated in animals treated with MCT and then given 4 hours later either a nontoxic dose of LPS (7.4 x 10^6 EU/kg) or it's Veh (Figure 2.9). When given alone, none of the doses of MCT tested (0 – 100 mg/kg) produced significant increases in plasma aminotransferases at 24 hours. However, when coadministered with LPS, increased plasma ALT and AST activities occurred at MCT doses of 50 mg/kg and greater. Mortality was approximately 15% in these groups. Each of the MCT doses that caused significantly elevated plasma ALT activity also produced midzonal and centrilobular lesions qualitatively similar to those described above.

When rats were cotreated with the smallest doses of MCT and LPS that produced significant hepatotoxicity in the dose-response studies above (i.e., 50 mg MCT/kg [Figure 2.9] and 1.85 x 10^6 EU LPS/Kg [Figure 2.8]), no increase in plasma ALT or substantial histopathologic change resulted (data not shown).

2.D.6. Effect of MCT and LPS on Hepatic Parenchymal Cells in Vitro

[This series of experiments was conducted by Shawn Kinser]. MCT and/or LPS were applied to isolated HPCs (Figure 2.10) following the treatment protocol described in Materials and Methods. Cytotoxicity was evaluated at 24 hours from ALT released into the medium. Although either MCT or LPS caused significant cytotoxicity at large concentrations, ANOVA revealed no significant interaction between these agents.

Figure 2.9. Hepatotoxicity from MCT/LPS Coadministration: MCT Dose-Response. 7.4 x 10^6 EU LPS/kg or its sterile saline Veh was given to animals 4 hours after MCT (0 – 100 mg/kg). Hepatotoxicity was assessed 24 hours after MCT administration from plasma ALT activity. N = 4 – 20 animals.

[a] Significantly different ($p \leq 0.05$) from value at the same dose of MCT.

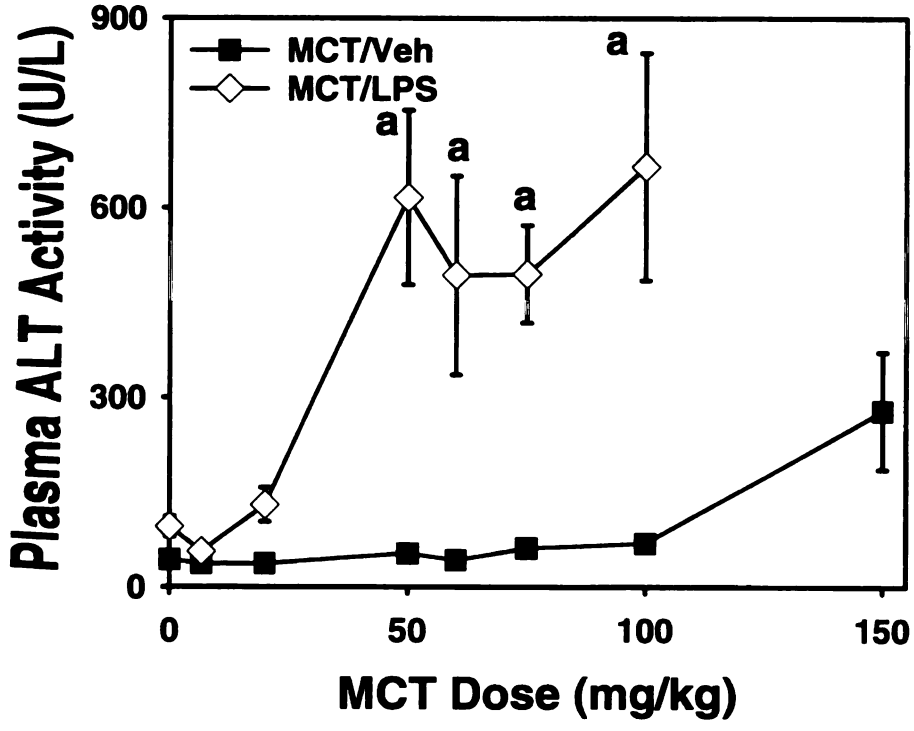
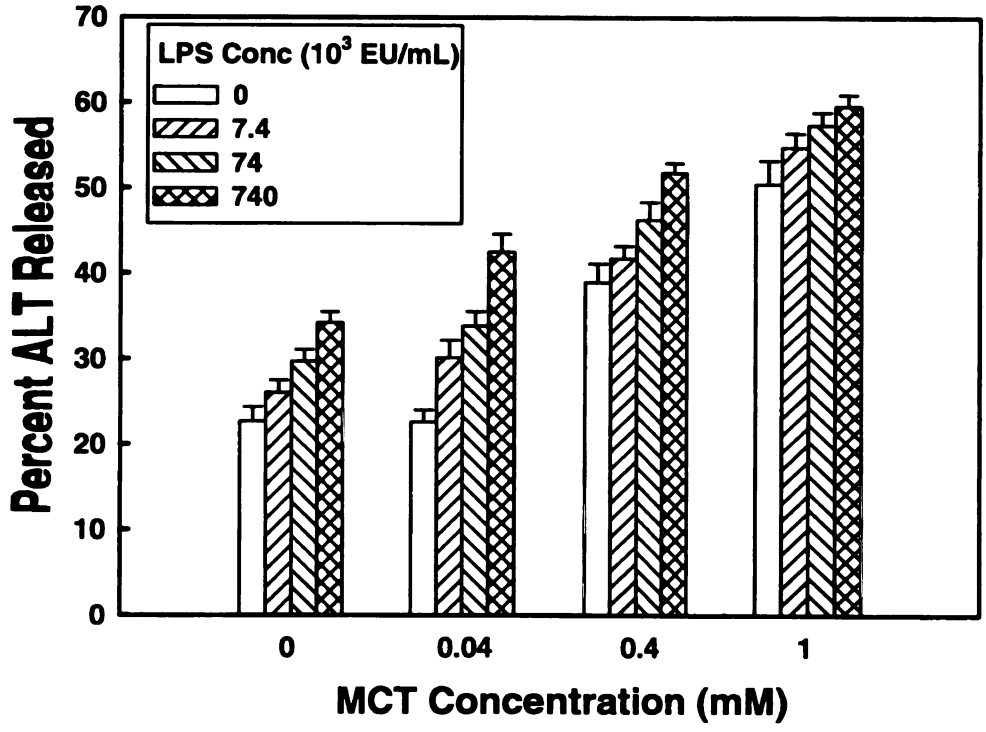

900
600
300
0
Plasma ALT Activity (U/L)
MCT/Veh
MCT/LPS
a
a
a
a
0
50
100
150
MCT Dose (mg/kg)

Figure 2.10. Cytotoxic Effects of MCT and LPS on Isolated, Hepatic Parenchymal Cells. HPCs were isolated and treated as described in Chapter 2 Materials and Methods. Cytotoxicity was evaluated as percent ALT release 24 hours after application of MCT to isolated HPCs. LPS (0 – 7.4 x 10^5 EU/mL) was administered to isolated hepatocytes 4 hours after MCT (0 – 1 mM). N = 7 liver isolations. ANOVA revealed that MCT and LPS each caused significant cytotoxicity ($p \leq 0.05$), but there was no significant interaction between the two treatments. [The work in this figure was completed by Shawn Kinser].


LPS Conc (10^3 EU/mL)
0
7.4
74
740
Percent ALT Released
70
60
50
40
30
20
10
0
0
0.04
0.4
1
MCT Concentration (mM)

2.D.7. Determination of Pyrrole Concentration in Livers from MCT-Treated Rats

Liver pyrrole concentration was measured to determine if LPS exposure altered the bioactivation of MCT to MCTP. Rats were treated with 7.4 x 10^6 EU LPS/kg or Veh 4 hours after 100 mg MCT/kg according to the protocol described in Materials and Methods. As described earlier (Figure 2.5), this regimen was hepatotoxic. Twenty-four hours after MCT treatment, livers were removed and homogenized for pyrrole determination by the Ehrlich assay. The administration of LPS did not increase the tissue Ehrlich activity that resulted from MCT treatment (Table 2.1).

2.E. Discussion

Large hepatotoxic doses of either MCT or LPS result in the release of liver enzymes such as ALT and AST into the plasma. However, MCT exposure at large doses results in an acute hepatic lesion distinct from that produced by LPS. In dose-ranging studies, MCT given at an hepatotoxic dose caused a centrilobular lesion characterized by predominantly apoptotic necrosis, hepatocellular degeneration and vascular injury. Parenchymal cell changes were associated with increased plasma aminotransferase activities, as has been reported in other models of liver injury in which apoptosis occurred (Levin *et al.*, 1999). The vascular injury included loss of intima in central veins and congestion and hemorrhage in sinusoids (Figure 2.2; Schoental and Head, 1955; McLean, 1970; DeLeve *et al.*, 1999). At doses equal to or greater than 200 mg MCT/kg,

Table 2.1. Influence of LPS on Hepatic MCT Bioactivation *in Vivo*. LPS (7.4×10^6 EU/kg; i.v.) or its saline vehicle (Veh) was administered to rats 4 hours after i.p. injection of 100 mg MCT/kg. Liver samples were taken 24 hours after MCT administration and homogenized for analysis of pyrrole concentration by the Ehrlich assay as described in Chapter 2 Materials and Methods. Resulting values were corrected for endogenous pyrrole concentration by subtracting values from animals given the Veh/Veh combination (i.e., 24.9 μg pyrrole/g liver). Data are expressed as mean ± SEM. N = 5 animals. There was no significant difference ($p > 0.05$) between MCT/Veh and MCT/LPS groups.

Treatment	Pyrrole (μg/g liver)
MCT/Veh	37 ± 4
MCT/LPS	35 ± 2

At doses equal to or greater than 200 mg MCT/kg, lesions radiated into the midzonal and periportal regions of the liver lobule. Some central veins were obscured by marked hemorrhage and centrilobular hepatocellular necrosis. This may have resulted from disruption of vascular intima and of sinusoidal endothelial cell integrity (DeLeve *et al.*, 1999). By contrast, LPS caused strictly midzonal lesions even at large doses. These comprised well-defined areas of coagulative necrosis of parenchymal cells with pronounced neutrophil infiltration. In summary, at toxic doses LPS produces a midzonal lesion with neutrophil involvement whereas MCT causes a distinctly different lesion that originates centrilobularly with more pronounced congestion and hemorrhage.

This study employed doses of LPS and MCT which, when administered separately to rats, were deemed noninjurious using as criteria a lack of elevation in plasma aminotransferase enzymes at 24 hours and minimal hepatic histopathological changes. Interestingly, a 100 mg/kg dose of MCT induced infrequent apoptotic necrosis of individual centrilobular hepatic parenchymal cells. TUNEL staining supported the morphological identification of apoptosis. This finding is in accord with evidence *in vitro* that MCT can induce apoptosis in other cell types (Thomas, *et al.*, 1998). When these small doses of MCT and LPS were coadministered to rats, pronounced liver injury resulted within 24 hours. This was reflected as increases in plasma ALT and AST activities. For these markers of injury, hepatotoxicity was evident between 12 and 18 hours after MCT administration. Histopathologically, hepatocellular injury appeared

infrequently as early as 12 hours in animals receiving the MCT/LPS cotreatment and became overt and typical by 18 hours.

In MCT/LPS cotreated rats, centrilobular areas showed enhanced hepatocellular apoptotic necrosis relative to controls treated with either agent alone. Congestion and hemorrhage within the centrilobular lesions were prevalent. Hepatocellular degeneration was also observed. Thus, the centrilobular lesion resembled that seen after a larger dose of MCT (between 150 – 200 mg MCT/kg) given alone, suggesting that LPS enhanced the hepatotoxicity of MCT. The midzonal region, however, was even more severely affected. Lesions midzonally revealed frequent, well defined areas of hepatocellular coagulative necrosis accompanied by neutrophil infiltration with congestion and hemorrhage. The midzonal lesion closely resembled that which occurred after a large dose of LPS (i.e., 30 x 10^6 EU LPS/kg) was given alone, suggesting profound, MCT-induced enhancement of the effects of LPS. Accordingly, the overall histopathological picture suggested that each of the agents enhanced the effects of the other, resulting in a "hybrid" lesion that favored midzonal injury.

To elucidate the dose-response relationship of the MCT/LPS interaction, a noninjurious dose of MCT (100 mg/kg) was coadministered with various doses of LPS. Significant hepatotoxicity was observed in these cotreated rats at LPS doses (1.85 – 2.8 x 10^6 EU/kg) which given alone produced no plasma aminotransferase increase or histopathologic change. The LPS threshold dose for this cotreatment was between 0.74 x 10^6 and 1.85 x 10^6 EU/kg (Figure 2.8). Therefore, in the presence of MCT, the threshold dose for LPS toxicity, as

determined by changes in plasma ALT activity, was 2 – 6% of that when LPS was given alone (i.e., 30 x 10^6 EU/kg).

In Figure 2.8, the dose-response relationship appeared to be bimodal, with a maximal effect occurring at an LPS dose of 3.7 x 10^6 EU/kg. It is important to note, however, that there were no statistically significant differences among the elevated ALT values (i.e., in the range of 3.7 – 11.6 x 10^6 EU LPS/kg). Moreover, mortality was considerably greater at the largest LPS dose studied. Since plasma ALT activity was measured only in the survivors, the animals that died and which may have had more severely affected livers were not represented in the data set. Thus, the bimodal appearance of the dose-response relationship depicted in Figure 8 may have resulted from greater mortality at greater LPS doses; furthermore, a bimodal relationship was not supported by statistical analysis.

When a noninjurious dose of LPS (7.4 x 10^6 EU/kg) was coadministered with various MCT doses, significant hepatotoxicity occurred with an MCT dose as small as 50 mg/kg. Thus, the threshold for MCT was between 20 and 50 mg/kg (Figure 2.9). Since 150 mg MCT/kg was near the threshold for toxicity for MCT when given alone, this indicates that LPS reduced the threshold for MCT, as determined by plasma ALT activities, to 13 – 33% of that which occurred in the absence of this inflammagen.

Although midzonal and centrilobular lesions were not quantified in the dose-response studies, it is clear that the threshold for appearance of each of these lesions was reduced when MCT and LPS were given in combination.

Hence, the threshold for toxicity for either agent was reduced in the presence of the other.

A study was performed *in vitro* to determine whether LPS and MCT act synergistically in causing damage to isolated, hepatic parenchymal cells. The same treatment protocol employed *in vivo* was used (i.e., MCT application was followed 4 hours later by LPS). Exposure either to MCT or to LPS resulted in concentration-dependent cytotoxicity, which was significant at larger concentrations. However, there was no significant interaction between MCT and LPS when they were given together. Thus, the cotreatment did not result in a synergistic response like that observed *in vivo*. This result suggests that other factors not present in the culture system, such as inflammatory cells and/or soluble inflammatory mediators, may play a role in the synergistic hepatotoxicity observed *in vivo*.

MCT-induced liver injury requires the bioactivation of MCT by cytochromes P450 to MCTP (Mattocks, 1968; McLean, 1970). This metabolic bioactivation of MCT to reactive pyrrole begins early after MCT administration and is evident as increased pyrrole concentration in liver for more than 24 hours (Allen *et al.*, 1972; Lame *et al.*, 1991). To test the possibility that the ability of LPS to augment MCT hepatotoxicity was the result of enhanced production of MCTP, an Ehrlich assay was performed to detect pyrrolic metabolites in the liver (Mattocks and White, 1970; Table 2.1). No significant effect of LPS treatment on the concentration of hepatic pyrroles occurred in rats given MCT. Although these results do not eliminate the possibility that LPS affects MCT metabolism, they do

suggest that LPS did not augment MCT hepatotoxicity by enhancing its bioactivation. This result might be expected from the observation that liver pyrrole concentration peaks after MCT administration (approximately 90 min) before the time when LPS was given (Allen *et al.*, 1972).

LPS and MCT each have effects on vascular endothelium, and it is possible that such effects contribute to the enhanced liver injury. Dose-dependent activation and destruction of endothelial cells is a well known effect of LPS (Pober and Cotran, 1990; Pohlman and Harlan, 1992; Hewett and Roth, 1993; Holst *et al.*, 1996; Mayeux, 1997). MCT can cause endothelial cell injury both *in vivo* (Schoental and Head, 1955; McLean, 1970; DeLeve *et al.*, 1999) and *in vitro* (DeLeve *et al.*, 1996). In MCT/LPS cotreated rats, the midzonal lesions appeared similar to those that occur from larger doses of LPS alone, except that hemorrhage was more pronounced. The greater hemorrhage suggests that MCT promoted sinusoidal endothelial cell injury (DeLeve *et al.*, 1996). It may have been that this disruption of the microvasculature that enhanced the potential for inflammatory injury to parenchymal cells was initiated by LPS. In the centrilobular areas, loss of venular intima and the collapse of surrounding sinusoidal architecture characterized damage from large doses of MCT. In animals receiving a small dose of MCT, such changes were enhanced by the administration of LPS. It seems possible that LPS-induced inflammation may have contributed to this vascular damage, with the result being disruption of local blood flow and consequent hepatocellular injury.

In summary, a small dose of LPS augments the hepatotoxicity of the food-borne hepatotoxin, MCT. Histologically, liver lesions that result from the cotreatment exhibit both LPS-like and MCT-like characteristics, suggesting that each agent enhances the hepatotoxic effects of the other. Results of a study in isolated, hepatic parenchymal cells suggest that the enhancement effect is mediated indirectly, perhaps through inflammatory and/or vascular events. These results provide evidence that modest LPS exposure may enhance sensitivity to pyrrolizidine alkaloids and possibly other hepatotoxins. The findings raise the possibility that people experiencing an inflammatory response who concurrently consume alternative medicines containing pyrrolizidine alkaloids may be particularly susceptible to liver injury.

CHAPTER 3

The Temporal Relationship between Bacterial Lipopolysaccharide and Monocrotaline Exposures Influences Toxicity: Shift in Response from Hepatotoxicity to Nitric Oxide-Dependent Lethality

3.A. Abstract

Liver injury from a variety of hepatotoxicants, including the food-borne phytotoxin monocrotaline (MCT), can be augmented by exposure to a noninjurious dose of the inflammagen, bacterial lipopolysaccharide (LPS). In a previous study, a nontoxic dose of LPS given four hours after MCT resulted in synergistic hepatotoxicity within 12-18 hours. The present study was designed to determine whether temporal differences in MCT and LPS exposure affect toxicity. When LPS (3.4×10^6 EU/kg; i.v.) was given one hour before MCT (100 mg/kg; i.p.), hepatotoxicity developed between 4 and 8 hours after MCT administration, and mortality was much greater than when LPS was administered 4 hours after MCT. To explore this difference, the temporal relationship between LPS and MCT exposure (7.4×10^6 EU/kg and 100 mg/kg, respectively) was altered. Twenty-four- hour survival was high in animals that received LPS 4 hours before (86%) or after (88%) MCT, but it decreased markedly when LPS was administered an hour before MCT (17%). Using this latter dosing regimen, animals became moribund as early as 4 hours after MCT administration. Since liver injury was similar from regimens that differed greatly in mortality, death appeared to result from extrahepatic causes. To explore a role for nitric oxide (NO)-induced shock in this regimen, animals were treated with aminoguanidine (AG), an inhibitor of inducible NO synthase, prior to administration of LPS given an hour before MCT. In the cotreated animals, AG significantly attenuated mortality and decreased plasma nitrate/nitrite concentrations, markers of NO

biosynthesis. Hence, the primary target of toxicity from MCT and LPS cotreatment appeared to shift from the liver to an extrahepatic site(s) as exposure to these agents occurred closer together temporally. NO appears to be causally involved in the deaths of animals treated with LPS an hour before MCT.

3.B. Introduction

In Chapter 2, it was demonstrated that synergistic liver injury resulted when a noninjurious dose of LPS was administered 4 hours after a small, non-hepatotoxic dose of MCT. Histopathological analysis revealed that liver lesions were both centrilobular and midzonal, exhibiting characteristics similar to lesions associated with larger, toxic doses of MCT or LPS, respectively. This study provided evidence that exposure to modest doses of LPS may enhance susceptibility to intoxication from PAs and perhaps other food-borne hepatotoxins.

In the present investigation, the interaction between LPS and MCT was characterized further. Specifically, the hypothesis was tested that toxicity was affected by changes in the temporal relationship between LPS and MCT exposures. Results showed that LPS and MCT administration led to substantially greater lethality when the agents were given concurrently than when separated by several hours. The increase in mortality did not appear to result directly from liver injury, and its nature suggested an enhanced sensitivity to

circulatory shock. Accordingly, the involvement of nitric oxide (NO) in the enhanced mortality was explored.

3.C. Materials and Methods

3.C.1. Materials

Aminoguanidine hemisulfate salt (AG), boron trifluoride in methanol, p-dimethylaminobenzaldehyde, heparin (Type II, disodium salt), LPS (*Escherichia coli,* serotype 0128:B12, 1.7 x 10^6 EU/mg), mercuric chloride, and sodium citrate were purchased from Sigma Chemical Company (St. Louis, MO). Diagnostic kits 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). Total Nitric Oxide (NO) Correlate Assay kit was acquired from Assay Design, Inc (Ann Arbor, MI). MCT was obtained 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).

3.C.2. Animals

Animals that were clearly moribund (i.e., recumbent, nonresponsive and lacking righting reflex) were euthanized. Otherwise, for more information on this section, refer to Chapter 2 Materials and Methods.

3.C.3. Treatment Protocol

AG (15 mg/kg; 2 ml/kg) or its saline vehicle was administered intravenously 10 minutes prior to LPS administration. Survival and enzymic markers were assessed at the times specified in the figures and tables. Otherwise, for more information on this section, refer to Chapter 2 Materials and Methods.

3.C.4. Enzyme Assays

Liver injury was assessed by analyzing plasma for increased activity of ALT and AST, two enzymic markers of hepatocellular damage. Otherwise, for more information on this section, refer to Chapter 2 Materials and Methods.

3.C.5. Nitric Oxide Determination

In plasma samples anticoagulated with sodium citrate, NO was determined from concentrations of nitrate and nitrite (NO_x) using a spectrophotometric method based on the Griess reaction (Green *et al.*, 1982). Using the Total Nitric Oxide Correlate Assay Kit, plasma samples were diluted 1:4 in reaction buffer (Hepes) and ultrafiltered through a 10,000 molecular weight cut-off filter. Samples and standards (sodium nitrate) were incubated in a 96-well microtiter plate for 30 minutes at 37°C with nitrate reductase and NADH to convert nitrate into nitrite. Griess reagent (a 1:1 mixture of sulfanilamide in 2M hydrochloric acid and N-(1-naphthyl)ethylenediamine in 2M hydrochloric acid)

was added to each of the wells and allowed to incubate for 10 minutes at room temperature. The absorbance of the colored azo-reaction product was read at 540 nm.

3.C.6. Ehrlich Assay

For more information on this section, refer to Chapter 2 Materials and Methods.

3.C.7. Histopathologic Evaluation

Livers were removed intact and fixed in 10% neutral buffered formalin for at least 3 days before being processed for histologic analysis. Serial transverse liver sections from the left and right lateral liver lobes were used for light microscopy. Paraffin-embedded sections were cut at 4 μm and stained with hematoxylin and eosin. Slides were coded, randomized, and evaluated by light microscopy. The following histopathology grading scale was used to score each of several categories: (0) none, (±) minimal, (+) modest, (++) moderate, (+++) marked, and (++++) severe effect. Categories included: hemorrhage/congestion, sinusoidal architecture loss, inflammation (neutrophil presence), parenchymal cell injury (centrilobular and midzonal) and disruption of central vein intima.

3.C.8. Statistical Analysis

Results are expressed as means ± S.E.M. When variances were not homogeneous, data were log-transformed before analysis. Single comparisons were analyzed by Student's t-test. Comparisons of percentages were analyzed by Fisher's exact test (Motulsky, 1995; Steel *et al.*, 1997). Multiple comparisons 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 (Steel *et al.*, 1997). The criterion for statistical significance was $p \leq 0.05$ for all comparisons.

3.D. Results

3.D.1. Hepatotoxicity Development: LPS Administered before MCT

Yee *et al.* (2000b) previously reported that 7.4 x 10^6 EU LPS/kg administered 4 hours after 100 mg MCT/kg resulted in synergistic hepatotoxicity within 12-18 hours after MCT administration (Yee *et al.*, 2000b). In the present study, 3.4 x 10^6 EU LPS/kg was administered one hour before 100 mg MCT/kg. Hepatotoxicity developed between 4 and 8 hours after MCT administration and was apparent until the final examination time of 18 hours (Figure 3.1A). Changes in plasma AST activities (data not shown) were similar to those shown for ALT. In LPS/MCT-cotreated animals, survival at 12 hours was 84%. However, survival decreased markedly to 31% by 18 hours after MCT administration (Figure 3.1B).

To test the possibility that liver injury from the cotreatment regimen of LPS an hour before MCT was the result of LPS-induced enhancement of MCT

Figure 3.1. Development of Hepatotoxicity in Rats Given LPS an Hour Before MCT. Rats were given 3.4 x 10^6 EU LPS/kg or saline Veh, i.v., an hour before 100 mg MCT/kg or saline Veh, i.p. (A) Hepatotoxicity was evaluated at various times after MCT administration as increases in plasma ALT activity. (B) Percent survival of animals cotreated with LPS and MCT was assessed up to 18 hours after MCT cotreatment. Error bars for SEM that are smaller than the symbols are not shown. N = 6 – 30 animals.

[a] Significantly different from all other groups at the same time.

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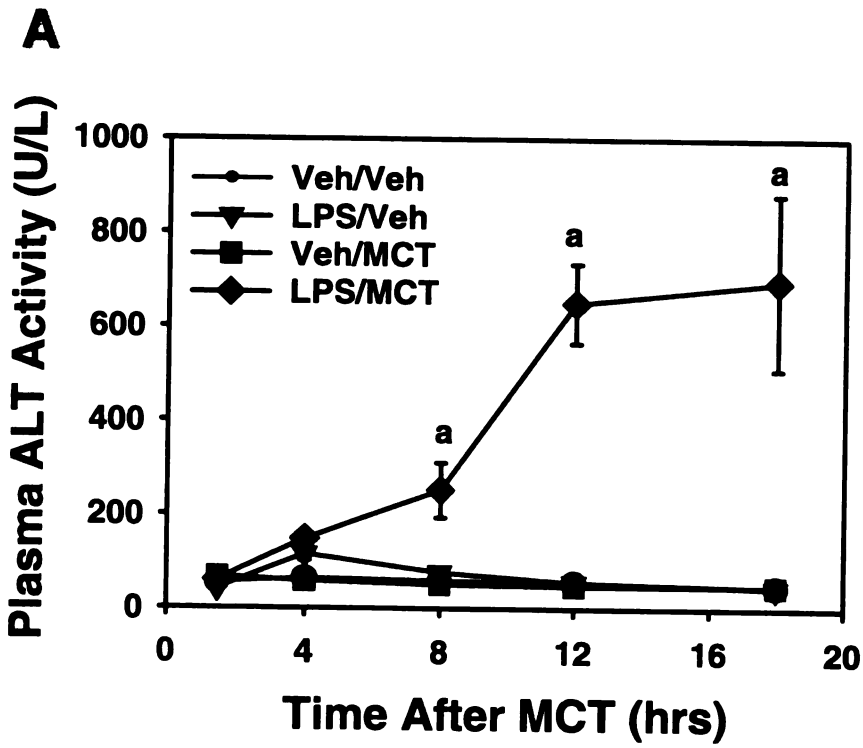

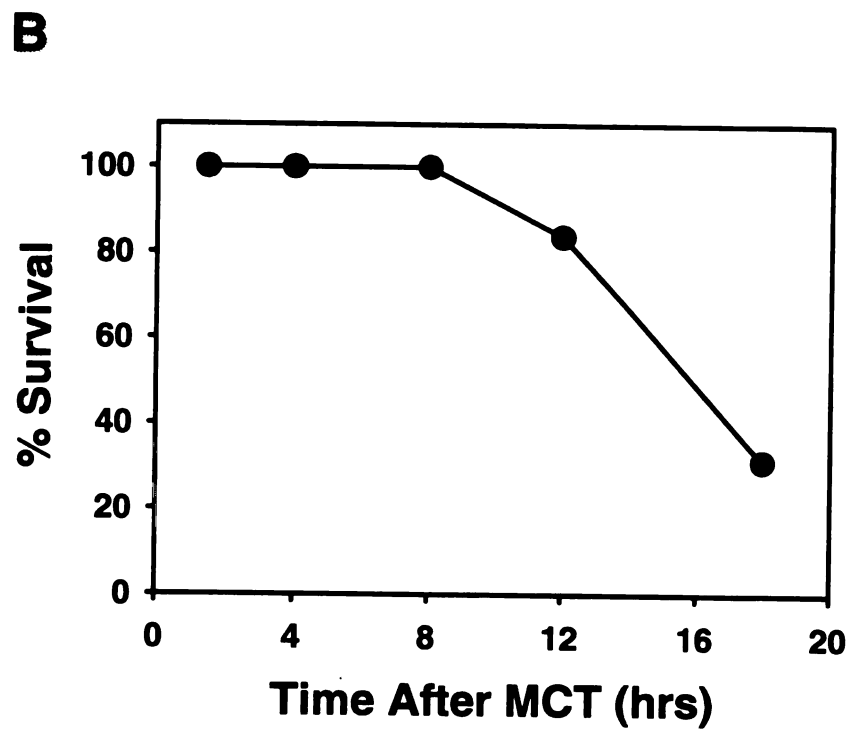

bioactivation, the concentration of pyrrolic metabolite(s) in the liver was determined. Peak production of MCTP occurs 90 minutes after MCT administration (Allen *et al.*, 1972), accordingly liver samples for analysis were collected at that time. A statistically significant increase in pyrrole production, an indicator of MCT bioactivation, was observed in livers from animals cotreated with LPS and MCT (Table 3.1).

Histopathologic lesions in livers from rats that received LPS an hour before MCT were similar to those observed in livers that had been given LPS 4 hours after MCT (data not shown [similar grading between two treatment regimens]). Both cotreatment regimens resulted in liver lesions that resembled a combination of centrilobular and midzonal lesions seen with MCT and LPS, respectively, when given by themselves at larger doses (Yee *et al.*, 2000b). The centrilobular lesion exhibited marked hepatocellular apoptotic and oncotic necrosis, degeneration, congestion/hemorrhage and vascular injury. The midzonal lesion consisted of well-defined areas of marked hepatocellular coagulative necrosis accompanied by neutrophil infiltration with congestion and hemorrhage.

To elucidate the dose-response relationship of the LPS/MCT interactions, various doses of LPS were administered an hour before MCT (100 mg/kg) or its saline vehicle. Hepatotoxicity was assessed 12 hours after MCT or vehicle administration. A dose-related increase in plasma ALT activity was observed in animals cotreated with LPS and MCT (Figure 3.2A). Plasma ALT activity was

Table 3.1. LPS Increases Hepatic MCT Bioactivation. LPS (3.4×10^6 EU/kg, i.v.) or its saline vehicle (Veh) was administered to rats an hour before MCT (100 mg/kg, i.p.). Liver samples were taken 1.5 hours after MCT administration and homogenized for analysis of pyrrole concentration by the Ehrlich assay. Resulting values were corrected for endogenous pyrrole by subtracting the average value (i.e., 10 µg pyrrole/g liver) from animals given the Veh/Veh combination. N = 5 animals.

[a] Significantly different from Veh/MCT group.

Treatment	Pyrrole (μg/g liver)
Veh/MCT	186 ± 12
LPS/MCT	270 ± 6[a]

Figure 3.2: Dose-Dependency of Hepatotoxicity from LPS and MCT Coadministration. Hepatotoxicity was assessed 12 hours after MCT administration. (A) To determine the dependence on LPS dose, rats were given the indicated doses of LPS an hour before 100 mg MCT/kg or its saline Veh. Plasma ALT activity for the Veh/Veh control group (not shown on graph) was 56 ± 11 U/L. (B) To examine the influence of MCT dose, animals were given 3.4×10^6 EU LPS/kg or its saline Veh an hour before the indicated MCT doses. Error bars for SEM that are smaller than the symbols are not shown. N = 6 – 30 animals.

[a] Significantly different from Veh/Veh control group.

[b] Significantly different from value at the same dose of LPS.

[c] Significantly different from value at the same dose of MCT.

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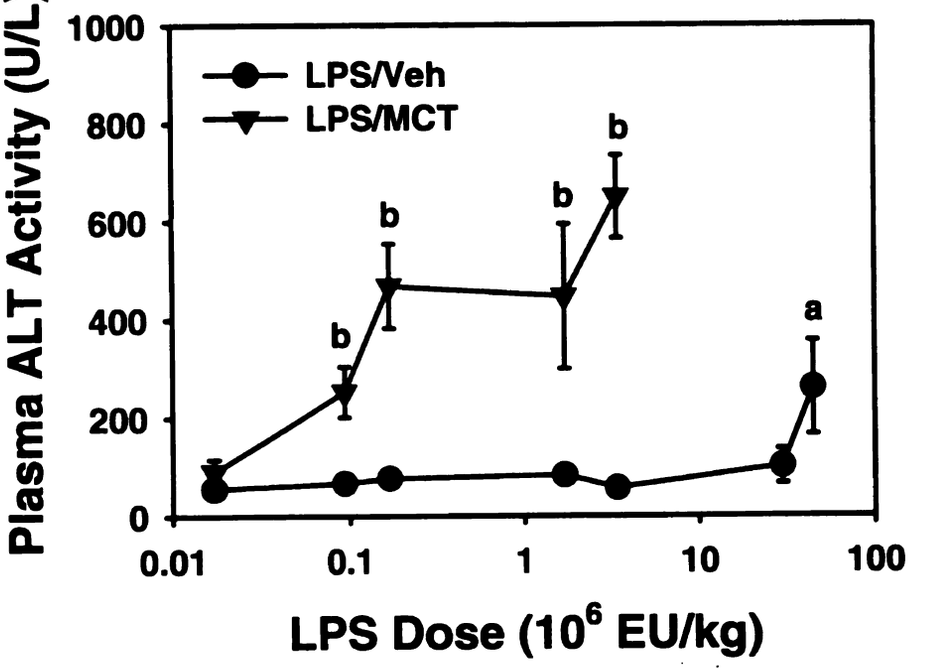
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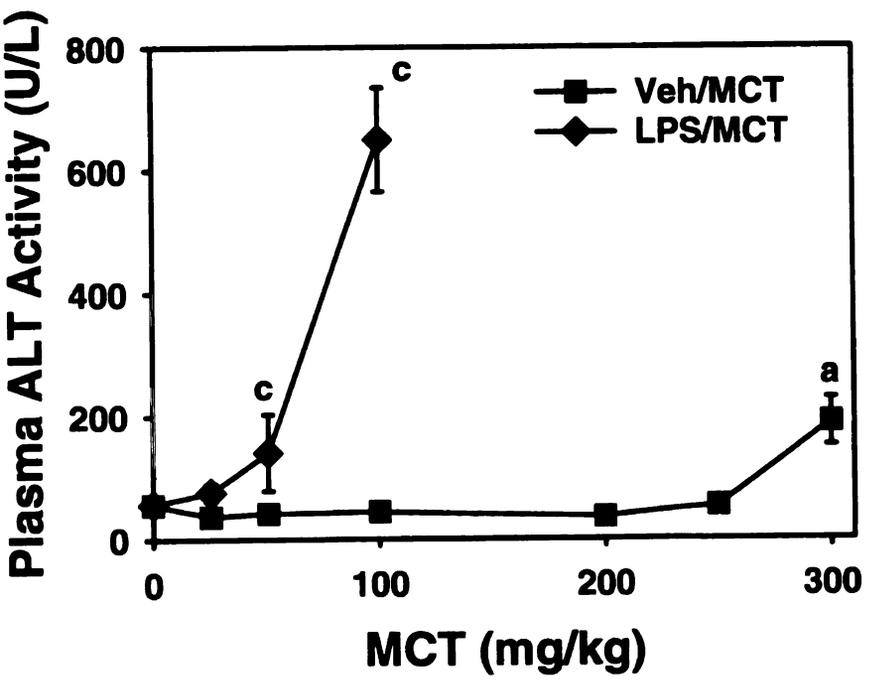
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significantly increased in MCT-treated animals pretreated with doses of LPS ranging from 0.09 – 3.4 x 10^6 EU/kg. Lethality in these cotreated animals did not exceed 30%. By comparison, there was no significant change in plasma ALT activity in the absence of MCT in rats receiving LPS up to 29.6 x 10^6 EU/kg. An LPS dose of 44.4 x 10^6 EU/kg, however, produced a significant increase in plasma ALT activity. No mortality occurred in animals that received LPS/saline. Thus, in the presence of MCT, the sensitivity to LPS toxicity increased about 500 fold.

When a noninjurious dose of LPS (3.4 x 10^6 EU/kg) was given an hour before various doses of MCT, significant toxicity was observed with MCT doses of 50 mg/kg or greater (Figure 3.2B). No mortality was observed at MCT doses of 50 mg/kg or less in conjunction with LPS pretreatment. In contrast, MCT was not hepatotoxic in the absence of LPS pretreatment at doses smaller than 300 mg/kg. Likewise, no mortality occurred in these Veh/MCT cotreated animals. In the presence of LPS, the no observed adverse effect level (NOAEL) for MCT was reduced to approximately 10% of the NOAEL for MCT alone.

3.D.2. MCT and LPS Temporal Relationship

The remarkably high mortality in this study (Figure 3.1B) prompted an examination of animal deaths in our attempts to explore hepatotoxic MCT/LPS treatment regimens. In these studies, animals were treated with the same doses of LPS (7.4 x 10^6 EU/kg) and MCT (100 mg/kg) but at different times between treatments. Percent survival was determined 24 hours after MCT administration

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(Table 3.2). When LPS was administered 4 hours before or after MCT, survival at 24 hours was about 86 and 88%, respectively. However, as the administrations of MCT and LPS were moved closer together temporally, survival significantly decreased. For example, when LPS was given an hour before MCT, only 17% of animals survived until 24 hours. Likewise, when LPS and MCT were administered concurrently, only 20% of animals survived to 24 hours.

3.D.3. Effect of AG on Animals Treated with LPS an Hour Before MCT

Observation of animals that received LPS an hour before MCT revealed that some of the animals became moribund as early as 4 hours after MCT administration. A gross examination of the brain, heart, kidneys, lungs, small intestine and spleen of animals euthanized at this time revealed no obvious abnormalities. Plasma ALT activity 4 hours after MCT was modestly, but significantly elevated over controls (Veh/Veh, 64 ± 12; LPS/Veh, 116 ± 4; Veh/MCT, 66 ± 7; LPS/MCT, 309 ± 45 U/L).

The modest nature of liver injury and lack of gross abnormalities in moribund animals given this treatment regimen suggested that death might have arisen from extrahepatic circumstances, perhaps circulatory shock. Inasmuch as shock caused by large doses of LPS is mediated by NO (Tracey *et al.*, 1995; Wu *et al.*, 1995; Takano *et al.*, 1997; Karima *et al.*, 1999), the effect of a NOS inhibitor on lethality from LPS/MCT coadministration was examined. AG (15 mg/kg, i.v.) or its saline Veh was administered 10 minutes before LPS (Wu *et al.*,

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Table 3.2. MCT and LPS Temporal Relationship Influences Lethality. Rats were given 7.4 x 10^6 EU LPS/kg (i.v.) and 100 mg MCT/kg (i.p.). Survival was evaluated 24 hours after MCT administration (i.e., time zero). LPS was administered at various times relative to MCT as specified in the table. Data represent findings which arose from several preliminary studies to determine hepatotoxic dosing regimens.

[a] Significantly different from survival when LPS was given 4 hours before or after MCT as determined by Bonferroni's Correction of the Fisher's Exact Test.

LPS Administration (Relative to MCT at t = 0 hour)	% Survival at 24 Hours	N
-4 hour	85.7	7
-1 hour	16.6[a]	53
0 hour	20.0[a]	5
1 hour	40.0	5
4 hour	87.5	32

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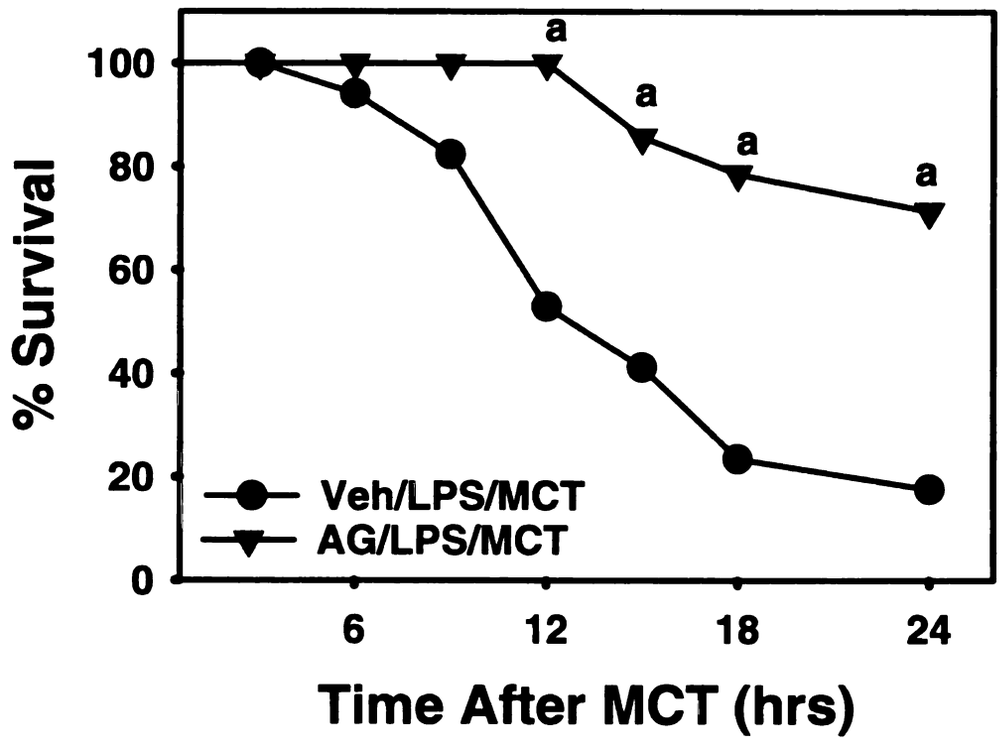
1995; Takano *et al.*, 1997), and LPS (7.4×10^6 EU/kg) was administered an hour before MCT (100 mg/kg). AG significantly improved survival (Figure 3.3). At 1.5 hours after MCT administration, plasma [NO_x] in LPS/MCT cotreated animals was small and unaffected by AG treatment (AG/LPS/MCT, 64 ± 14; Veh/LPS/MCT, 60 ± 13 μM). By 6 hours, LPS/MCT treatment resulted in a large increase in plasma [NO_x], which was significantly attenuated by AG pretreatment (Table 3.3). The AG-mediated reduction in plasma [NO_x] remained through 24 hours (i.e., Veh/LPS/MCT, 801 ± 186; AG/LPS/MCT, 223 ± 43 μM). Although AG markedly attenuated lethality, it had no significant effect on plasma ALT activity 6 hours after LPS/MCT administration (Table 3.3).

Because AG afforded protection from LPS/MCT-induced lethality, it was possible that AG decreased MCT bioactivation. Pretreatment with AG did not affect Ehrlich activity in liver samples taken either at 1.5 or 6 hours after LPS/MCT (at 1.5 hours after MCT administration: Veh/LPS/MCT, 255 ± 28, and AG/LPS/MCT, 296 ± 40 μg pyrrole/g liver; at 6 hours after MCT administration: Veh/LPS/MCT, 309 ± 20, and AG/LPS/MCT, 309 ± 35 μg pyrrole/g liver). Furthermore, when AG was given to rats 10 minutes before an hepatotoxic dose of MCT (300 mg/kg [This series of experiments was conducted by Bryan L. Copple]), no change in MCT-induced liver injury was observed (plasma ALT values: Veh/MCT, 1151 ± 282; AG/MCT, 1131 ± 192 U/L; measured 18 hours after MCT administration). At this dose of MCT, plasma [NO_x] did not increase over 18 hours (data not shown).

Figure 3.3: Effect of AG on Animal Survival Following a Lethal Combination of LPS an Hour Before MCT. Animals were given 15 mg AG/kg or its saline Veh i.v. 10 minutes before LPS administration. 7.4×10^6 EU LPS/kg (i.v.) was administered an hour before 100 mg MCT/kg (i.p.). Percent survival was assessed up to 24 hours after MCT administration. N = 14 for AG/LPS/MCT and 17 for Veh/LPS/MCT cotreated animals.

[a] Significantly different from other group.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% Survival
Veh/LPS/MCT
AG/LPS/MCT
6
12
18
24
Time After MCT (hrs)

Table 3.3. AG Reduces Plasma [NO_x] at 6 hours in Animals That Received LPS an Hour Before MCT. Rats were given 15 mg AG/kg or its saline Veh (i.v.) ten minutes before LPS administration. LPS (7.4×10^6 EU/kg; i.v.) was administered an hour before MCT (100 mg/kg; i.p.), and plasma [NO_x] (μM) was assessed 6 hours after MCT administration. N = 3 – 6 animals/group

[b] Significantly different from Veh/LPS/MCT group.

Treatment Group	Plasma [NO_x] (μM)	Plasma ALT (U/L)
Veh/Veh/Veh	39 ± 6	64 ± 12
AG/Veh/Veh	28 ± 5	96 ± 17
Veh/LPS/MCT	409 ± 37	377 ± 32
AG/LPS/MCT	268 ± 40[a]	348 ± 91

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3.E. Discussion

Exposure to modest amounts of LPS enhances the susceptibility to chemical intoxication (Roth *et al.*, 1997). Treatment with LPS (7.4×10^6 EU/kg) 4 hours after MCT (100 mg/kg) results in synergistic hepatotoxicity within 12-18 hours after MCT administration and minimal lethality (i.e., 12%) (Yee *et al.*, 2000b). In the present study, a smaller dose of LPS (3.4×10^6 EU/kg) administered an hour before MCT (100 mg/kg) caused liver injury that developed between 4 and 8 hours after MCT administration (Figure 3.1A). By 18 hours, ALT activity remained elevated and mortality was pronounced (i.e., 69%; Figure 3.1B). Thus, although a smaller dose of LPS was given in the present study, liver injury was earlier in onset. In addition, plasma ALT activities increased to a similar degree in both treatment regimens, yet mortality was considerably greater when the exposures to MCT and LPS occurred closer in time.

Histopathological analysis of livers of rats that had received LPS an hour before MCT revealed both a centrilobular and a midzonal component to the lesions. These lesions were similar to lesions observed when LPS was given 4 hours after MCT (Yee *et al.*, 2000b). However, unlike the latter treatment regimen, in which the midzonal lesion (i.e., the LPS-like lesion) was more pronounced, neither lesion predominated in size nor frequency when LPS was administered an hour before MCT, suggesting that a greater degree of MCT-induced liver injury occurred using this regimen. Why the MCT-like lesion was

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relatively more apparent in this regimen remains unclear, but the timing of the production and exposure to critical inflammatory factors may be involved.

In light of the increase in the severity of the centrilobular lesion and of the more rapid development of liver injury compared to when LPS was given 4 hours after MCT, an Ehrlich assay was used to determine whether MCT metabolism was affected under this altered regimen. Tissue Ehrlich activity is an indirect marker of MCT bioactivation to its toxic metabolite, MCTP (Mattocks and White, 1970). A statistically significant increase in liver pyrrole was observed at 1.5 hours after LPS/MCT cotreatment compared to treatment with MCT alone (Table 3.1). Although the enhanced pyrrole concentration in this nonspecific assay might be attributed to sources other than MCTP, the values presented in Table 3.1 were corrected for endogenous pyrrole, and the LPS-treatment control showed minimal pyrrole formation (6 ± 2 μg pyrrole/g liver). The result suggests that more MCTP was produced and/or remained in the liver after pretreatment of rats with LPS. This is in contrast to the regimen in which LPS was given 4 hours after MCT, which yielded no LPS-associated change in hepatic pyrrole concentration (Yee *et al.*, 2000b).

Cytochrome P450 (CYP) 3A has been identified as the predominant isozyme responsible for the metabolism of MCT to MCTP in Sprague-Dawley rats (Kasahara *et al.*, 1997). CYP 3A2 mRNA and protein expression appear to decrease within twenty-four hours after LPS treatment in Fischer 344 rats (Sewer and Morgan, 1998). CYP 3A metabolic activity also decreases in Sprague-Dawley rats after treatment with LPS (Muller *et al.*, 1996). Indeed, several

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studies in rats have indicated that within 6-24 hours after LPS administration CYP 3A expression is not induced (Muller *et al.*, 1996; Sewer *et al.*, 1996; Sewer and Morgan, 1998). However, the apparent increase in liver pyrrole in the present study occurred much earlier (i.e., 2.5 hours after LPS administration) than the times evaluated by these investigators, so it is possible that enzyme expression or activity is increased by LPS at this earlier time. Moreover, the activity of the LPS used in these studies was not reported, making it difficult to compare doses among studies on the basis of LPS activity (Shedlofsky *et al.*, 1994). Accordingly, it is not possible to rule out that the increased liver pyrrole concentrations after MCT administration (Table 3.1) arose from an effect of LPS on MCT bioactivation and/or a decreased loss of MCTP from the liver.

Alteration in the sequence of MCT and LPS administration resulted in a profound effect on lethality (Table 3.2). This effect has been observed in other models of LPS augmentation of xenobiotic toxicity, such as with cadmium (Cook *et al.*, 1974, 1975), lead (Selye *et al.*, 1966; Trejo *et al.*, 1972), and galactosamine (Galanos *et al.*, 1979). In these models, the greatest mortality occurred when rats were treated with xenobiotic and LPS simultaneously rather than hours apart. In a primate model, 100% mortality was observed upon the simultaneous administration of nonlethal doses of LPS and lead acetate (Holper *et al.*, 1973). Death occurred in one animal as early as three hours after LPS administration, and the others exhibited hypotension between 6 and 24 hours, suggesting that death may have been due to circulatory shock.

Approximately 10% of animals given LPS an hour before MCT became moribund within 6 hours after MCT administration. A few became moribund as early as 4 hours. Analysis of liver injury at 4 hours after MCT revealed that, although liver injury was present, it was less pronounced than that which occurred in MCT/LPS regimens in which mortality was minimal. Accordingly, it seems unlikely that liver injury was the cause of the profound and relatively rapid lethality. This suggests that rats that died upon cotreatment with LPS an hour before MCT succumbed to an extrahepatic effect.

The lack of grossly apparent injury to tissues and the early deaths suggest that animals may have succumbed to rapidly developing, severe hypotension. The pathophysiological responses to larger doses of LPS may provide a clue to the underlying cause of the pronounced lethality that was observed. Large doses of LPS lead to systemic hypotension that can progress quickly to circulatory failure and death (Tracey *et al.*, 1995; Wu *et al.*, 1995; Mayeux, 1997; Takano *et al.*, 1997; Karima *et al.*, 1999). Enhanced formation of NO following the expression of inducible nitric oxide synthase (iNOS) is known to occur in the pathogenesis of hypotensive shock (Ruetten and Thiemermann, 1996; Mayeux, 1997; Karima *et al.*, 1999). LPS is a potent inducer of iNOS, the activity of which leads to production of vasodilatory NO and to hypotension (Mayeux, 1997; Wolkow, 1998; Karima *et al.*, 1999). Indeed, deaths from large doses of LPS appear to occur from enhanced NO production (Karima *et al.*, 1999; Takano, 1997), although this conclusion remains controversial (Wolkow, 1998; Karima *et al.*, 1999).

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Treatment with LPS an hour before MCT resulted in significantly greater plasma [NO_x] than occurred in vehicle-treated animals (Table 3.3). This indicated that NO was produced after LPS/MCT cotreatment. AG is a selective inhibitor of iNOS and prevents hypotension and death in animals treated with large, lethal doses of LPS (Wu *et al.*, 1995; Southan and Szabo, 1996; Takano *et al.*, 1997). A dose of AG was used that provides protection from LPS-induced hypotension (Tracey *et al.*, 1995; Wu *et al.*, 1995; Takano *et al.*, 1997) and that is selective for iNOS inhibition (Scott and McCormack, 1999). Six hours after LPS/MCT coadministration, plasma [NO_x] was significantly attenuated by treatment with AG (Table 3.3), and it remained attenuated at 24 hours. AG markedly enhanced survival in animals given the lethal treatment of LPS an hour before MCT (Figure 3.3). Survival was significantly enhanced starting at 12 hours, and protection continued throughout the 24-hour examination period. At 24 hours, 71% of AG-cotreated rats had survived compared to 18% in the LPS/MCT group that did not receive AG. The protective effect of AG did not arise from inhibition of MCT bioactivation because AG did not decrease the concentration of pyrrolic metabolites in liver that arose from MCT administration and because AG did not protect against liver injury from a larger dose of MCT. Indeed, others have reported that AG does not inhibit CYP 3A activity (Muller *et al.*, 1996; Kasahara *et al.*, 1997; Sewer and Morgan, 1998). Together, our results suggest that vasodilatory NO may cause lethal hypotension in this LPS/MCT model.

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It is possible that increased but nonlethal hypotension also occurred in the LPS/MCT treatment regimen of 3.4 x 10^6 EU LPS/kg an hour before 100 mg/kg. This hypotension may have resulted in decreased MCTP transport out of the liver thereby accounting for the increased pyrrole observed in Table 3.1.

It is tempting to further speculate that another contributor to the lethality is disseminated intravascular coagulation (DIC). DIC has been characterized as a clotting defect that simultaneously results in the activation of the coagulation and fibrinolytic systems, leading to the formation of microthrombi, vessel obstruction, slowing of blood flow and internal hemorrhage (Korbut *et al.*, 1994). DIC, along with systemic hypotension, has been implicated as a factor responsible for circulatory shock and death evoked by LPS and in septic shock (Korbut *et al.*, 1994; Hardaway and Williams, 1996). Interestingly, a study by Korbut *et al.* (1994) indicated that the use of the NOS inhibitor N^G-nitro-L-arginine methyl ester in rats given a large dose of LPS attenuated symptoms of DIC, including a decrease in hemorrhage/congestion in the lungs, heart, spleen, kidneys and intestines. Thus, it is possible that the treatment with AG, a specific inhibitor of iNOS, in the LPS/MCT model resulted in a decrease in vasodilatory NO (and hence in systemic hypotension) and a decrease in DIC, which together improved animal survival. Since the liver injury observed in this model exhibited a distinct LPS component, it seemed possible that DIC may have been a component of the lethality. However, the absence of gross hemorrhagic lesions in the organs examined argues against this. Additionally, no hemorrhage/congestion was observed by light microscopic examination of the lungs, heart or kidneys of

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animals receiving the lethal LPS/MCT cotreatment. Hence, it seems unlikely that DIC was solely responsible for the lethality in this model; however, it cannot be conclusively ruled out as a contributor.

Concurrent exposure to inflammation increases the susceptibility to chemical intoxication from numerous xenobiotic agents (Roth *et al.*, 1997; Sneed *et al.*, 1997; Barton *et al.*, 2000b; Yee *et al*, 2000b; Labib *et al.*, 2002). This may have particular implication in assessing risk from chemical exposure and in treatment of chemical intoxications. In particular, MCT is a food-borne contaminant that affects both animals and people; it is also found in alternative medicines such as Nong ji li, Zi xiao rong and others (Roeder, 2000). Individuals who consume PAs may be particularly susceptible to toxicity during times of inflammatory conditions, and this should perhaps be a precaution for people when using alternative medicines (Yee *et al.*, 2000b; Ganey and Roth, 2001).

In summary, the temporal relationship of exposures to MCT and LPS has an important influence on lethality. That is, lethality is much greater when the exposures occur close together in time than when they are separated by several hours. The predominant target of toxicity shifts from liver when LPS and MCT are administered several hours apart to an extrahepatic site(s) when they are administered in close temporal proximity. NO appears to have a causal role in MCT/LPS-induced lethality, and this suggests the possibility that animals die from enhancement by MCT of LPS-induced circulatory collapse.

CHAPTER 4

Endothelial Cell Injury and Coagulation System Activation During Synergistic Hepatotoxicity from Monocrotaline and Bacterial Lipopolysaccharide Coexposure

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4.A. Abstract

A small, noninjurious dose of bacterial lipopolysaccharide (LPS; 7.4 x 10^6 EU/kg) administered 4 hours after a small, nontoxic dose of monocrotaline (MCT; 100 mg/kg) produces synergistic hepatotoxicity in rats within 6 to 12 hours after MCT exposure. The resulting centrilobular (CL) and midzonal (MZ) liver lesions are characterized by hepatic parenchymal cell (HPC) necrosis. Pronounced hemorrhage, disruption of sinusoidal architecture and loss of central vein intima suggest that an additional component to injury may be the liver vasculature. In the present investigation, the hypothesis that sinusoidal endothelial cell (SEC) injury and coagulation system activation occur in this model was tested. Plasma hyaluronic acid (HA) concentration, a biomarker for SEC injury, was significantly increased in cotreated animals before the onset of HPC injury and remained elevated through the time of maximal HPC injury (i.e., 18 hours). SEC injury was confirmed by immunohistochemistry and electron microscopy. Pyrrolic metabolites were produced from MCT by SECs *in vitro*, which suggests that MCT may injure SECs directly through the formation of its toxic metabolite, monocrotaline pyrrole. Inasmuch as SEC activation and injury can promote hemostasis, activation of the coagulation system was evaluated. Coagulation system activation, as marked by a decrease in plasma fibrinogen, occurred before the onset of HPC injury. Furthermore, extensive fibrin deposition was observed immunohistochemically within CL and MZ regions after MCT/LPS cotreatment. Taken together, these results suggest that SEC injury and

coagulation system activation are components of the synergistic liver injury resulting from MCT and LPS coexposure.

4.B. Introduction

Yee *et al.* (2000b) recently demonstrated that a small, noninjurious dose of LPS given to rats 4 hours after a small, nonhepatotoxic dose of MCT results in synergistic liver injury that is maximal 18 hours after MCT administration. Liver lesions were both centrilobular (CL) and midzonal (MZ), exhibiting characteristics similar to lesions associated with larger, toxic doses of MCT or LPS given separately. MCT-like, CL lesions consisted of moderate to marked hepatocellular apoptotic and oncotic necrosis, hemorrhage and loss of central vein (CV) intima. LPS-like, MZ lesions comprised marked but more frequent and well defined areas of hepatocellular coagulative necrosis accompanied by PMN infiltration, disruption of sinusoidal architecture, and hemorrhage. Vascular injury can be inferred from the loss of central vein intima (i.e., central vein endothelial cell; CVEC) in the CL lesion and the disruption of sinusoidal architecture and hemorrhage in both the CL and MZ lesions in this model (Yee *et al.*, 2000b).

Injury to endothelial cells in the microvasculature can result in activation of the coagulation system (Machovich, 1985; Ryan, 1986, Hirata *et al.*, 1989). Coagulation system activation is critical for the development of hepatic parenchymal cell (HPC) injury in other liver injury models (Perry, 1984; Fujiwara *et al.* 1988; Yamada *et al.* 1989; Hewett and Roth, 1995; Aria *et al.*, 1996). In

addition, microcirculatory disturbances from hemorrhage and/or intrasinusoidal fibrin deposition have been postulated to contribute to HPC injury (Shibayama, 1987; DeLeve *et al.*, 1996; Ba *et al.*, 2000; Saetre *et al.*, 2000; Copple *et al.*, 2002a, 2002b). CVEC and SEC injury occurs in the livers of rats after administration of a large, acutely toxic dose of MCT (DeLeve *et al.*, 1996; Copple *et al.*, 2002a), leading to coagulation system activation and fibrin deposition (Copple *et al.*, 2002a). Similar changes at more modest MCT doses in the absence or presence of additional susceptibility factors have not been reported. Accordingly, the present investigation was designed to test the hypothesis that injury to SECs and activation of the coagulation system occur during coexposure to normally nontoxic doses of MCT and LPS.

4.C. Materials and Methods

4.C.1. Materials

Isopentane, LPS (*Escherichia coli,* serotype 0128:B12, 1.7 x 10^6 endotoxin units (EUs)/mg), phosphate buffered saline (PBS), sodium citrate and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Company (St. Louis, MO). Mouse anti-rat endothelial cell antigen (RECA)-1 was acquired from Serotec, Inc. (Raleigh, NC). Rabbit anti-rat CYP4503A2 was obtained from BD Gentest (Woburn, MA). Horseradish peroxidase (HRP) labeled anti-rabbit secondary antibody was acquired from Santa Cruz Biotech (Santa Cruz, CA). Goat anti-rat fibrinogen was purchased from ICN Pharmaceuticals (Aurora, OH).

Goat and horse sera were obtained from Vector Laboratories (Burlingame, CA). Goat anti-mouse secondary antibody conjugated to Alexa 594 and donkey anti-goat secondary antibody conjugated to Alexa 594 were acquired from Molecular Probes (Eugene, OR). Enhanced chemiluminescence (ECL) for immunoblotting was obtained from Aversham Bioscience (Piscataway, NJ). Acrylamide/Bis solution was purchased from Bio-Rad Laboratories (Hercules, CA). Nitrocellulose membrane was procured from Schleicher and Schuell Inc. (Keene, NH). 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 procured from Surgipath Medical Industries, Inc. (Richmond, IL). Sodium cacodylate buffer was acquired from Electron Microscopy Sciences (Fort Washington, PA). Liver perfusion, liver digestion and hepatocyte wash mediums were purchased from Life Technologies, Inc. (Rockville, MD). Endothelial cell medium (EGM-2) was acquired through Biowhittaker, Inc. (Walkersville, MD). Diagnostic kits 59 UV and 886-A for the determination of alanine aminotransferase (ALT) activity and fibrinogen concentration, respectively, and Total Hemoglobin Kits were 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). All other materials were purchased from Sigma Chemical Company (St. Louis, MO)

4.C.2. Animals

Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR, Charles River, Portage, MI) weighing 175-200 g or 200-300 g were used for studies *in vitro* and *in vivo*, respectively. Otherwise, for more information on this section, refer to Chapter 2 Materials and Methods.

4.C.3. Treatment Protocol In Vivo

No mortality occurred in MCT/LPS-cotreated animals within 12 hours after MCT administration, but approximately 20% of rats died by 18 hours. No animals that received saline Veh, MCT or LPS alone died. Otherwise, for more information on this section, refer to Chapter 2 Materials and Methods.

4.C.4. Assessment of HPC Injury and Plasma Fibrinogen and HA Concentrations

At the times indicated in the figure legends, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). 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 plasma ALT activity. A BBL fibrometer (Becton, Dickson and Company, Hunt Valley, MD) and a fibrinogen diagnostic kit were used to determine plasma fibrinogen concentration. An ELISA kit was used to measure plasma HA concentration, a biomarker of hepatic SEC injury (Deaciuc *et al.*, 1993b, 1994; Copple *et al.*, 2002a).

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4.C.5. Histopathologic Evaluation

A one-cm^3 portion of the liver for immunohistochemical staining was cut from the middle of the left lateral lobe and frozen in isopentane immersed in liquid nitrogen. Further, a second portion of this lobe was snap frozen in liquid nitrogen for evaluation of tissue hemoglobin. The remaining portions of the liver were fixed by immersion in 10% neutral buffered formalin for at least 3 days before being processed for histologic analysis. Serial transverse sections from the left lateral liver lobe were processed for light microscopy. Paraffin-embedded sections were cut at 4 μm, stained with hematoxylin and eosin, and evaluated for lesion size and severity. Tissue sections were analyzed using a light microscope without knowledge of the treatment group.

Additionally, a thin section (1-2 mm) of the liver from the middle of the left lateral lobe was fixed in 4% glutaraldehyde in 0.1 M phosphate buffer for 24 hours, processed, and analyzed by transmission electron microscopy (TEM) using a Philips 301 transmission electron microscope (FEI Company, Hillsboro, OR). [All transmission electron micrographs were taken by Steven Yee with the assistance of Ralph Commons and Donna Craft].

4.C.6. Immunohistochemistry

For endothelial cell immunostaining, 8 μm-thick sections of frozen liver were fixed in acetone (4°C) for 5 minutes. Sections were incubated for 30 minutes with PBS containing 10% goat serum (i.e., blocking solution) and then with mouse anti-rat 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 antibody stains both SECs and endothelial cells from larger vessels. After incubation with RECA-1 antibody, the sections were incubated for 3 hours with goat anti-mouse secondary antibody conjugated to Alexa 594 (1:500) in blocking solution containing 2% rat serum. Sections were washed 3 times for 5 minutes each with PBS and visualized using fluorescence microscopy.

For fibrin immunostaining, 8 μm-thick sections of frozen tissue were fixed in 10% buffered formalin containing 2% acetic acid for 30 minutes at room temperature. This fixation protocol solubilizes all fibrinogen and fibrin species except for cross-linked fibrin. Thus, only cross-linked fibrin is stained in liver sections (Schnitt *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 hours in blocking solution with donkey anti-goat secondary antibody conjugated to Alexa 594 (1:1000). Liver sections were washed 3 times for 5 minutes each with PBS and visualized using fluorescence microscopy.

For both protocols, no staining was observed in the controls in which the primary or secondary antibody was omitted from the staining protocol. All morphometrically compared treatment groups were stained at the same time and evaluated on the same day.

4.C.7. Quantification of Liver Endothelial Cells and Fibrin Deposition

Endothelial cells and fibrin deposition in the liver were quantified morphometrically by analyzing the area of immunohistochemical staining for each liver section (Copple *et al.*, 2002a). A decrease in the endothelial cell staining suggests a loss of these cells from the liver. An increase in the area of fibrin staining in the liver indicates fibrin deposition. Fluorescent staining of liver sections was visualized using an Olympus AX-80T microscope (Olympus, Lake Success, NY). For morphometric analysis of total endothelial cell or fibrin deposition in a liver section, digital images of 5 randomly chosen 100X fields 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 mm^2 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 endothelial cell quantification, a density slice was taken from an inverted, gray-scale digital image of a liver section from a

Veh/Veh-treated rat. A density slice allows analysis of pixels in a defined range of gray values (i.e., densities). Next, the threshold was adjusted so that background staining was eliminated from the analysis and only endothelial staining was visualized. For quantification of fibrin deposition, the threshold was selected so that minimal positive staining was present in Veh/Veh-treated controls. The same threshold value was used to analyze digital images from all treatment groups. For quantification of endothelial cells or fibrin, the area of positive staining was measured and divided by the total area of the image. Analysis of endothelial cells or fibrin deposition in the CL, MZ and PP regions was conducted by drawing a 145 μm-diameter circle around the CV or vessels of the portal triad. The circumference of the circle was about 4-6 hepatocytes away from the CV or portal triad vessels, and this area was arbitrarily defined as the CL and PP region. The MZ region was defined as the center of area between the CL and PP region using the same circle circumference, without having overlap of these arbitrary circles. The area of endothelial cells or 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). For the time-course studies, Veh/Veh-treated rats at various times were combined into one group for statistical analysis, since no differences occurred among these groups.

4.C.8. Liver Hemoglobin

Liver hemoglobin concentration was used as a biomarker for hemorrhage (Jaeschke *et al.*, 2000; Copple *et al.*, 2002a). A 20% homogenate was made from samples of frozen liver in 50mM sodium phosphate buffer (120 mM sodium chloride, 10 mM ethylenediaminetetraacetic acid). Samples were centrifuged (16,000 X gravity) for 10 min at 4°C, and 200 µl of the supernatant was diluted in Drabkin's solution. Following a 15-minute incubation, the absorbance was measured at 540 nm, and the hemoglobin concentration was determined from a standard curve.

4.C.9. SEC Studies In Vitro

Primary SECs were isolated from rat liver as described in Braet *et al.* (1994). Briefly, the liver from an anaesthetized rat was digested by *in situ* sequential perfusion with liver perfusion and liver digestion media. Cells were released by gentle scraping and suspended in hepatocyte wash medium. The cell suspension was centrifuged (100 X gravity) for 5 minutes, and the supernatant containing the nonparenchymal (NP) fraction was collected. The NP fraction was layered on top of a two-layer percoll gradient (50% and 25%) and centrifuged (900 X gravity) for 20 minutes. The SEC-enriched zone between the percoll gradients was collected, washed with PBS and plated. Additionally, a rat liver sinusoidal endothelial cell line, NP-26, was used in studies *in vitro*. The NP-26 cell line was established from an enriched fraction of liver SECs transfected with SV40 large-T antigen (Maru *et al.*, 1998).

4.C.10. Scanning Electron Microscopy

Primary SECs and NP-26 cells were plated on 12 mm-diameter collagen-coated coverslips and cultured for 24 hours in EGM-2. Scanning electron microscopy was performed as described in Braet *et al.* (2002), with minor modifications. Briefly, cells were rinsed twice with PBS and fixed for 12 hours with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.1 M sucrose (pH 7.4). The fixed cells were subsequently treated with filtered 1% tannic acid in 0.15 M sodium cacodylate buffer (pH 7.4) for 1 hour and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.4) for another hour. The samples were further dehydrated in a graded ethanol series, critical point dried and sputter-coated with gold. The cells were examined using a JSM 6400V scanning electron microscope (JEOL USA Inc., Peabody, MA).

[Scanning electron photomicrographs were taken by Umesh M. Hanumegowda with the assistance of Ewa Danielewicz].

4.C.11. MCT treatment In Vitro and Ehrlich Assay

The ability of SECs to convert MCT to pyrrolic metabolite(s) was evaluated *in* vitro. Isolated primary SECs were routinely greater than 90% pure as determined by morphology; they were contaminated with approximately 10% hepatocytes. To correct for the contribution of these contaminating hepatocytes to MCT metabolism in SEC cultures, MCT metabolism in an equivalent number of hepatocytes was examined. Hepatocytes were isolated by the collagenase perfusion as described previously by Seglen (1973) and Klaunig *et al.* (1981).

Primary SECs, NP-26 cells or hepatocytes were plated on collagen-coated, 12-well plates and cultured for 24 hours. MCT was added to the serum-free culture medium to a final concentration of 0, 1, 2 or 4 mM, and cells were incubated for 4 hours. Medium was then collected and evaluated for pyrrole concentration by a modified Ehrlich reaction as described previously by Mattocks and White (1970). Briefly, one ml of incubation medium was mixed with one ml of 5% ascorbic acid in 80% ethanol. One ml of Ehrlich reagent was added, and the mixture was heated in a waterbath for 1 minute. After cooling to room temperature, 0.1 ml of $FeCl_3$ solution was added to inhibit fading of the Ehrlich-pyrrole complex color. Absorbance was measured at 565 nm and compared against a standard curve. The amounts of pyrrolic MCT metabolite(s) formed by primary SECs was corrected for pyrroles produced from contaminating hepatocytes by subtracting the amount of pyrrolic MCT metabolite(s) formed from an equal number of hepatocytes. Results are expressed as µg pyrrole per mg of cellular protein. Protein concentration was evaluated as described by Bradford (1976) in cells that were solubilized with 1% Triton X-100 and sonicated.

4.C.12. Preparation of Microsomal Fraction and Immunoblotting

Microsomes were prepared as described by Coffman *et al.* (1999). Briefly, cultured NP-26 cells or isolated rat hepatocytes were suspended in homogenization buffer (0.25 M sucrose in 5 mM HEPES buffer, pH 7.4). Cells were disrupted by sonication and then centrifuged (9,000 X gravity) for 20 min. The supernatant was recovered and then centrifuged (100,000 X gravity) for 1 h.

The resulting pellet was resuspended in homogenization buffer. Protein concentrations were measured as described by Bradford (1976), and CYP3A2 protein was identified by immunoblotting as described by Sharp *et al.* (2001). Microsomal proteins (0.5 μg from hepatocytes and 5 μg from NP-26 cells) were electrophoresed on a 10% polyacrylamide gel with SDS and then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat dry milk, washed with PBS-Tween, probed with anti-CYP3A2 antibody (1:1000) and subsequently reacted with HRP-conjugated anti-rabbit secondary antibody (1:5000). The protein bands were visualized by ECL.

4.C.13. Statistical Analysis

All results are expressed as mean ± S.E.M. Data expressed as fractions were arc sine square root-transformed before analysis. When variances were not homogeneous, data were log-transformed before analysis. Homogeneous data were analyzed by one-way or two-way analysis of variance (ANOVA), as appropriate, and group means were compared using Student-Newman-Keuls *post hoc* test (Steele *et al.*, 1997). The criterion for significance was $p \leq 0.05$ for all comparisons.

4.D. Results

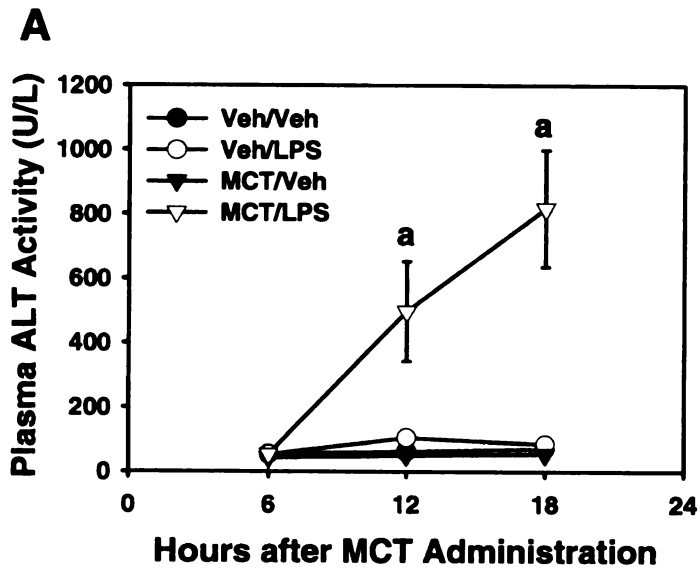
4.D.1. Liver Endothelial Cell Injury in MCT/LPS-Cotreated Animals

As marked by increased ALT activity in plasma, HPC injury from MCT/LPS-coexposure occurred between 6 and 12 hours and was maximal by 18 hours after MCT administration (Figure 4.1A). MCT-like, CL and LPS-like, MZ liver lesions developed as previously described by Yee *et al.* (2000b). Although no parenchymal lesions were apparent 6 hours after MCT administration, some disruption of central vein intima was observed in MCT/LPS-cotreated animals at this time. Loss of central vein intima, disruption of sinusoidal architecture and the presence of hemorrhage within lesions at 12 and 18 hours suggested vascular injury.

Hepatic SECs remove HA from the circulation, and impairment of this function results in increased HA concentration in the plasma (Deaciuc *et al.*, 1993b, 1994; Copple *et al.*, 2002a). Accordingly, to estimate hepatic SEC dysfunction plasma HA concentration was measured. No elevation in plasma HA concentration was observed for Veh/Veh or MCT/Veh treatment groups at any of the times analyzed (Figure 4.1B). Although no elevation in plasma HA concentration was observed in the Veh/LPS treatment group at 6 and 12 hours, a modest but significant increase occurred at 18 hours. By 6 hours, plasma HA concentration was significantly elevated in MCT/LPS-cotreated animals, and this increase became progressively more pronounced with time (Figure 4.1B). This elevation in plasma HA concentration occurred before the onset of HPC injury.

Figure 4.1. MCT/LPS-Induced Liver Injury. LPS (7.4×10^6 EU/kg) or saline vehicle (Veh) was administered to rats i.v. 4 hours after i.p. administration of MCT (100 mg/kg) or Veh. Rats were killed at 6, 12, and 18 hours after administration of MCT or its Veh. HPC injury (A) was measured as increases in plasma ALT activity. SEC injury (B) was estimated as increases in plasma HA concentration. N = 4 – 7 animals per group at each time.

[a] Significantly different from all other groups at the same time.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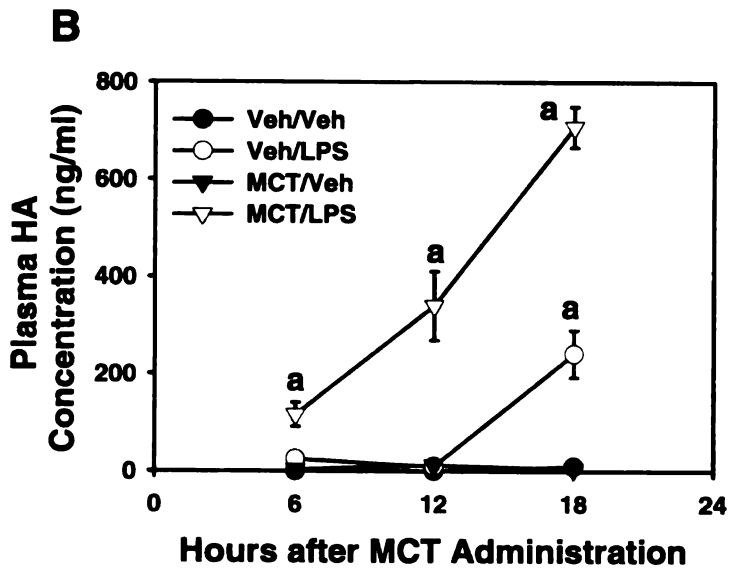
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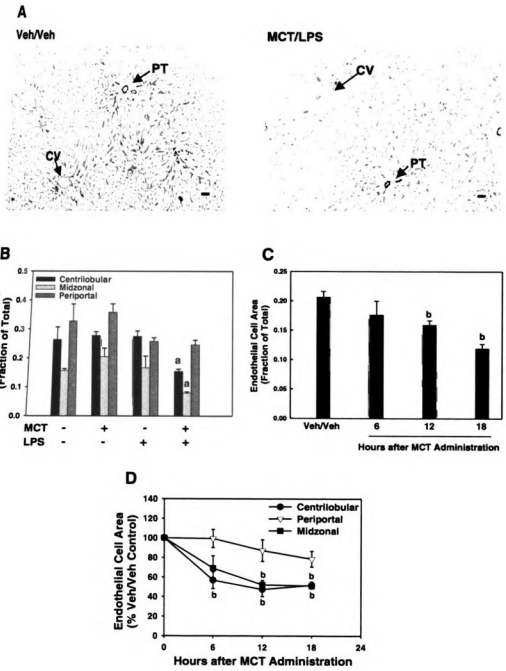
RECA-1 immunostaining of liver sections has been used to visualize and quantify the loss of endothelial cells in the liver (Copple *et al.*, 2002a). Representative photomicrographs of RECA-1 immunostaining in livers from a Veh/Veh-treated and a MCT/LPS-cotreated rat at 18 hours can be seen in Figure 4.2A. The liver section from a Veh/Veh treated rat exhibited panlobular RECA-1 staining of sinusoids and the intima of CVs and vessels of the portal triad. RECA-1 staining decreased in the sinusoids and in CV intima in livers from MCT/LPS-cotreated rats but not in the endothelium in the portal triad. Morphometry conducted on 18 hour liver samples (Figure 4.2B) revealed a significant decrease in RECA-1 staining in the CL and MZ regions of livers from MCT/LPS-cotreated animals. No decrease in RECA-1 immunostaining was observed in the livers from Veh, MCT or LPS treatment groups. A time-dependent decrease in total RECA-1 immunostaining was observed in the livers of MCT/LPS-cotreated animals (Figure 4.2C). This decrease was statistically significant by 12 hours and remained at 18 hours. Zonal analysis of the liver lobules from MCT/LPS-cotreated animals revealed a significant decrease in RECA-1 immunostaining within the CL region of the liver lobule starting at 6 hours and within the MZ region by 12 hours (Figure 4.2D).

Figure 4.2. Liver RECA-1 Immunohistochemical Staining. Rats were treated with MCT and/or LPS and killed at the indicated times, as described in Figure 4.1 legend. Livers were removed and processed for RECA-1 immunohistochemistry. RECA-1 is an antibody selective for endothelial cells. (A) Representative photomicrographs of RECA-1-immunostained liver sections from a Veh/Veh- and a MCT/LPS-cotreated rat at 18 hours. RECA-1 immunostaining (shown in black) is prominent in the central vein (CV) and sinusoidal endothelium and vessels of the portal triad (PT). Bar = 50 μm. (B) The zonal distribution of RECA-1 immunostaining was evaluated at 18 hours for all treatment groups. Additionally, timecourses for total liver RECA-1 immunostaining (C) and its zonal distribution (D) in the livers of MCT/LPS-cotreated animals were examined. N = 4 – 7 animals. For (C) and (D), Veh/Veh-treated animals were combined into one group (N = 12), since no differences occurred among Veh/Veh-treated groups at the various times.

[a] Significantly different from all other groups in same lobular region.

[b] Significantly different from Veh/Veh group.


A
Veh/Veh
PT
CV
MCT/LPS
CV
PT
B
Centrilobular
Midzonal
Periportal
Endothelial Cell Area
(Fraction of Total)
MCT
LPS
C
Endothelial Cell Area
(Fraction of Total)
Veh/Veh
Hours after MCT Administration
D
Centrilobular
Periportal
Midzonal
Endothelial Cell Area
(% Veh/Veh Control)
Hours after MCT Administration

Representative TEM photomicrographs from livers of Veh/Veh and MCT/LPS-cotreated animals at 18 hours are shown in Figure 4.3. CVECs as well as SECs in CL and MZ regions of liver lobules from Veh/Veh-treated rats exhibited normal morphology (Figure 4.3A). CVECs formed a distinct, continuous barrier with well-defined organelles. MZ regions had largely continuous SECs with clearly defined spaces of Disse. HPCs within CL and MZ regions were well defined and had distinct, normal-appearing organelles. By comparison, CVECs as well as SECs from both CL and MZ regions of MCT/LPS-cotreated rats were markedly altered or absent (Figure 4.3B). The CVs in CL regions had highly vacuolated remnants of CVECs without discernible organelles, or were completely denuded of CVECs. HPCs contained numerous fat droplets, rounded mitochondria and indistinct plasma membranes. Within the MZ regions, SECs were indistinct and discontinuous with attenuated and electron-lucent cytoplasm. In other MZ areas, the SECs were completely lost. The sinusoids were narrowed and contained fibrin deposits, red blood cells (RBCs) and PMNs. RBCs and cell fragments were visible in the remaining space of Disse. HPCs had indistinct plasma membranes and disrupted mitochondria. Hence, in the livers of MCT/LPS-cotreated animals, CVECs, SECs and HPCs were injured by 18 hours after MCT administration. Such injury was also evident at 12 hours (data not shown).

Figure 4.3. Representative TEM Photomicrographs from Liver Sections of Veh/Veh- and MCT/LPS-Cotreated Rats. Rats were treated with MCT and LPS as described in Figure 4.1 legend. They were killed 18 hours after administration of MCT or its Veh, and livers were removed and fixed for TEM as described in Materials and Methods. (A) The central vein (CV) and a midzonal (MZ) sinusoid from a Veh/Veh-treated rat have normal endothelial morphology. (B) The CV and a MZ sinusoid from a MCT/LPS-cotreated rat show a highly vacuolated remnant of a central vein endothelial cell (CVEC) and absence of sinusoidal endothelial cells (SECs), respectively. RBC, red blood cell; PMN, polymorphonuclear leukocyte. Magnification in (A) is 12,180X for CV region and 14,820X for MZ region and in (B) is 19,170X for CV region and 12,150X for MZ region.

aphs from Liver Sections

were treated with MCT

y were killed 18 hours

re removed and fixe

The central vein (CV

ed rat have norma

from a MCT/LPS-

a central ver

ndothelial cell

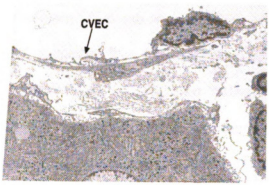
morphonuclear

14,820X for

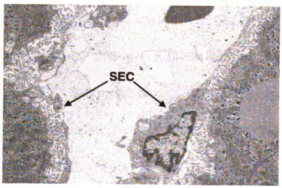
MZ region.

A

Veh/Veh – CV

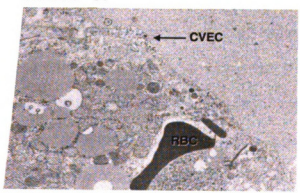


Veh/Veh – MZ

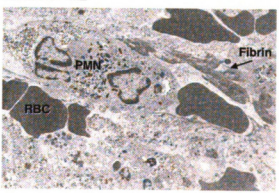


B

MCT/LPS – CV



MCT/LPS – MZ



4.D.2. SECs and NP-26 Cells are Morphologically Similar and Form Pyrrolic Metabolites From MCT

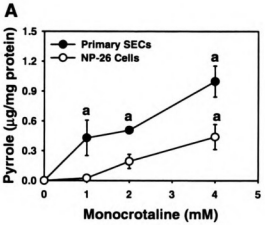
[This series of experiments was conducted by Umesh M. Hanumegowda.] Previously, DeLeve *et al.* (1996) demonstrated that MCT is toxic to SECs *in vitro* and suggested that the toxicity might be due to the production of a toxic metabolite (i.e., MCTP). To determine if MCT is metabolized by SECs, an Ehrlich assay was used to estimate the formation of pyrrolic metabolite(s) in primary SEC isolates and in a transformed rat SEC line (i.e., NP-26 cells).

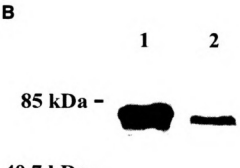
After incubation with MCT, primary SECs produced pyrrole in a concentration-dependent manner (Figure 4.4A). The amount of pyrrole produced from primary SECs was corrected for the contribution by contaminating hepatocytes. Scanning electron microscopic analysis of NP-26 cells revealed fenestrations (Figure 4.4C), a hallmark of SEC morphology (Figure 4.4D). Pyrrole production also occurred in NP-26 cells in a MCT concentration-dependent manner (Figure 4.4A). Immunoblotting of microsomal proteins with an anti-CYP3A2 specific antibody revealed a protein of ~72 kDa in both NP-26 cells and rat hepatocytes (Figure 4.4B).

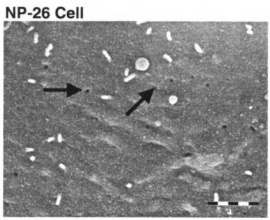
Figure 4.4. Pyrrole Formation from MCT in SECs *In Vitro*. (A) During incubation with various concentrations of MCT, pyrrolic metabolites are formed by primary SECs and NP-26 cells. Pyrrole formation in primary SECs was corrected for the contribution of contaminating hepatocytes (see Materials and Methods). (B) Representative immunoblot revealing CYP3A2 protein in the microsomal fraction of hepatocytes (Lane 1) and NP-26 cells (Lane 2). Representative scanning electron microscopic photomicrographs are depicted for NP-26 cells (C) and primary SECs (D), demonstrating fenestrae on the cell surface (solid black arrows). Bar = 2 μm. N = 3 – 4 animals.

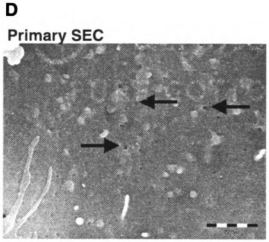
[a] Significantly different from Veh control.

[The work in this figure was completed by Umesh M. Hanumegowda].


A
Primary SECs
NP-26 Cells
Pyrrole (µg/mg protein)
1.5
1.2
0.9
0.6
0.3
0.0
a
a
a
a
0
1
2
3
4
5
Monocrotaline (mM)


B
1
2
85 kDa -
40.7 kDa -


C
NP-26 Cell


D
Primary SEC

4.D.3. Liver Hemorrhage

To quantify the degree of hemorrhage in the livers of MCT/LPS-cotreated animals, the concentration of tissue hemoglobin was determined. A time-dependent increase in liver hemoglobin began within 12 hours in the MCT/LPS-cotreated group and remained significant at 18 hours (Figure 4.5A). No other treatment group exhibited increased concentration of liver hemoglobin.

4.D.4. Coagulation System Activation

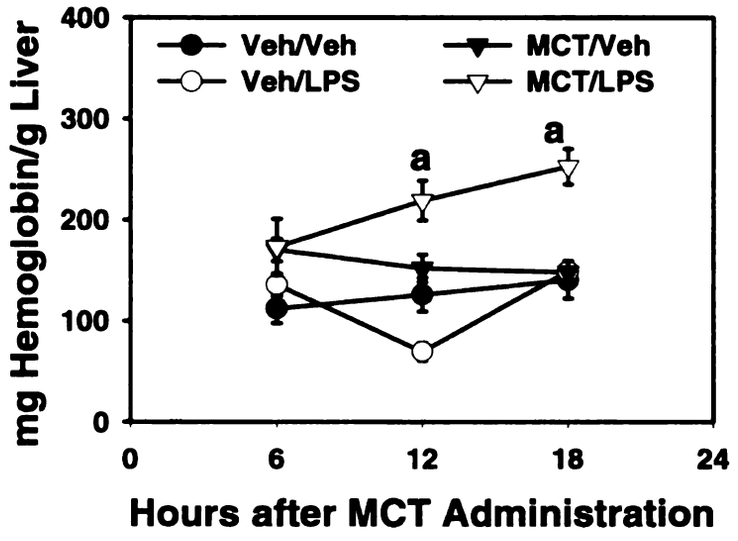
During activation of the coagulation system, fibrinogen is converted to fibrin, resulting in a decrease in plasma fibrinogen concentration. Hence, plasma fibrinogen is a biomarker for activation of the coagulation system. No decrease in plasma fibrinogen was observed at any time in groups treated with Veh, LPS or MCT alone. By contrast, plasma fibrinogen concentration decreased significantly in MCT/LPS-cotreated animals by 6 hours (Figure 4.5B). This decrease occurred before the onset of HPC injury (Figure 4.1A). Plasma fibrinogen concentration from MCT/LPS-cotreated animals remained significantly depressed at 12 and 18 hours.

To determine if the decrease plasma fibrinogen was associated with deposition of insoluble fibrin in the liver, hepatic fibrin was examined immunohistochemically. Little fibrin immunostaining was observed in the sinusoids of liver sections from Veh/Veh-treated rats at 18 hours (Figure 4.6A). However, some staining occurred in the intima of larger vessels, which results from fibrin deposition that occurs after animal sacrifice (Copple *et al.*, 2002a). By

Figure 4.5. Timecourse for Liver Hemoglobin and Coagulation System Activation in MCT/LPS-Coexposed Rats. Rats were treated with MCT and/or LPS, and killed at the indicated times, as described in Figure 4.1 legend. (A) Livers were removed and analyzed for tissue hemoglobin, a biomarker of hemorrhage. (B) Plasma fibrinogen, a biomarker for coagulation system activation, was also analyzed. N = 3 – 7 animals.

[a] Significantly different from all other groups at the same time.

A


mg Hemoglobin/g Liver
400
300
200
100
0
Veh/Veh
Veh/LPS
MCT/Veh
MCT/LPS
a
a
0
6
12
18
24
Hours after MCT Administration

B

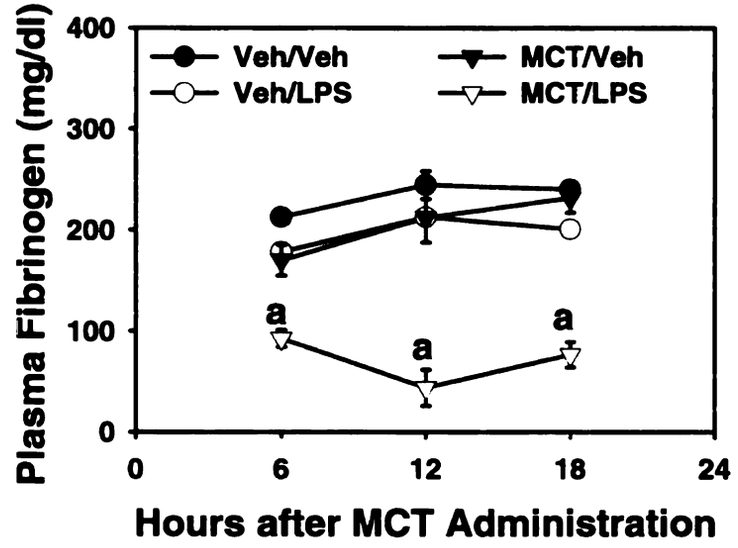
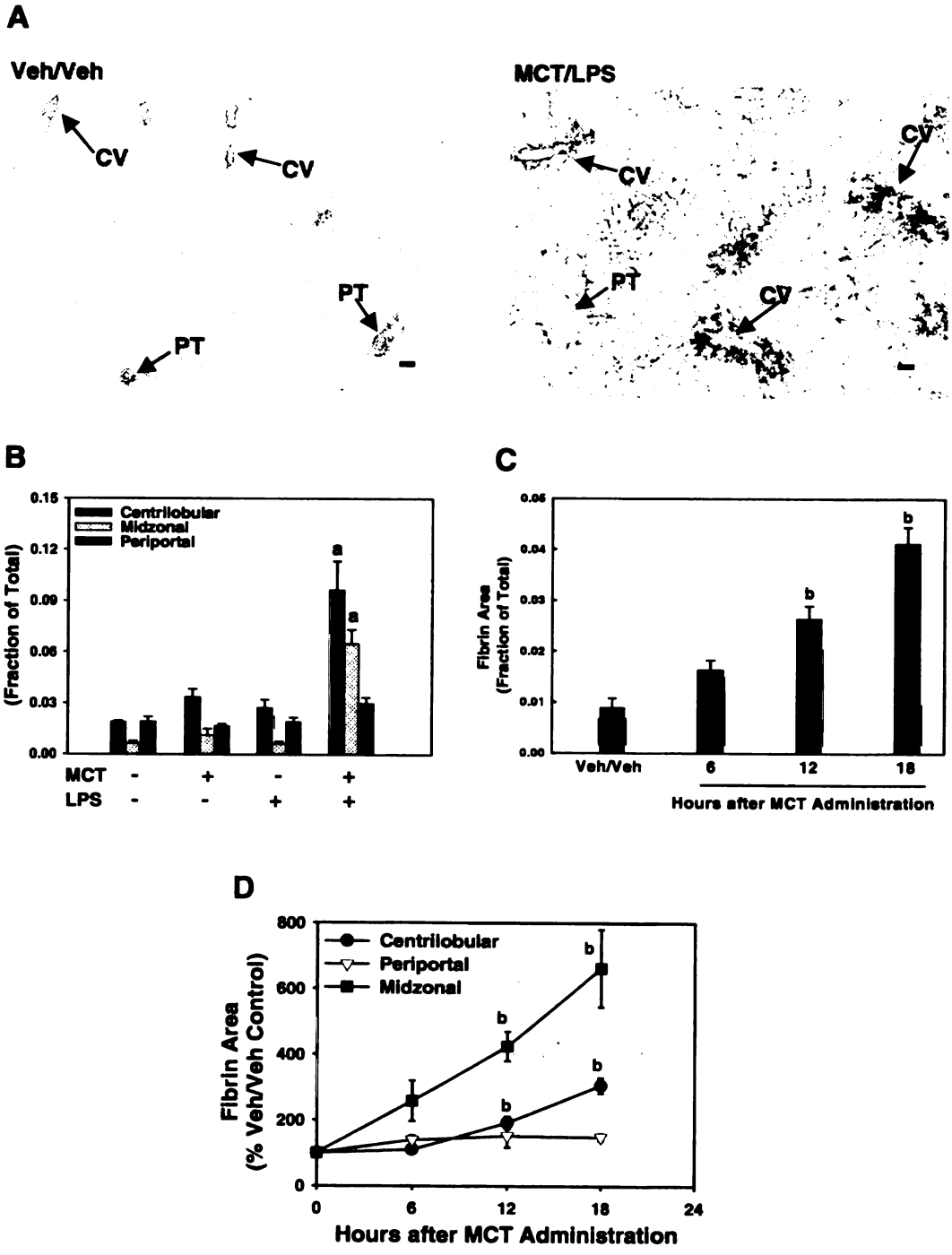

Plasma Fibrinogen (mg/dl)
400
300
200
100
0
Veh/Veh
Veh/LPS
MCT/Veh
MCT/LPS
a
a
a
0
6
12
18
24
Hours after MCT Administration

Figure 4.6. Hepatic Fibrin Deposition. Animals were treated with MCT and/or LPS, and killed at the indicated times, as described in Figure 1 legend. Livers were removed and processed for fibrin immunohistochemistry. (A) Representative photomicrographs of liver sections from a Veh/Veh- and a MCT/LPS-cotreated rat at 18 hours immunostained for fibrin deposits, which appear black. CV, central vein; PT, portal triad. Bar = 50 μm. (B) Zonal distribution of fibrin immunostaining was evaluated at 18 hours. Timecourses for total liver fibrin immunostaining (C) and its zonal fibrin distribution (D) in the livers of MCT/LPS-cotreated animals were determined. N = 4 – 7 animals. For (C) and (D), Veh/Veh-treated animals were combined into one group (N = 12), since no differences occurred among Veh/Veh-treated groups at the various times.

[a] Significantly different from all other groups in same lobular region.

[b] Significantly different from Veh/Veh group.


A
Veh/Veh
MCT/LPS
CV
PT
B
Centrilobular
Midzonal
Periportal
Fibrin Area
(Fraction of Total)
MCT
LPS
C
Veh/Veh
Hours after MCT Administration
D
Fibrin Area
(% Veh/Veh Control)
Hours after MCT Administration

contrast, intense fibrin staining occurred in the sinusoids of CL and MZ regions of livers from rats treated with MCT/LPS (Figure 4.6A). Fibrin staining was minimal in the PP regions. Morphometric analysis confirmed a significant increase in fibrin staining at 18 hours in the CL and MZ regions in livers of MCT/LPS-cotreated animals (Figure 4.6B). No increase in fibrin staining was observed in the other treatment groups at this time. Furthermore, a time-dependent increase in total fibrin staining occurred in the livers of MCT/LPS-cotreated animals; this was significant at 12 hours and remained elevated at 18 hours (Figure 4.6C). Zonal analysis of hepatic fibrin staining revealed a time-dependent increase that became significant at 12 hours in both CL and MZ regions of livers from MCT/LPS-cotreated animals (Figure 4.6D).

4.E. Discussion

Large, acutely toxic doses of LPS cause dose-dependent activation and injury to endothelial cells (Pober and Coltran, 1990; Pohlman and Harlan, 1992; Seto *et al.*, 1998). Acutely hepatotoxic doses of MCT also cause endothelial cell injury both *in vivo* (Schoental and Head, 1955; DeLeve *et al.*, 1999; Copple *et al.* 2002a) and *in vitro* (DeLeve *et al.*, 1996). To explore hepatic endothelial cell injury in a model of synergistic hepatotoxicity involving coexposure to small, nontoxic doses of MCT and LPS, a plasma biomarker for SEC injury, immunohistochemistry and TEM were used.

HA is a glycosaminoglycan component of the extracellular matrix that is tonically released into blood (Laurent and Frazier, 1992). Over 90% of circulating HA is removed and degraded by hepatic SECs through receptor-mediated endocytosis (Laurent and Frazier, 1992; Kobayashi *et al.*, 1999). SEC injury impairs the ability of these cells to clear HA, resulting in increased plasma HA concentration. Consequently, such increases have been used *in vivo* as a biomarker for SEC dysfunction and injury (Shimizu *et al.*, 1994; Copple *et al.*, 2002a). Increases 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.* 2002a) or LPS (Deaciuc *et al.*, 1994; Seto *et al.*, 1998; Spapen *et al.*, 1999). In the present study, a significant increase in plasma HA concentration occurred in MCT/LPS-cotreated animals within 6 hours, and the concentration remained elevated until 18 hours after MCT administration (Figure 4.1B). The pronounced rise in plasma HA concentration preceded the onset of HPC injury (Figure 4.1A), suggesting that SEC dysfunction might be causally involved in HPC injury. Additionally, a modest increase in plasma HA concentration was observed at 18 hours in rats given only LPS (Figure 4.1B). This increase suggests functional impairment of SECs (Deaciuc *et al.*, 1993a; Takeuchi *et al.*, 1994; Sarphie *et al.*, 1996), but in rats treated only with LPS this was not accompanied by HPC injury at 18 hours or times thereafter (Yee *et al.*, 2000b, 2003c). Therefore, either the extent of SEC dysfunction that occurred in rats given LPS alone was of insufficient magnitude to produce HPC injury or HPC injury depended on factors in addition to SEC dysfunction that are

absent in animals treated with LPS alone. Alternatively, HPC injury in MCT/LPS-cotreated animals may occur independently of SEC injury.

RECA-1 immunostaining of liver sections revealed that endothelial cell injury was extensive in MCT/LPS-cotreated animals. Approximately 25% of endothelial cell staining was lost within 12 hours after MCT administration, and this loss increased to 40% by 18 hours. Reduction in RECA-1 staining was pronounced within CL regions by 6 hours and within MZ regions by 12 hours (Figure 4.2D). The CL and MZ location of this decrease corresponds to the regions wherein HPC necrosis occurs. The reduction in RECA-1 staining, however, was not observed in the group given LPS alone at 18 hours, and there was no reduction in total RECA-1 staining in the MCT/LPS group at 6 hours. Since increases in plasma HA occurred under both of these conditions, plasma HA concentration appears to be a more sensitive biomarker for SEC injury than loss of RECA-1 staining. A similar difference in sensitivity was observed in a study of hepatotoxic doses of MCT (Copple *et al.*, 2002a).

TEM of livers from MCT/LPS-cotreated animals was used to confirm hepatic vascular injury. By 18 hours, the sinusoids and CVs from the livers of MCT/LPS-cotreated animals either contained remnants of SECs or CVECs or were devoid of endothelial cells altogether (Figure 4.3). Fibrin deposition, RBCs in the space of Disse (i.e., hemorrhage) and PMNs were found in sinusoids within CL and MZ regions. These changes were apparent by 12 hours (data not shown). Accordingly, endothelial damage, as determined by plasma HA

concentration, immunohistochemistry and TEM, is a pronounced component of synergistic liver injury induced by MCT/LPS-cotreatment.

The cause of SEC injury in MCT/LPS-cotreated rats is not fully understood. DeLeve *et al.* (1996) postulated that SECs *in vitro* can bioactivate MCT to its toxic metabolite (i.e., MCTP), resulting in SEC injury. Although SECs are known to express some CYPs (Lester *et al.*, 1993; DeLeve *et al.*, 1997), it is not known if the particular CYP subfamily (i.e., CYP 3A) responsible for MCT bioactivation is expressed in these cells (Kasahara *et al.*, 1997; Reid *et al.*, 1998). However, both isolated primary SECs and the NP-26 rat SEC cell line produced pyrrolic metabolite(s) from MCT in a concentration-dependent manner (Figure 4.4A). In addition, a ~72 kDa protein recognized by the CYP3A2 specific antibody is expressed in the microsomal fractions in NP-26 cells (Figure 4.4B). This confirms that an isozyme from the CYP3A subfamily is present in these cells (i.e., NP-26 cells). Moreover, it has been demonstrated that MCTP causes injury to endothelial cells *in vitro.* For example, treatment of pulmonary artery endothelial cells with low concentrations of MCTP *in vitro* results in enhanced cell detachment (i.e., disruption of monolayer integrity), derangement of actin polymerization, and apoptotic necrosis (Reindel and Roth, 1991; Thomas *et al.*, 1998; Wilson *et al.*, 1998). Hence, SECs may be able to metabolize MCT. The consequent production of MCTP would have adverse effects on these cells that could explain widespread SEC destruction *in vivo.*

No SEC injury was observed *in vivo* when the dose of MCT used in the present study was given by itself. This suggests that the degree of bioactivation

by SECs and HPCs combined was insufficient to cause overt injury (Figure 4.1B). However, at the small MCT dose used, MCT bioactivation might alter SEC homeostasis to render these cells more susceptible to toxicity from inflammatory factors evoked by concurrent LPS exposure (Ward and Varani, 1990; Jaeschke and Farhood, 1991; Springer, 1994; Sarphie *et al.,* 1996; Yachida *et al.*, 1998). Yee *et al.* (2003a, 2003c) demonstrated that Kupffer cell inactivation, TNF-α neutralization and PMN depletion were each effective in attenuating HPC injury in MCT/LPS-cotreated animals, but these manipulations were only modestly successful in attenuating SEC injury. Hence, it is probable that SEC injury in this model develops in part from the interaction of MCT with LPS or with an unknown inflammatory mediator(s) evoked by LPS. One possible interaction could involve depletion of glutathione (GSH) in endothelial cells by bioactivated MCT (Pan *et al.*, 1993; Reid *et al.*, 1999). DeLeve *et al.* (1996) showed that MCT is more toxic to SECs than to HPCs *in vitro* and that toxicity may require profound GSH depletion. It is tempting to speculate that the small dose of MCT used in this model decreases GSH concentration in SECs without resulting in overt injury. Prooxidant inflammatory mediators evoked by LPS might precipitate overt injury to those SECs in which GSH has been decreased by MCT. Further study is required to explore this possibility.

Activation of SECs by LPS or LPS-induced inflammatory mediators (e.g., TNF-α) results in SECs becoming procoagulant (Stern *et al.*, 1985; Takeuchi *et al.*, 1994). Moreover, SEC injury or destruction can result in the activation of the coagulation system (Machovich, 1985; Ryan, 1986; Hirata *et al.*, 1989; Copple *et*

al., 2002a). In MCT/LPS-cotreated animals, plasma fibrinogen concentration decreased significantly by 6 hours after MCT administration and remained depressed through 18 hours (Figure 4.5B). A consequence of coagulation system activation is the formation of insoluble fibrin. Fibrin deposition increased significantly in the livers of MCT/LPS cotreated animals by 12 hours and remained elevated at 18 hours (Figure 4.6C). Both CL and MZ regions of the liver lobule experienced fibrin deposition at these times (Figure 4.6D). TEM confirmed the immunohistochemical evidence of fibrin deposition in the sinusoids (Figure 4.3B). The observations that the reduction in plasma fibrinogen concentration preceded HPC damage and that fibrin deposits localized to CL and MZ regions that ultimately experience damage suggest the possibility of a causal relationship between activation of the coagulation system and HPC injury.

Hemorrhage and fibrin deposition caused by injury or loss of SECs can result in the impairment of sinusoidal blood flow (Hirata *et al.*, 1989; Vollmar *et al.* 1993; Yachida *et al.*, 1998). Moreover, activation of SECs by TNF-α or other inflammatory mediators results in adhesion of PMNs to these cells and to cell swelling that can narrow sinusoidal lumens and contribute to such impairment of blood flow (Springer, 1994; Vollmar *et al.*, 1996a; Yachida *et al.*, 1998). It has been postulated that consequent sinusoidal hypoperfusion leads to ischemic/hypoxic injury to HPCs within affected regions (Fujiwara *et al.*, 1988; Hirata *et al.*, 1989; DeLeve *et al.*, 1996; Mochida *et al.*, 1999; Yee *et al.*, 2000b; Copple *et al.*, 2002a). Consistent with this hypothesis is the observation in MCT/LPS-treated rats that both SEC injury (Figure 4.1B) and coagulation system

activation (Figure 4.5B) precede liver hemorrhage (Figure 4.5A), formation of fibrin deposits and the onset of HPC injury. In addition, thrombin and other coagulation factors might act on protease-activated receptors to promote liver injury, as occurs after an hepatotoxic dose of LPS (Copple *et al.*, 2003). Therefore, damage to SECs may lead to HPC injury through activation of the coagulation system resulting in fibrin deposition in the liver and hypoxic injury to HPCs.

Other mechanisms exist by which SEC injury could contribute to HPC injury. For example, SECs perform various functions in the liver, including filtration, endocytosis and putative regulation of sinusoidal blood flow (Wisse *et al.*, 1996; Arii and Imamura, 2000). As described above, treatment of endothelial cells with MCTP results in detrimental cytoskeletal, junctional barrier and permeability changes (Reindel and Roth, 1991; Thomas *et al.*, 1998; Wilson *et al.*, 1998). If similar changes occur in SECs *in vivo* after MCT/LPS coexposure, such disruption of the SEC barrier could permit access to HPCs of injurious products from activated PMNs and Kupffer cells, resulting in HPC injury (Aria *et al.*, 1993; Farhood *et al.*, 1995; Jaeschke *et al.*, 1996; Yachida *et al.*, 1998; Yoshidome *et al.*, 2000). Hence, injury or destruction of SECs might lead to HPC injury through a variety of mechanisms. Accordingly, SECs may be an important initial target in the development of liver injury in this model, as it appears to be in others (Deaciuc and Spitzer, 1996; DeLeve *et al.*, 1996; Sarphie *et al.*, 1997; Copple *et al.*, 2002a). However, further studies will be needed to determine if

SECs have a causal role in the development of HPC injury in MCT/LPS-cotreated animals.

In summary, MCT/LPS cotreatment results in extensive, time-dependent injury to hepatic endothelial cells. Destruction of CVECs is evident by light and electron microscopy as well as in the loss of intimal RECA-1 immunostaining. Similarly, elevated plasma HA concentration, decreased sinusoidal RECA-1 immunostaining and TEM results all indicate injury to SECs. SEC injury precedes hepatic fibrin deposition, hemorrhage and HPC injury. Overall, the results indicate that synergistic hepatotoxicity from coexposure to small, noninjurious doses of MCT and LPS involves pronounced SEC dysfunction and injury and activation of the coagulation system.

ROLE OF INFLAMMATION IN THE SYNERGISTIC HEPATOTOXICITY OF MONOCROTALINE AND BACTERIAL LIPOPOLYSACCHARIDE

VOLUME II

By

Steven Byron Yee

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

2003

CHAPTER 5

The Role of Kupffer Cells and TNF-α in Monocrotaline and Bacterial Lipopolysaccharide-Induced Liver Injury

5.A. Abstract

Coexposure to small, non-injurious doses of the pyrrolizidine alkaloid phytotoxin monocrotaline (MCT) and bacterial lipopolysaccharide (LPS) results in synergistic hepatotoxicity. Both centrilobular and midzonal liver lesions occur and are similar to those seen from large, toxic doses of MCT and LPS, respectively. The nature of the lesions *in vivo* and results from studies *in vitro* suggest that injury is mediated indirectly rather than from a simple interaction of MCT and LPS with hepatic parenchymal cells. Accordingly, the role of inflammatory factors, such as Kupffer cells and TNF-α, in the development of MCT/LPS-induced liver injury was investigated. In Sprague-Dawley rats, MCT (100 mg/kg, i.p.) was administered 4 hours before LPS (7.4×10^6 EU/kg, i.v.). Pretreatment of these animals with gadolinium chloride, an inhibitor of Kupffer cell function, attenuated liver injury 18 hours after MCT administration. An increase in plasma TNF-α preceded the onset of hepatic parenchymal cell injury, raising the possibility that this inflammatory cytokine contributes to toxicity. Either pentoxifylline, an inhibitor of cellular TNF-α synthesis, or anti-TNF-α serum coadministered to MCT/LPS-treated animals significantly attenuated liver injury. These results suggest that Kupffer cells and TNF-α are important mediators in the synergistic hepatotoxicity resulting from MCT and LPS coexposure.

5.B. Introduction

Yee *et al.* (2000b) recently demonstrated that synergistic liver injury resulted when a noninjurious dose of LPS was administered 4 hours after a small, nonhepatotoxic dose of MCT. Failure to reproduce synergistic injury in isolated hepatic parenchymal cells (HPCs) *in vitro* suggested that the enhanced toxicity resulted not from direct interaction of MCT and LPS with HPCs, but rather from an indirect mechanism (Yee *et al.*, 2000b). Inasmuch as LPS is an inflammagen, the present study was designed to identify inflammatory factors critical to the synergistic hepatotoxicity in this model.

KCs are the resident macrophages of the liver. When activated by LPS, they produce and release numerous mediators, including cytokines (TNF-α, IL-1, and IL-6) and reactive oxygen species (Michie *et al.*, 1988; Holst *et al.*, 1996). Cyclooxygenase-2 (COX-2) is also induced, leading to the enhanced synthesis of prostaglandins (PGs) that have proinflammatory actions (Brouwer *et al.*, 1995; Dieter *et al.*, 1999). Many of these mediators further activate or modulate the effects of nearby cells involved in the inflammatory process and can contribute to the development of injury (Hewett *et al.*, 1993; Holst *et al.*, 1996). Inactivation of KCs with gadolinium chloride ($GdCl_3$) in rats given a large, hepatotoxic dose of LPS significantly attenuates hepatocellular necrosis, suggesting that KCs are critical to the pathogenesis of LPS-induced injury (Brown *et al.*, 1997).

TNF-α is a potent inflammatory cytokine produced by activated macrophages and to a lesser degree by other cell types (Vasselli, 1992; Bradham *et al.*, 1998). This cytokine can exert a variety of effects on cells ranging from mitochondrial damage and oncotic or apoptotic necrosis to cell

proliferation (Bradham *et al.*, 1998). TNF-α may also prompt the accumulation of neutrophils (PMNs) by activating endothelial cells (Vasselli, 1992; Bradham *et al.*, 1998). It can indirectly promote toxicity by priming PMNs to release reactive oxygen and nitrogen species and proteases that damage nearby cells (Nagaki *et al.*, 1991; Vasselli, 1992). Inhibition of TNF-α synthesis or activity attenuates injury caused by an hepatotoxic dose of LPS, indicating that TNF-α is a critical factor in the pathogenesis (Hewett *et al.*, 1993).

The purpose of this study was to investigate the role of inflammatory factors in the synergistic liver injury that occurs following coexposure to small doses of MCT and LPS. Specifically, the hypothesis that KCs and TNF-α play causal roles in the hepatotoxicity was tested. In addition, the importance of COX-2 products for the development of injury was evaluated.

5.C. Materials and Methods

5.C.1. Materials

LPS (*Escherichia coli,* serotype 0128:B12, 1.7 x 10^6 endotoxin units (EUs)/mg), pentoxifylline, and sodium citrate were purchased from Sigma Chemical Company (St. Louis, MO). Serum directed against TNF-α (anti-rat TNF-α serum; ATS) was produced in New Zealand White rabbits (Hewett *et al.*, 1993). $GdCl_3$-$6H_2O$ was purchased from Aldrich Chemical Company (St. Louis, MO). The selective COX-2 inhibitor *N*-(2-cyclohexyloxy-4-nitro-phenyl)methane sulfonamide (NS-398) was acquired from Cayman Chemical Co. (Ann Arbor, MI).

MCT was obtained 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, were also purchased from Sigma Chemical Company (St. Louis, MO). ELISA kit for hyaluronic acid was acquired from Corgenix, Inc. (Westminster, CO). ELISA kit for rat TNF-α was purchased from Biosource International, Inc. (Camarillo, CA).

5.C.2. Animals

Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR, Charles River, Portage, MI) weighing 200-300 g were used in all studies. Otherwise, for more information on this section, refer to Chapter 2 Materials and Methods.

5.C.3. Treatment Protocol

For more information on this section, refer to Chapter 4 Materials and Methods.

5.C.4. Treatment with Gadolinium Chloride

Rats were treated with 10 mg $GdCl_3$-$6H_2O$/kg (pH 3.5) or saline vehicle (Veh) intravenously 24 hours before the administration of LPS or its vehicle. This treatment protocol has been shown to inactivate KC function (Brown *et al.*, 1997).

5.C.5. Treatment with Pentoxifylline

Rats received 100 mg PTX/kg or its saline Veh intravenously 1 hour before LPS treatment. This treatment regimen prevents the LPS-induced increase in plasma TNF-α activity (Barton *et al.*, 2001).

5.C.6. Treatment with Anti-TNF-α Serum

Rats were treated intravenously with rabbit ATS (1 ml) or control rabbit serum (CS; 1 ml) 1 hour before LPS administration. This treatment protocol has been shown to prevent LPS-induced increase in plasma TNF-α activity (Hewett *et al.*, 1993; Barton *et al.*, 2001).

5.C.7. Treatment with NS-398

NS-398 is a selective inhibitor of COX-2 (Futaki *et al.*, 1994, 1997). Rats were given 5 mg NS-398/kg or olive oil intraperitoneally 1 hour before LPS administration.

5.C.8. Assessment of Liver Injury, Plasma TNF-α and Plasma Hyaluronic Acid.

HPC injury was estimated by increases in the activities of plasma ALT and AST. An ELISA was used to measure plasma hyaluronic acid (HA), a marker of hepatic SEC injury (Deaciuc *et al.*, 1993b, 1994; Copple *et al.*, 2002a). Plasma TNF-α concentration was determined via a rat TNF-α ELISA kit.

5.C.9. Histopathologic Evaluation and Morphometry

Serial transverse sections from the left lateral liver lobe were processed for light microscopy. Paraffin-embedded sections were cut at 4 μm, stained with hematoxylin and eosin, and evaluated for lesion size and severity. Tissue sections were analyzed histologically without knowledge of the treatment group.

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 centrilobular 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; Majno and Joris, 1995; Levin *et al.*, 1999). 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):

$$Area_{Interest} = \Sigma\ Points_{Interest}\ X\ Area/Point$$

$$Area/Point = (Distance\ Between\ Points)^2$$

Distance between points was 55 μm. Accordingly, the area represented by each point was 3025 μm^2. 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 10^{th} 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:

$$[Area_{Lesion\ of\ Interest}/(Area_{All\ Lesions} + Area_{Parenchyma})] \times 100.$$

5.C.10. Statistical Analysis

Results are expressed as means ± S.E.M. 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). Homogeneous data were analyzed by one-way or two-way analysis of variance (ANOVA), as appropriate, and group means were compared using Student-Newman-Keuls post hoc test (Steele *et al.*, 1997). When variances were not homogeneous, data were analyzed using the Kruskal-Wallis nonparametric ANOVA, and Dunn's Multiple Comparison test was used to compare group means (Steele *et al.*, 1997). The criterion for significance was $p \leq 0.05$ for all comparisons.

5.D. Results

5.D.1. Effect of $GdCl_3$ on MCT/LPS-Induced Liver Injury

To test the hypothesis that KCs are important for the development of liver injury in the MCT/LPS model, rats were pretreated with the KC inactivator $GdCl_3$, at a dose that inhibits KC-mediated phagocytosis (Brown *et al.*, 1997). As further verification of $GdCl_3$ efficacy, plasma TNF-α concentration was monitored (Figure 5.1A). Plasma TNF-α concentration was elevated in the MCT/LPS-cotreated animals, and this increase was reduced with $GdCl_3$ pretreatment. Plasma TNF-α concentration was not elevated in animals receiving either vehicles or $GdCl_3$ alone.

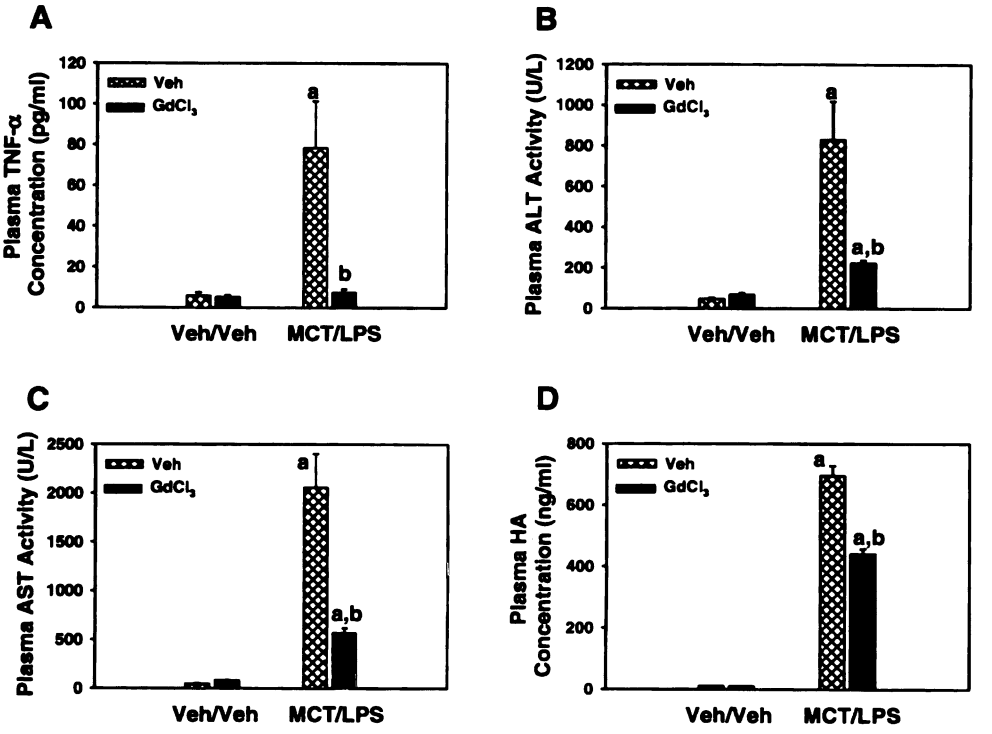
Plasma ALT activity was elevated in MCT/LPS-cotreated animals (Figure 5.1B). $GdCl_3$ pretreatment markedly attenuated this increase. Similar results were observed with plasma AST activity (Figure 5.1C). Plasma HA concentration was also elevated in animals that received MCT/LPS cotreatment. This increase was attenuated slightly but significantly in $GdCl_3$-pretreated rats (Figure 5.1D). No elevated plasma ALT or AST activity or plasma HA concentration was observed in control animals that received vehicles or $GdCl_3$ alone.

MCT/LPS-cotreated control animals exhibited centrilobular and midzonal liver lesions as previously described by Yee *et al.* (2000b). Centrilobular lesions consisted of moderate to marked hepatocellular apoptotic and oncotic necrosis, degeneration, hemorrhage and vascular injury. Midzonal lesions in the MCT/LPS-cotreated group comprised marked but more frequent and well-defined

Figure 5.1. $GdCl_3$ Protects Against MCT/LPS-Induced Liver Injury. LPS (7.4×10^6 EU/kg) or saline vehicle (Veh) was administered i.v. to rats 4 hours after i.p. administration of MCT (100 mg/kg) or saline vehicle. Rats were pretreated with 10 mg $GdCl_3$-$6H_2O$/kg or saline vehicle, i.v., 24 hours before LPS administration. TNF-α concentration (A), ALT (B) and AST (C) activities, and HA concentration (D) were evaluated in plasma 18 hours after MCT administration. N = 4 for Veh/Veh/Veh, 4 for $GdCl_3$/Veh/Veh, 14 for Veh/MCT/LPS, 13 for $GdCl_3$/MCT/LPS.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from Veh/MCT/LPS group.


A
Plasma TNF-α Concentration (pg/ml)
120
100
80
60
40
20
0
Veh
GdCl3
a
b
Veh/Veh
MCT/LPS
B
Plasma ALT Activity (U/L)
1200
1000
800
600
400
200
0
Veh
GdCl3
a
a,b
Veh/Veh
MCT/LPS
C
Plasma AST Activity (U/L)
2500
2000
1500
1000
500
0
Veh
GdCl3
a
a,b
Veh/Veh
MCT/LPS
D
Plasma HA Concentration (ng/ml)
800
600
400
200
0
Veh
GdCl3
a
a,b
Veh/Veh
MCT/LPS

areas of hepatocellular coagulative necrosis accompanied by neutrophil infiltration with congestion and hemorrhage. Liver lesions from animals treated with $GdCl_3$/MCT/LPS exhibited qualitatively similar centrilobular and midzonal lesions; however, these lesions were both smaller in size and less frequent. The $GdCl_3$-induced reduction in lesion size and frequency was more pronounced in the midzonal regions. Morphometric analysis of liver lesions supported the observation that $GdCl_3$ pretreatment reduced the liver area affected by centrilobular and midzonal lesions (Table 5.1). Furthermore, the number of hepatic PMNs was smaller in MCT/LPS-treated animals that received $GdCl_3$ pretreatment compared to MCT/LPS-treated animals that did not. Histologically, no evidence of liver injury was observed in animals treated with vehicles or $GdCl_3$ alone.

5.D.2. Plasma TNF-α Concentration

Plasma TNF-α concentration was assessed at various times after the administration of MCT or its saline vehicle (Table 5.2). It did not increase after Veh/Veh or MCT/Veh cotreatments at any of the times evaluated but was elevated 2 hours after LPS administration irrespective of MCT-treatment. Whereas plasma TNF-α concentration in Veh/LPS-cotreated animals returned to baseline within the next 8 hours, plasma TNF-α concentration in MCT/LPS-cotreated animals remained modestly but significantly elevated 14 hours after LPS administration.

Table 5.1. Morphometric Analysis of Liver Lesions from MCT/LPS-Cotreated Rats Following Administration of $GdCl_3$, PTX, or ATS. 7.4×10^6 EU LPS/kg or its saline vehicle (Veh) was administered 4 hours after 100 mg MCT/kg or saline vehicle. In addition, rats were treated with $GdCl_3$, PTX, ATS or their vehicles as described in Chapter 5 Materials and Methods. All livers were removed from rats 18 hours after MCT treatment, fixed in formalin and processed for light microscopy. Morphometric analysis was performed as described in Chapter 5 Materials and Methods. N = 9 for Veh/MCT/LPS, 13 for $GdCl_3$/MCT/LPS, 8 for MCT/Veh/LPS, 8 for MCT/PTX/LPS, 6 for MCT/CS/LPS, and 5 for MCT/ATS/LPS.

[a] Significantly different from respective value in the absence of pharmacological treatment (i.e., $GdCl_3$, PTX or ATS).

Treatment	Percent Lesion Area	
	Centrilobular	Midzonal
$GdCl_3$ Study		
Veh/MCT/LPS	3.7 ± 0.8	9.3 ± 1.8
$GdCl_3$/MCT/LPS	1.2 ± 0.2[a]	1.4 ± 0.3[a]
PTX Study		
MCT/Veh/LPS	2.4 ± 0.6	10.3 ± 1.3
MCT/PTX/LPS	0.8 ± 0.2[a]	1.1 ± 0.4[a]
ATS Study		
MCT/CS/LPS	3.3 ± 0.8	12.0 ± 1.6
MCT/ATS/LPS	0.6 ± 0.2[a]	1.5 ± 0.4[a]

Table 5.2. Plasma TNF-α Concentration after MCT/LPS Administration in Sprague-Dawley Rats. LPS (7.4×10^6 EU/kg) or saline vehicle (Veh), i.v., was administered 4 hours after MCT (100 mg/kg) or saline vehicle, i.p. Rats were killed at the times indicated after MCT or MCT vehicle administration. Plasma TNF-α concentrations were determined by ELISA. At 6 and 12 hours N = 4 for Veh/Veh, Veh/LPS and MCT/Veh, and 6 for MCT/LPS. At 18 hours N = 4 for Veh/Veh, 6 for Veh/LPS and MCT/Veh, and 12 for MCT/LPS.

[a] Significantly different from respective values in the absence of LPS.

[b] Significantly different from all other groups at the indicated time.

Hours After MCT or its Vehicle	Plasma TNF-α Concentration (pg/ml)
6 Hours	
Veh/Veh	13 ± 9
Veh/LPS	1578 ± 141[a]
MCT/Veh	32 ± 15
MCT/LPS	1301 ± 162[a]
12 Hours	
Veh/Veh	20 ± 9
Veh/LPS	23 ± 5
MCT/Veh	17 ± 2
MCT/LPS	170 ± 37[b]
18 Hours	
Veh/Veh	9 ± 3
Veh/LPS	4 ± 0.3
MCT/Veh	16 ± 5
MCT/LPS	87 ± 21[b]

5.D.3. Effect of PTX on Hepatotoxicity from MCT/LPS Cotreatment

PTX decreases the synthesis of TNF-α at the mRNA level (Dezube *et al.*, 1993) and was used to determine whether TNF-α has a causal role in the development of liver injury. Control animals treated with vehicles or PTX alone did not have detectable plasma TNF-α concentrations at 18 hours (Figure 5.2A). The rise in plasma TNF-α concentration in MCT/LPS-treated control animals was markedly attenuated by PTX treatment.

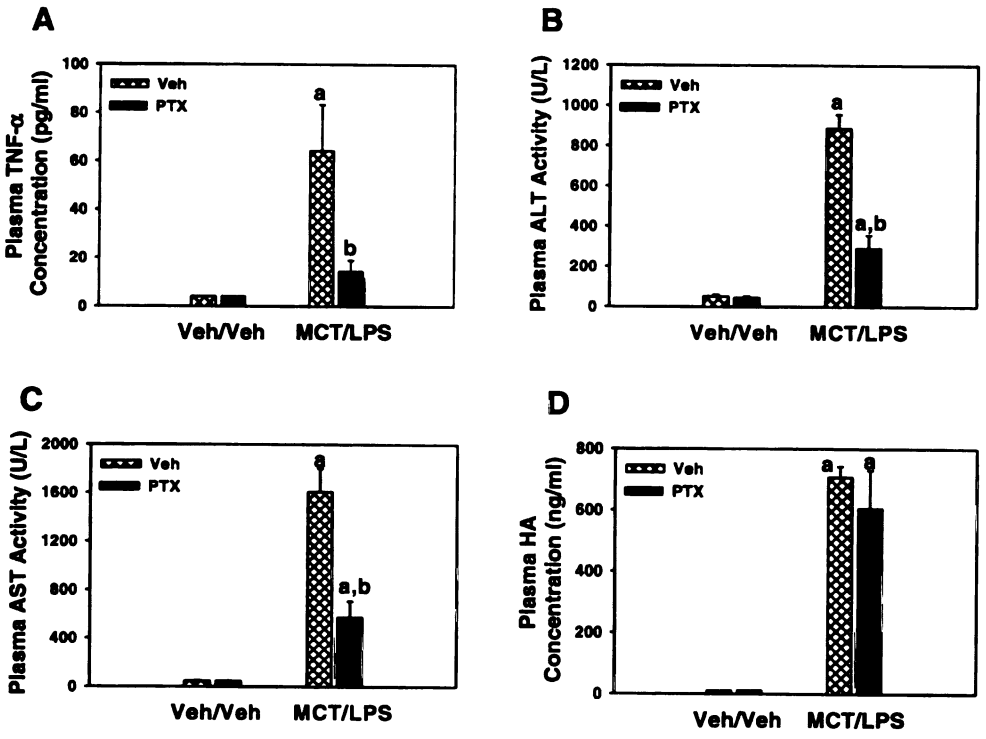
As determined by plasma ALT (Figure 5.2B) and AST (Figure 5.2C) activities, HPC injury was significantly reduced in animals that received PTX. Plasma HA concentration, however, was not significantly affected by PTX in MCT/LPS-treated animals (Figure 5.2D). No elevated plasma ALT or AST activity or plasma HA concentration was observed in control animals that received vehicles or PTX alone.

Histological examination of the livers from animals that received the MCT/PTX/LPS treatment revealed lesions qualitatively similar to those observed in animals that received MCT/LPS alone, but they were smaller in size and less frequent. Midzonal lesions exhibited the greatest reduction in size and frequency and demonstrated less congestion and hemorrhage. These observations were consistent with the mophometric analysis of lesions in this study (Table 5.1). Hepatic PMN accumulation was also reduced by PTX treatment. No lesions were observed in livers from animals that were given vehicles or PTX alone.

Figure 5.2. Effect of PTX on MCT/LPS-Induced Liver Injury. LPS (7.4 x 10^6 EU/kg) or its saline vehicle was administered i.v. to rats 4 hours after i.p. administration of MCT (100 mg/kg) or its vehicle. PTX (100 mg/kg) or saline vehicle was given i.v. to rats 1 hour before LPS. Plasma TNF-α concentration (A), ALT (B) and AST (C) activities, and HA concentration (D) were evaluated 18 hours after MCT administration. N = 4 for Veh/Veh/Veh, 4 for Veh/PTX/Veh, 13 for MCT/Veh/LPS, and 8 for MCT/PTX/LPS.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from MCT/Veh/LPS group.


A
Plasma TNF-α Concentration (pg/ml)
100
80
60
40
20
0
Veh
PTX
a
b
Veh/Veh
MCT/LPS
B
Plasma ALT Activity (U/L)
1200
1000
800
600
400
200
0
Veh
PTX
a
a,b
Veh/Veh
MCT/LPS
C
Plasma AST Activity (U/L)
2000
1600
1200
800
400
0
Veh
PTX
a
a,b
Veh/Veh
MCT/LPS
D
Plasma HA Concentration (ng/ml)
800
600
400
200
0
Veh
PTX
a
a
Veh/Veh
MCT/LPS

5.D.4. Effect of Anti-TNF-α Serum on Liver Injury from MCT/LPS Cotreatment.

MCT/LPS-cotreated animals were given ATS before LPS treatment to confirm the causal relationship between TNF-α and MCT/LPS-induced liver injury. ATS given to MCT/LPS-treated animals prevented the increase in plasma TNF-α concentration at 18 hours (Figure 5.3A).

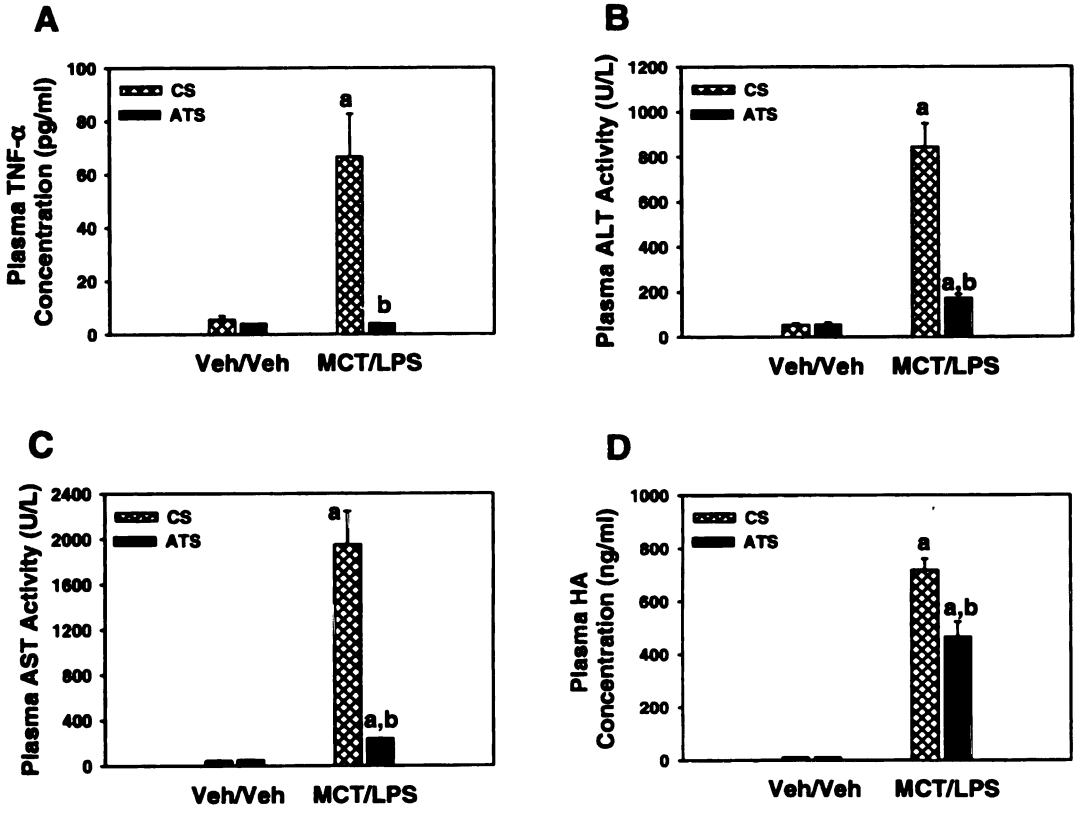
ATS treatment before LPS administration markedly attenuated liver injury in MCT/LPS-cotreated animals. Plasma ALT (Figure 5.3B) and AST (Figure 5.3C) activities were significantly less in MCT/ATS/LPS-treated animals compared to controls that received MCT/CS/LPS. ATS treatment caused a small but significant reduction in plasma HA concentration in MCT/LPS-treated rats (Figure 5.3D). No elevation in plasma ALT or AST activity or plasma HA concentration was observed in control animals that received vehicles or ATS alone.

Animals treated with MCT/ATS/LPS had liver lesions qualitatively similar to those in rats given MCT/CS/LPS, although the lesions were reduced in size and frequency. Midzonal liver lesions from MCT/ATS/LPS-treated animals exhibited less congestion and hemorrhage than livers from MCT/CS/LPS animals. Morphometric analysis of liver lesions supports the observation that ATS treatment lessened the centrilobular, MCT-like and midzonal, LPS-like lesions (Table 5.1). Furthermore, hepatic PMN accumulation was reduced in MCT/ATS/LPS-treated animals compared to animals given MCT/CS/LPS. No

Figure 5.3. Anti-TNF-α Serum Protects Against Hepatic Injury from MCT/LPS-Cotreatment. LPS (7.4×10^6 EU/kg) or saline vehicle was administered i.v. to rats 4 hours after i.p. administration of MCT (100 mg/kg) or saline vehicle. A 1 ml dose of either ATS or CS was administered i.v. 1 hour before LPS. Plasma TNF-α concentration (A), ALT (B) and AST (C) activities, and HA concentration (D) were evaluated 18 hours after MCT administration. N = 4 for Veh/CS/Veh, 4 for Veh/ATS/Veh, 8 for MCT/CS/LPS, and 5 for MCT/ATS/LPS.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from MCT/Veh/LPS group.


A
Plasma TNF-α Concentration (pg/ml)
100
80
60
40
20
0
CS
ATS
a
b
Veh/Veh
MCT/LPS
B
Plasma ALT Activity (U/L)
1200
1000
800
600
400
200
0
CS
ATS
a
a,b
Veh/Veh
MCT/LPS
C
Plasma AST Activity (U/L)
2400
2000
1600
1200
800
400
0
CS
ATS
a
a,b
Veh/Veh
MCT/LPS
D
Plasma HA Concentration (ng/ml)
1000
800
600
400
200
0
CS
ATS
a
a,b
Veh/Veh
MCT/LPS

injury was observed histologically in the livers of animals that received treatment with vehicles or ATS alone.

5.D.5. Effect of NS-398 on MCT/LPS-Induced Liver Injury

Among the various mediators released by LPS-activated KCs are metabolites of arachadonic acid, including PGE_2 and PGD_2. (Decker, 1990; Ganey *et al.*, 2001). These PGs are produced via the inflammation-inducible COX-2 enzyme and have a variety of effects on other inflammatory cells and on HPCs that can contribute to pathophysiological alterations in tissues (Casteleijn, *et al.*, 1988c; Decker, 1990; Ganey *et al.*, 2001).

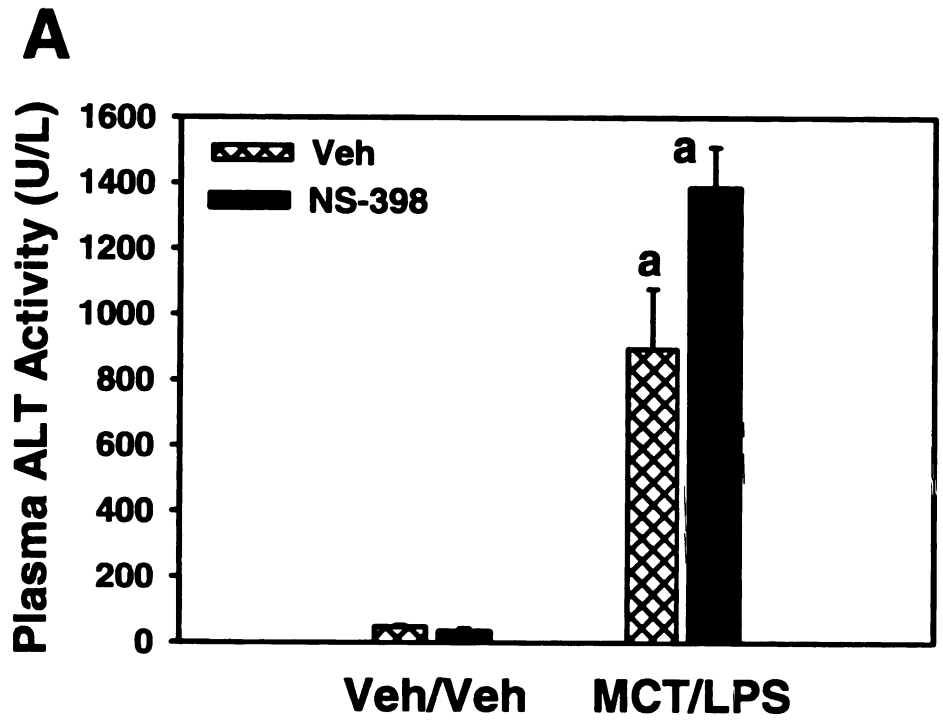
NS-398, a selective COX-2 inhibitor, was administered 1 hour before LPS at a dose that reduces PG production *in vivo* (Ganey *et al.*, 2001). NS-398 did not attenuate plasma ALT (Figure 5.4A) or AST (Figure 5.4B) activity in MCT/LPS-treated animals. Plasma ALT or AST activity was unaffected in animals receiving treatment with NS-398 alone. Histologically, liver lesions appeared the same in the MCT/LPS-cotreated animals given NS-398 or its vehicle. No liver lesions were observed in control animals that received vehicles or NS-398 alone.

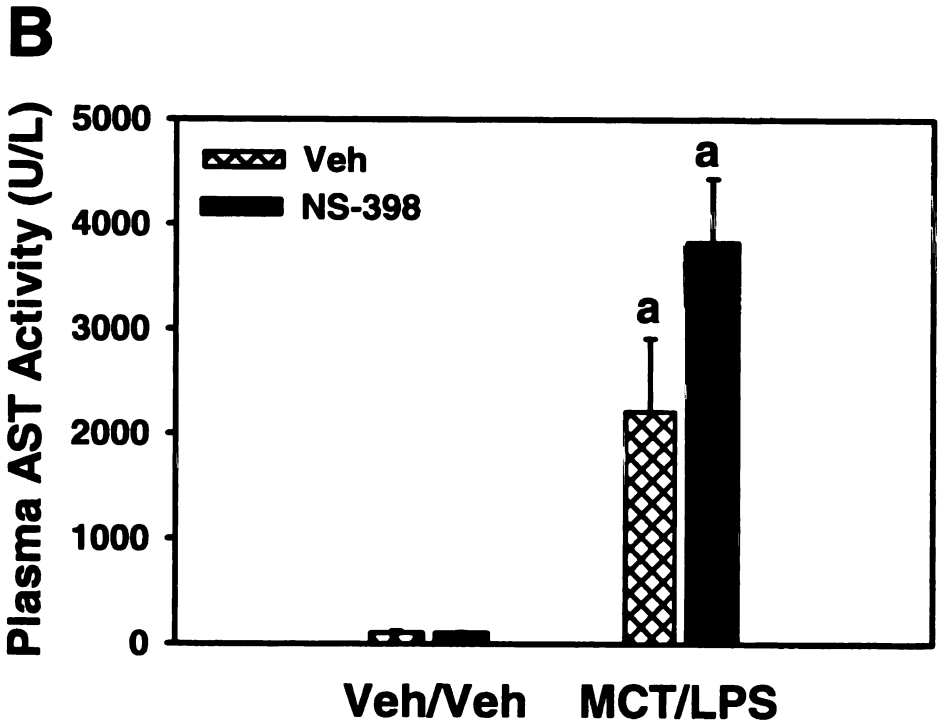
5.D.6. Mortality

Mortality ranged from 19 to 27% in MCT/LPS-cotreated control animals and from 0 to 33% in MCT/LPS-cotreated animals that received administration of pharmacologic agent (i.e., $GdCl_3$, PTX, ATS or NS-398.) None of the

Figure 5.4. NS-398 does Not Protect Against MCT/LPS-Induced Liver Injury. LPS (7.4×10^6 EU/kg) or its saline vehicle was administered i.v. to rats 4 hours after i.p. administration of MCT (100 mg/kg) or its vehicle. Rats were given 5 mg NS-398/kg or olive oil vehicle i.p. 1 hour before LPS administration. Plasma ALT (A) and AST (B) activities were evaluated 18 hours after MCT administration. N = 3 for Veh/Veh/Veh, 3 for Veh/NS-398/Veh, 7 for MCT/Veh/LPS, and 4 for MCT/NS-398/LPS.

[a] Significantly different from respective value in the absence of MCT/LPS.


A
Plasma ALT Activity (U/L)
1600
1400
1200
1000
800
600
400
200
0
Veh
NS-398
a
a
Veh/Veh
MCT/LPS


B
Plasma AST Activity (U/L)
5000
4000
3000
2000
1000
0
Veh
NS-398
a
a
Veh/Veh
MCT/LPS

pharmacologic agents significantly affected survival in MCT/LPS-cotreated animals. No animals died that received vehicles or any pharmacologic agent alone.

5.E. Discussion

Inasmuch as KCs have proven to be critical in the pathogenesis of some inflammatory tissue injuries (Adachi *et al.*, 1994; Stachlewitz *et al.*, 1999), the hypothesis that KCs are causally important in the synergistic hepatotoxicity from MCT/LPS cotreatment was tested. Inhibition of KC function by $GdCl_3$ in MCT/LPS-cotreated animals resulted in pronounced attenuation of HPC injury (Figures 5.1B and 5.1C), suggesting that KCs are causally involved in the pathogenesis. The dose of $GdCl_3$ used in this study was effective in inactivating KCs (Brown *et al.*, 1997) and inhibiting the increase in plasma TNF-α concentration (Figure 5.1A).

In this model of liver injury, plasma TNF-α concentration is elevated before the onset of HPC injury, raising the possibility that this cytokine might have a causal role in injury development (Table 5.2). Either inhibition of TNF-α synthesis by PTX (Figures 5.2B and 5.2C) or neutralization of TNF-α activity by ATS (Figures 5.3B and 5.3C) resulted in the attenuation of HPC injury. Both PTX and ATS decreased plasma TNF-α concentration in MCT/LPS cotreated animals, confirming that the agents were effective in reducing TNF-α (Figures 5.2A and

5.3A). These results suggest that TNF-α is causally involved in the hepatocellular injury.

In rats treated with a small, noninjurious dose of LPS 4 hours after a small, nontoxic dose of MCT, morphologic evidence of liver lesions develop within 12-18 hours after MCT administration, consistent with elevated plasma ALT and AST activities (Yee *et al.*, 2000b). Both centrilobular and midzonal liver lesions result and are qualitatively similar to lesions that characterize larger, toxic doses of MCT and LPS, respectively. Centrilobular liver lesions comprise moderate to marked hepatocellular apoptotic and oncotic necrosis, degeneration, congestion, hemorrhage and vascular injury. Midzonal lesions consist of frequent, well-defined areas of marked hepatocellular coagulative necrosis, accompanied by neutrophil infiltration, congestion and hemorrhage (Yee *et al.*, 2000b). SEC injury is apparent in both lesions (Yee *et al.*, 2002). The observation that both MCT-like and LPS-like lesions develop suggests that each agent enhances the effect of the other in this model (Yee *et al.*, 2000b).

Administration of $GdCl_3$, PTX or ATS to MCT/LPS-treated animals reduced the size and frequency of liver lesions. Morphometric analysis of these lesions demonstrated that Kupffer cell inactivation or TNF-α depletion in MCT/LPS-cotreated animals significantly reduced the area of the centrilobular, MCT-like and midzonal, LPS-like lesions (Table 5.1). This suggests that pharmacologic manipulation ameliorated the interactive effect of these agents.

In this model of synergistic liver injury, LPS caused an expected, early rise in plasma TNF-α concentration. As seen previously (Barton *et al.*, 2001), this

increase was transient in rats treated with LPS alone. Unexpectedly, however, plasma TNF-α concentration remained significantly elevated for at least 12 hours in rats treated with MCT/LPS (Table 5.2). This prolonged elevation contrasts with another model of LPS potentiation of hepatotoxicity in which the LPS-induced increase was not prolonged by aflatoxin B_1 cotreatment (Barton *et al.*, 2001). Even at a larger, hepatotoxic dose of LPS, the elevation in plasma TNF-α concentration remains transient (Iimuro *et al.,* 1994; Pearson, *et al.*, 1996a). It is noteworthy that MCT given by itself to rats, either at the nontoxic dose used in this study (Table 5.2) or at a larger, hepatotoxic dose (300 mg/kg; unpublished observation), does not increase plasma TNF-α concentration. Thus, the interaction of these two agents is required for this prolonged effect in MCT/LPS treated rats.

Under normal conditions, approximately 90% of HA circulating in the blood is removed and degraded by SECs in the liver (Kobayashi *et al.*, 1999). SEC injury impairs the ability of these cells to clear HA from the circulation, and this results in increased plasma HA concentration. Such increases have been used as a biomarker of SEC injury *in vivo* after exposure of animals to toxicants. An increase in plasma HA concentration correlates with histopathologic evidence of SEC injury and destruction in rats given an hepatotoxic dose of either MCT (Copple *et al.*, 2002a) or LPS (Deaciuc *et al.,* 1994; Spapen *et al.*, 1999). Similarly, MCT/LPS-coexposure results in increased plasma HA concentration in rats, and this correlates with SEC injury (Yee *et al.*, 2002). The elevation in plasma HA concentration was slightly but significantly smaller after

coadministration of $GdCl_3$ or ATS to MCT/LPS-treated animals (Figures 5.1D and 5.3D). Administration of PTX to MCT/LPS-treated animals resulted in a trend toward a decrease that was not statistically significant (Figure 5.2D). These results suggest that KC inactivation or TNF-α neutralization in MCT/LPS-treated animals significantly but incompletely attenuated SEC injury.

The attenuation of HPC and SEC injury in this model was likely not the result of $GdCl_3$, PTX or ATS administration interfering with MCT bioactivation. In animals given a large, hepatotoxic dose of MCT (300 mg/kg) neither $GdCl_3$ nor PTX pretreatment of rats altered liver injury (unpublished observation), suggesting that these agents do not interfere with MCT bioactivation. Badger *et al.* (1997) demonstrated that $GdCl_3$ pretreatment of rats caused a modest decrease in hepatic cytochrome P450 (CYP); however, this reduction could be explained by a decrease in the CYP 2E1 isoform (Badger *et al.*, 1997), and since MCT is bioactivated by a different isoform (i.e., CYP 3A family) (Kasahara *et al.*, 1997), it is unlikely that $GdCl_3$ reduced injury in this model by decreasing CYP concentration. In the present study, PTX and ATS were given to MCT/LPS-cotreated animals at a time when most of the administered MCT has been metabolized (Allen *et al.*, 1972). Neither $GdCl_3$ (Mimura *et al.*, 1995; Vollmar *et al.,* 1996b) nor PTX (Heller *et al.*, 1999) interferes with LPS clearance. Accordingly, it is unlikely that the pharmacological agents used in this study reduced injury by interfering with MCT bioactivation or affecting LPS metabolism.

Rose *et al.* (2001) demonstrated that $GdCl_3$ caused a transient activation of NFκB and enhanced hepatocellular proliferation in the liver. However, NFκB

activation returned to baseline within 24 hours after $GdCl_3$ administration; therefore, it is unlikely that NFκB activation influenced the results in this study, since MCT/LPS-cotreatment commenced at this time. The possibility that $GdCl_3$ had effects independent of its ability to inactivate Kupffer cells cannot be excluded; however a similar degree of hepatotoxicity from a large, toxic dose of MCT (300 mg/kg) was unaffected by $GdCl_3$ (unpublished observation), suggesting that it is unlikely to protect through nonselective modes of action such as enhancing cell proliferation.

TNF-α can promote liver injury in a number of ways. For example, *in vitro* TNF-α renders hepatocytes more susceptible to toxicity (Adamson and Billings, 1992; ElSisi *et al.*, 1993; Hoek and Pastorino, 2002). Likewise, HPCs altered homeostatically by the actions of hepatotoxicants may become more sensitive to TNF-α-induced cell killing (Lawson *et al.*, 1998; Jaeschke *et al.*, 1998). In addition, TNF-α can prime PMNs to release toxic products (i.e., ROS and proteases) that can damage nearby cells (Nagaki *et al.*, 1991; Vasselli, 1992; Kushimoto *et al.,* 1996). Further study will be required to understand how TNF-α acts to promote hepatotoxicity in this model.

In addition to TNF-α, other inflammatory mediators are released by KCs that may have deleterious effects on liver (Holst *et al.*, 1996). For example, COX-2 products mediate liver injury in other models. NS-398, a selective COX-2 inhibitor, was administered to rats using a treatment protocol that inhibits COX-2 *in vivo* and affords protection from liver injury in a PG-dependent model (Ganey *et al.*, 2001). The increases in plasma ALT and AST activities in MCT/LPS-

cotreated animals were not attenuated by NS-398, which suggests that COX-2 products are not needed for HPC injury in this model (Figure 4A and 4B). A similar result was observed in a model of LPS-potentiated aflatoxin B_1 hepatotoxicity (Barton *et al.*, 2001); in contrast, a significant protective effect occurred in rats treated with LPS and allyl alcohol (Ganey *et al.*, 2001). These contrasting results suggest that the critical mediators of LPS-potentiated hepatotoxic responses differ with different hepatotoxicants. Interestingly, inhibition of COX-2 in the MCT/LPS-cotreatment model resulted in a trend toward an increase in toxicity, which would suggest that COX-2 products (e.g., PGE_2) might have a protective effect. Further study will be required to elucidate this finding.

In summary, $GdCl_3$ administered to MCT/LPS-treated rats at a dose that inhibits KC function reduced HPC and SEC injury. Moreover, the administration of TNF-α depleting agents to MCT/LPS-cotreated rats also protected against HPC injury and caused a modest attenuation of SEC injury. Accordingly, KCs and TNF-α appear to play important roles in the synergistic hepatotoxicity from MCT/LPS exposure in rats.

CHAPTER 6

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

6.A. Abstract

Synergistic liver injury develops in Sprague-Dawley rats from administration of a small, noninjurious dose of bacterial lipopolysaccharide (LPS; 7.4 x 10^6 EU/kg) given 4 hours after a nontoxic dose of the pyrrolizidine alkaloid, monocrotaline (MCT; 100 mg/kg). Previous studies demonstrated that liver injury is mediated through inflammatory factors, such as Kupffer cells and tumor necrosis factor (TNF)-α, rather than through simple interaction between MCT and LPS. In the present study, the hypothesis that neutrophils (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 hours. PMN depletion did not, however, protect against early SEC injury that occurred before the onset of HPC injury at 6 hours. 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.

6.B. Introduction

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 (Mavier *et al.,* 1988; Hewett *et al.*, 1992; Ganey *et al.,* 1994; Sakamoto *et al.*, 1997; Ohtsuka *et al.*, 2000).

The present study was designed to investigate the role of PMNs in this model of inflammation-enhanced hepatotoxicity. The timecourses for appearance of chemoattractant in plasma and hepatic PMN accumulation and localization were examined. 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.*

6.C. Materials and Methods

6.C.1. 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 x 10^6 endotoxin units (EUs)/mg), magnesium sulfate,

mercuric chloride, pentoxifylline (PTX), phosphate buffered saline (PBS), pyrrole, sodium citrate and sodium bicarbonate 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-rat 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). Mouse anti-rat endothelial cell antigen (RECA)-1 was purchased from Serotec, Inc. (Raleigh, NC). Goat serum was obtained from Vector Laboratories (Burlingame, CA). Goat anti-mouse 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 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 acquired 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).

6.C.2. 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. Otherwise, for more information about this section, refer to Chapter 2 Materials and Methods.

6.C.3. Treatment Protocol

For more information about this section, refer to Chapter 2 Materials and Methods.

6.C.4. Neutrophil Depletion Protocol

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

6.C.5. Assessment of Hepatic Injury and Plasma TNF-α and CINC-1

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 (Deaciuc *et al.*, 1993b, 1994; Copple *et al.*, 2002a). 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. Otherwise, for more information about this section, refer to Chapter 4 Materials and Methods.

6.C.6. Ehrlich Assay

Pyrrole was used to generate the standard curve. Otherwise, for more information about this section, refer to Chapter 2 Materials and Methods.

6.C.7. Histopathologic Evaluation and Morphometry

For more information about histopathologic evaluation, refer to Chapter 4 Materials and Methods. For more information about morphometry, refer to Chapter 5 Materials and Methods.

6.C.8. 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 anti-rabbit IgG, avidin-conjugated alkaline

phosphatase, and Vector Red substrate to stain PMNs. [PMN immunostaining was conducted by the Histotechnology Center, Division of Human Pathology, Michigan State University]. Hepatic PMN accumulation was assessed by averaging the numbers of PMNs enumerated in 30 randomly selected, high power fields (HPFs; 400X) 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 unbiasly 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 timecourse 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 $GdCl_3$/kg was administered to rats 24 hours before LPS administration. In the TNF-α depletion studies, either PTX (100 mg/kg) or ATS (1 ml/rat), were administered 1 hour 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.*, 2003a).

6.C.9. PMN Distribution

Hepatic PMN distribution was assessed in MCT/LPS-treated animals 6 hours after MCT administration. PMNs were enumerated in 15 randomly

selected, low power fields (LPFs; 100X) 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.

6.C.10. Immunohistochemistry

For more information about RECA-1 immunohistochemistry, refer to Chapter 4 Materials and Methods.

6.C.11. Quantification of Liver Endothelial Cells

For more information about this section, refer to Chapter 4 Materials and Methods.

6.C.12. PMN Isolation and Treatment Protocol

PMNs were isolated from the peritoneal cavity of retired breeder rats by glycogen elicitation as described by Ganey *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 $CaCl_2$, 0.68 mM $MgSO_4$ and 14 mM $NaHCO_3$. The final concentration of cells in

all studies was 2.5 X 10^6 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.

6.C.13. 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 µl of phosphate buffer (pH 6.0) containing 0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide was added to 10 µl 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 and 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.

6.C.14. Statistical Analysis

Results are expressed as means ± S.E.M. 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.

6.D. Results

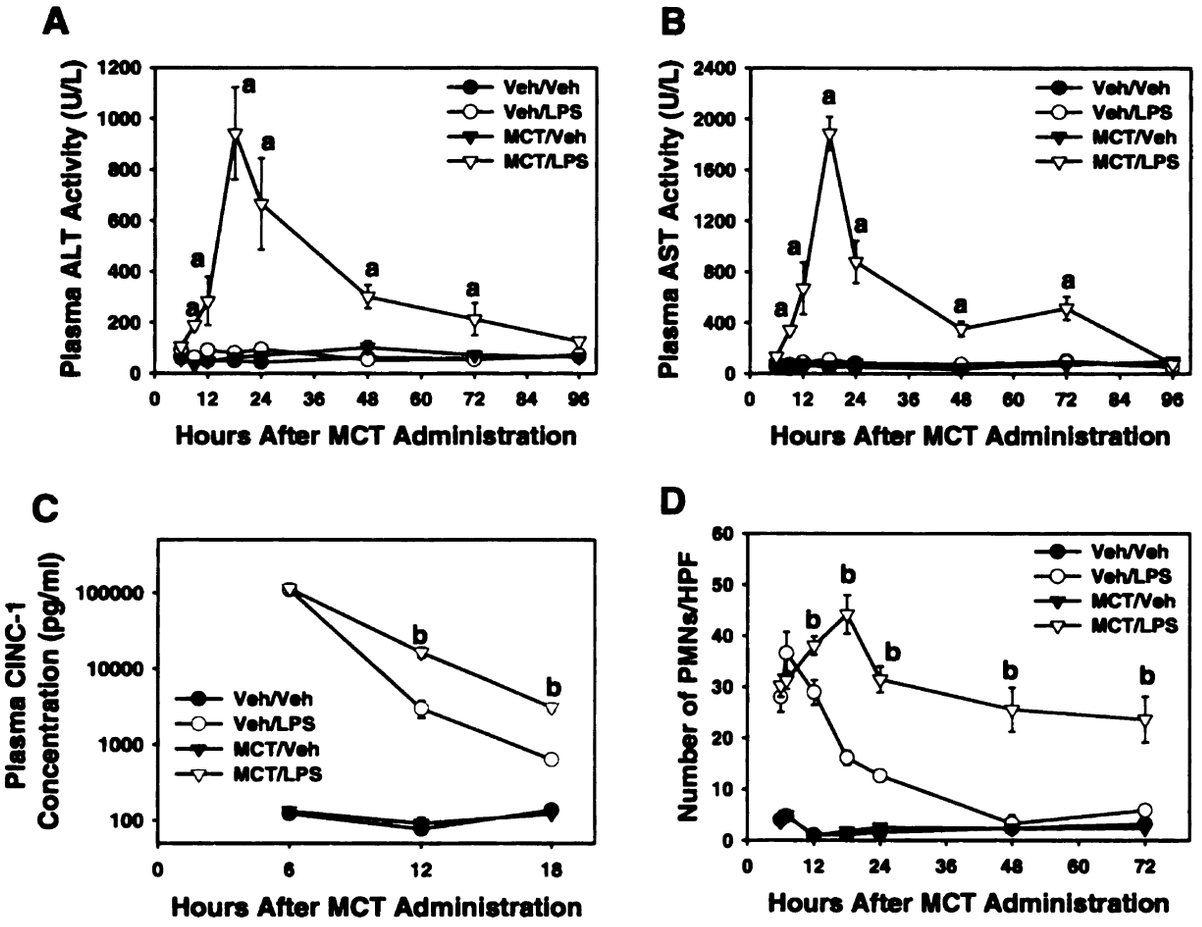
6.D.1. MCT/LPS-Cotreatment Time Course

Synergistic liver injury from a small, noninjurious dose of LPS (7.4×10^6 EU/kg) administered 4 hours after a small, nontoxic dose of MCT (100 mg/kg) is maximal by 18 hours 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 hours and continued to be significant until 72 hours after MCT administration (Figures. 6.1A and 6.1B). By 96 hours, 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 hours and were still apparent by 72 hours. During this time, coagulative necrotic subserosal

Figure 6.1. Timecourse of Events after MCT/LPS Exposure. LPS (7.4 x 10^6 EU/kg) or saline vehicle (Veh) was administered i.v. to rats 4 hours after i.p. administration of MCT (100 mg/kg) or saline vehicle. Panels A and B present the timecourse 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. Panels C and D depict timecourse changes in the plasma concentration of the PMN chemoattractant CINC-1 and in hepatic PMN accumulation, respectively. In Panel C, both groups that received LPS were significantly different from those groups that did not at all times evaluated. In Panel D, the group receiving Veh/LPS was significantly different from both groups not receiving LPS at times encompassing 6 to 24 hours, whereas the group receiving MCT/LPS was significantly different from these two groups at all times. N = 4 – 24 animals.

[a] Significantly different from all other groups.

[b] Significant difference between Veh/LPS and MCT/LPS groups.


A
Plasma ALT Activity (U/L)
Hours After MCT Administration
Veh/Veh
Veh/LPS
MCT/Veh
MCT/LPS
B
Plasma AST Activity (U/L)
Hours After MCT Administration
C
Plasma CINC-1 Concentration (pg/ml)
Hours After MCT Administration
D
Number of PMNs/HPF
Hours After MCT Administration

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 oncotic 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 frank lesions were observed at 6 hours, 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 hours after MCT administration, but approximately 20% of rats died by 24 hours, with no deaths thereafter. No animals that received saline vehicles, MCT or LPS alone died.

6.D.2. Plasma CINC-1 Concentration in MCT/LPS-Treated Animals

CINC-1, the rat equivalent to human interleukin-8, is a PMN chemoattractant (Zhang *et al.,* 1995; Luster, 1998). Plasma CINC-1 concentration was assessed at various times after administration of MCT or its vehicle (Veh) (Figure 6.1C). 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 hours 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 hours

plasma CINC-1 concentration was five times as great in MCT/LPS-treated rats (note log scale in Figure 6.1C).

6.D.3. 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 hours in livers from animals treated with either LPS alone or MCT/LPS (Fig 6.1D). PMN numbers in the livers of animals treated with LPS alone had begun to decline by 12 hours and returned to baseline by 48 hours. By contrast, PMN accumulation remained significantly elevated in livers from MCT/LPS-treated animals until the final analysis time of 72 hours.

Hepatic neutrophil distribution at 6 hours was panlobular in livers from animals treated with either LPS alone or MCT/LPS (Figure 6.2). Whereas PMN numbers were elevated at 6 hours throughout all the regions of the liver lobules in MCT/LPS-treated animals, by 9 hours most PMNs were localized within lesions (i.e., centrilobular, and midzonal).

6.D.4. 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 (Figure 6.3B) and practically eliminated the increases in plasma ALT and AST activities caused by MCT/LPS exposure 18 hours after

Figure 6.2. Hepatic PMN Distribution in Rat Livers at 6 Hours. Rats were treated with MCT and/or LPS as described in Figure 6.1 legend. Hepatic PMN distribution in rat livers was determined 6 hours after MCT administration. N = 4 – 6 animals.

[a] Significantly different from both Veh/Veh and MCT/Veh groups in same lobular region.

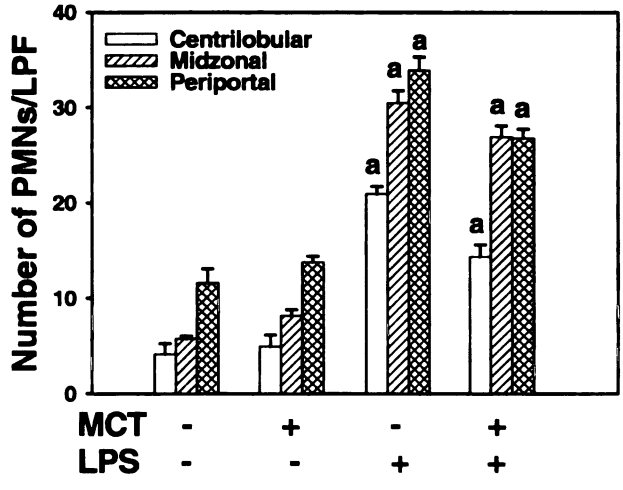
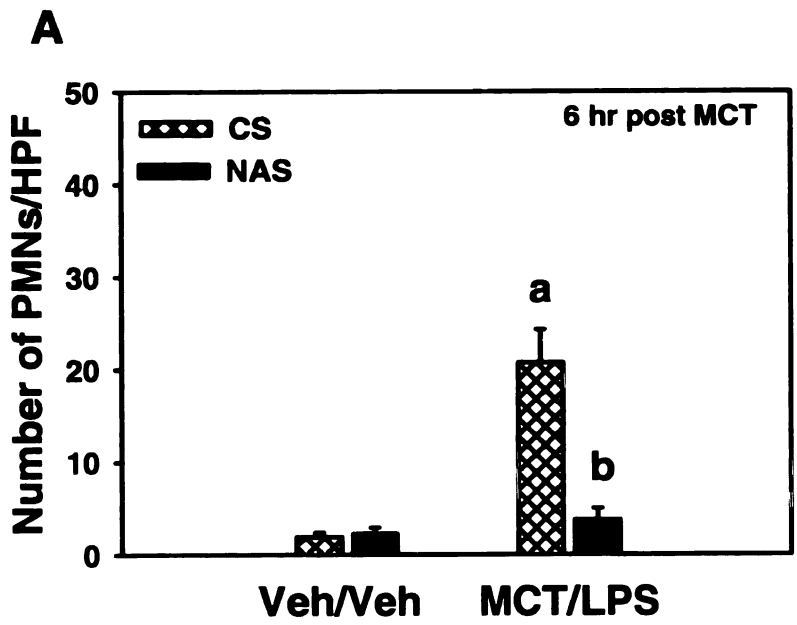
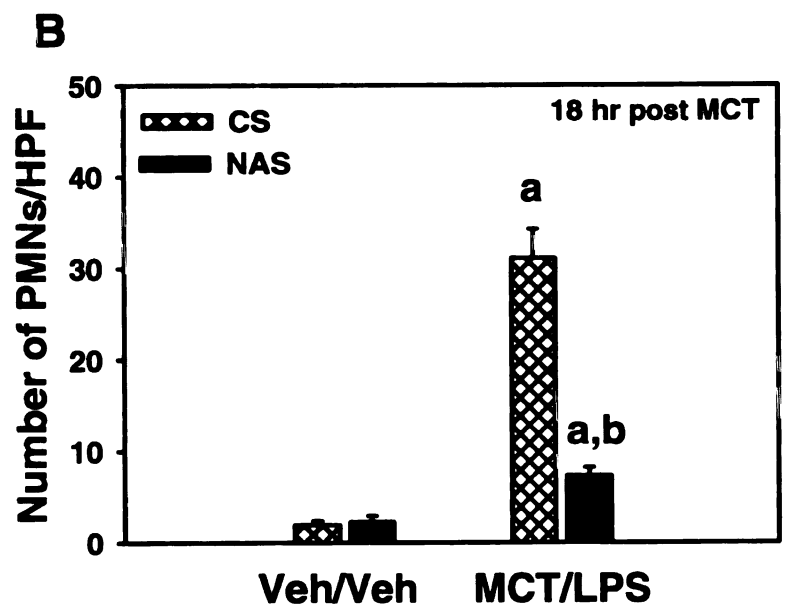

Number of PMNs/LPF
40
30
20
10
0
Centrilobular
Midzonal
Periportal
a
a
a
a
a
a
MCT - + - +
LPS - - + +

Figure 6.3. Hepatic PMN Accumulation after PMN Depletion in Animals Exposed to MCT and LPS. Neutrophil anti-serum (NAS) or control (nonimmune) serum (CS) was given i.p. to rats 24 and 8 hours before LPS. Rats were treated with MCT and/or LPS as described in Figure 6.1 legend. Panels A and B present hepatic PMN accumulation at 6 and 18 hours after MCT administration, respectively. Hepatic PMN accumulation was evaluated to determine the effectiveness of PMN depletion by NAS. N = 4 – 13 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from CS/MCT/LPS group.


A
Number of PMNs/HPF
50
40
30
20
10
0
CS
NAS
6 hr post MCT
a
b
Veh/Veh
MCT/LPS


B
Number of PMNs/HPF
50
40
30
20
10
0
CS
NAS
18 hr post MCT
a
a,b
Veh/Veh
MCT/LPS

MCT administration (Figures 6.4A and 6.4B). 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 6.1). 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 6.2).

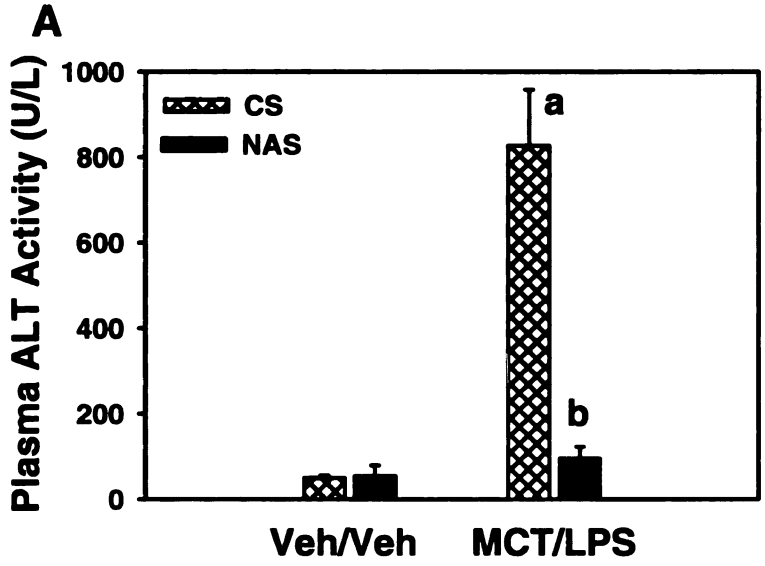
6.D.5. Effect of PMN Depletion on SEC injury

Hepatic SEC injury has been quantified though 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.*, 2002a) or LPS (Deacuic *et al.*, 1994; Spapen *et al.*,

Figure 6.4. Effect of PMN Depletion on MCT/LPS-Induced HPC Injury. NAS or CS was given to rats 24 and 8 hours before LPS. Rats were treated with MCT and/or LPS as described in Figure 6.1 legend. Panels A and B depict plasma ALT and AST activities as two biomarkers of HPC injury, 18 hours after MCT administration. N = 4 – 13 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from CS/MCT/LPS group.


A
Plasma ALT Activity (U/L)
1000
800
600
400
200
0
CS
NAS
a
b
Veh/Veh
MCT/LPS

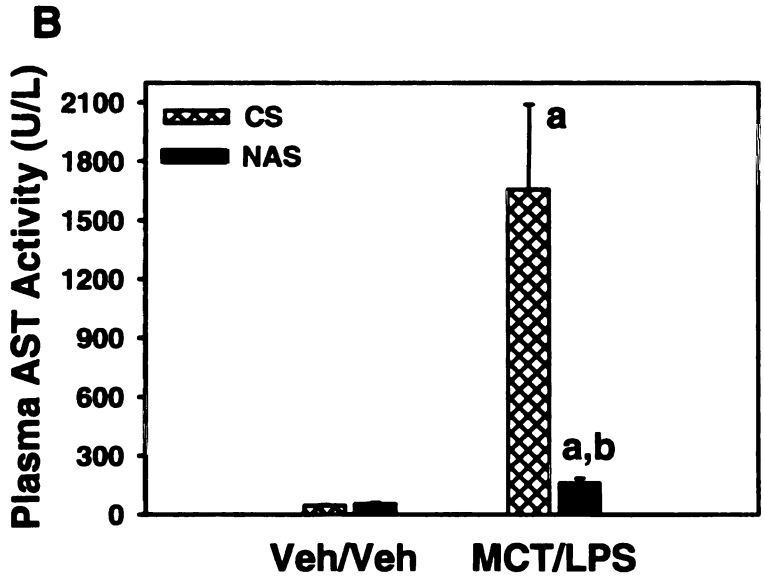

B
Plasma AST Activity (U/L)
2100
1800
1500
1200
900
600
300
0
CS
NAS
a
a,b
Veh/Veh
MCT/LPS

Table 6.1. PMN Depletion Reduces Liver Lesions in MCT/LPS-Cotreated Rats. Neutrophil anti-serum (NAS) or control (nonimmune) serum (CS) was given i.p. to rats 24 and 8 hours before LPS. LPS (7.4 x 10^6 EU/kg) was administered i.v. to rats 4 hours after i.p. administration of MCT (100 mg/kg). Livers were taken 18 hours after MCT administration and processed for morphometric analysis. N = 8 animals.

[a] Significantly different from CS/MCT/LPS group.

Treatment	Percent Lesion Area	
	Centrilobular	Midzonal
CS/MCT/LPS	2.3 ± 0.3	8.2 ± 1.6
NAS/MCT/LPS	0.4 ± 0.1[a]	1.0 ± 0.3[a]

Table 6.2. NAS does not Influence MCT Bioactivation. NAS or CS was given i.p. to rats 24 and 8 hours before LPS. Rats were treated with MCT and/or LPS as described in Table 6.1 legend. Liver samples were taken 18 hours 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.

Treatment	Pyrrole (μg/g liver)
CS/MCT/LPS	6.3 ± 0.4
NAS/MCT/LPS	6.4 ± 0.2

1999). In MCT/LPS-treated animals SEC injury occurs before the onset of HPC injury (Yee *et al.*, 2002).

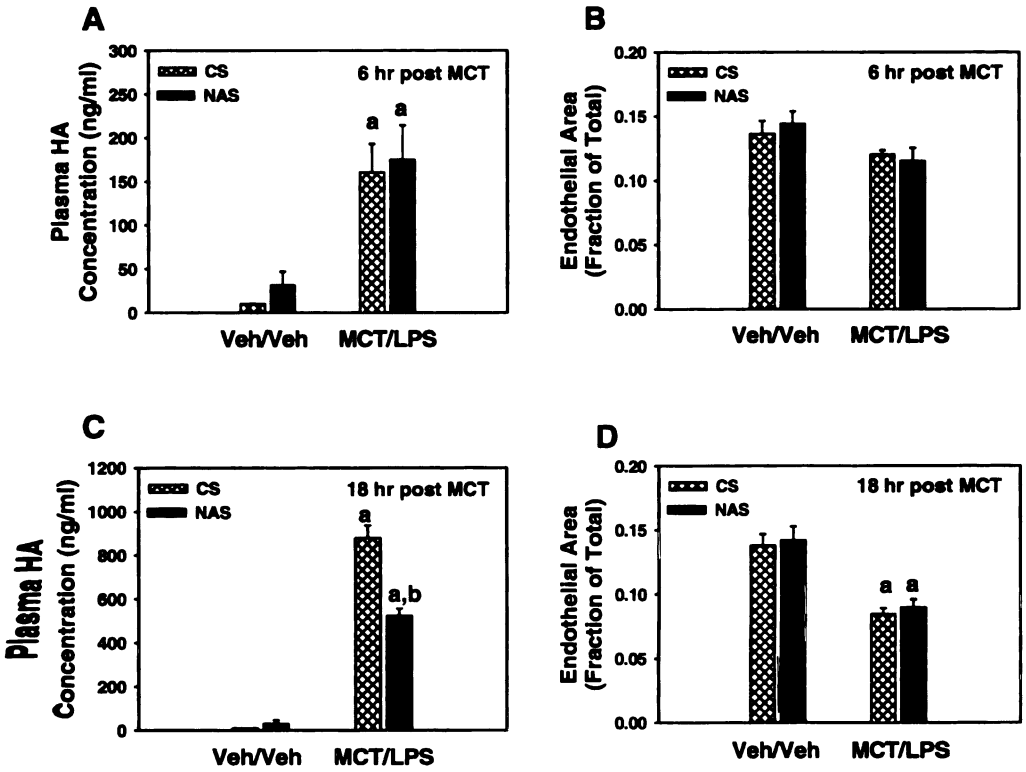
To define the importance of PMNs in SEC injury in MCT/LPS-coexposed animals, rats were pretreated with NAS, and injury was assessed 6 hours after MCT administration (i.e., before the onset of hepatocellular injury). NAS pretreatment eliminated hepatic PMN accumulation in MCT/LPS-cotreated animals (Figure 6.3A). By 6 hours, plasma HA concentration was increased in MCT/LPS cotreated animals; however, NAS pretreatment failed to affect this increase (Figure 6.5A). No mortality occurred in control or cotreated animals at this time. At 18 hours, the increase in plasma HA concentration persisted in MCT/LPS-cotreated animals, and the increase remained in NAS pretreated rats but was less pronounced (Figure 6.5C).

To confirm the effect of PMN depletion on SEC injury, endothelial cells were stained immunhistochemically 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 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 hours (Figure 6.5B); however, it decreased significantly by 18 hours (Figure 6.5D). 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

Figure 6.5. Effect of PMN Depletion on MCT/LPS-Induced SEC Injury. NAS or CS was given to rats 24 and 8 hours before LPS. Rats were treated with MCT and/or LPS as described in Figure 6.1 legend. Panels 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 hours after MCT administration, respectively. Panels C and D present the same markers 18 hours after MCT administration. Note ordinal scale differences in Panel 5A and Panel 5C. N = 3 – 6 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from CS/MCT/LPS group.


A
Plasma HA Concentration (ng/ml)
300
250
200
150
100
50
0
CS
NAS
6 hr post MCT
a
a
Veh/Veh
MCT/LPS
B
Endothelial Area (Fraction of Total)
0.20
0.15
0.10
0.05
0.00
CS
NAS
6 hr post MCT
Veh/Veh
MCT/LPS
C
Plasma HA Concentration (ng/ml)
1200
1000
800
600
400
200
0
CS
NAS
18 hr post MCT
a
a,b
Veh/Veh
MCT/LPS
D
Endothelial Area (Fraction of Total)
0.20
0.15
0.10
0.05
0.00
CS
NAS
18 hr post MCT
a
a
Veh/Veh
MCT/LPS

hours. No effect of NAS pretreatment on liver zonal distribution (i.e., centrilobular, midzonal, or periportal) of RECA-1 staining intensity was observed in MCT/LPS-cotreated animals at 6 or 18 hours (data not shown).

6.D.6. Relationship Between TNF-α and PMNs in MCT/LPS-Treated Rats

LPS can activate KCs to release inflammatory cytokines such as TNF-α (Michie *et al.*, 1988; Hewett and Roth, 1993). In an earlier study (Yee *et al.*, 2003a), KC depletion by $GdCl_3$ 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 hours (Figures 6.6A and 6.6B).

6.D.7. Effect of MCT on PMNs In Vitro

[This series of experiments was conducted by Umesh M. Hanumegowda.] 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 (Figure 6.7A) from PMNs without a concurrent increase in LDH release (Figure 6.7B).

Table 6.3. $GdCl_3$, PTX or ATS Reduce Hepatic PMN Accumulation in MCT/LPS-Treated Rats. Rats were treated with MCT and/or LPS as described in Table 6.1 legend. In addition, rats were treated with $GdCl_3$ (KC inactivator), PTX (inhibitor of TNF-α synthesis) or ATS (inactivator of TNF-α) or their vehicles as described in Materials and Methods. PMNs were counted in immunohistochemically stained sections of liver removed from rats 18 hours after MCT treatment. N = 4 – 13 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from the respective value in the absence of pharmacological treatment (i.e., $GdCl_3$, PTX or ATS).

Treatment	PMNs per HPF
$GdCl_3$ Study	
Veh/Veh/Veh	2.2 ± 0.4
$GdCl_3$/Veh/Veh	2.2 ± 0.2
Veh/MCT/LPS	30.1 ± 3.1[a]
$GdCl_3$/MCT/LPS	18.3 ± 1.0[a,b]
PTX Study	
Veh/Veh/Veh	2.1 ± 0.3
Veh/PTX/Veh	2.0 ± 0.4
MCT/Veh/LPS	37.0 ± 2.1[a]
MCT/PTX/LPS	17.2 ± 1.5[a,b]
ATS Study	
Veh/CS/Veh	2.3 ± 0.4
Veh/ATS/Veh	2.1 ± 0.2
MCT/CS/LPS	53.1 ± 11.7[a]
MCT/ATS/LPS	22.1 ± 3.2[a,b]

Figure 6.6. Effect of PMN Depletion on TNF-α Concentration in MCT/LPS-Cotreated Animals. NAS or CS was given to rats 24 and 8 hours before LPS. Rats were treated with MCT and/or LPS as described in Figure 6.1 legend. Panels A and B present plasma TNF-α concentration at 6 and 18 hours after MCT administration, respectively. Note the ordinal scale differences in these panels. N = 3 – 13 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

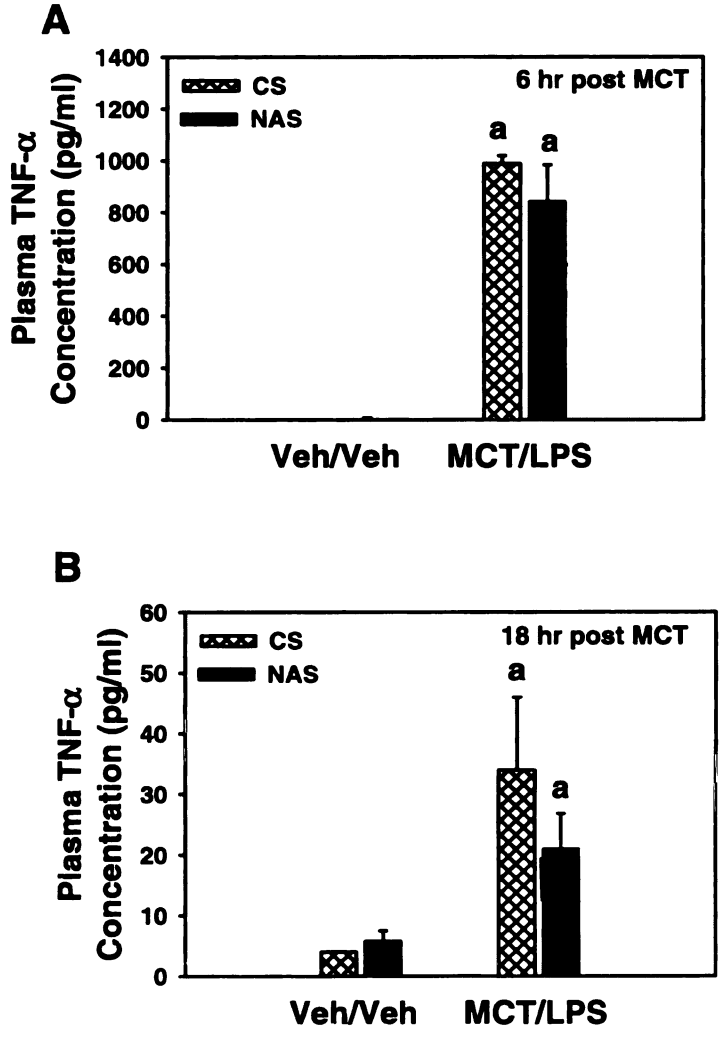
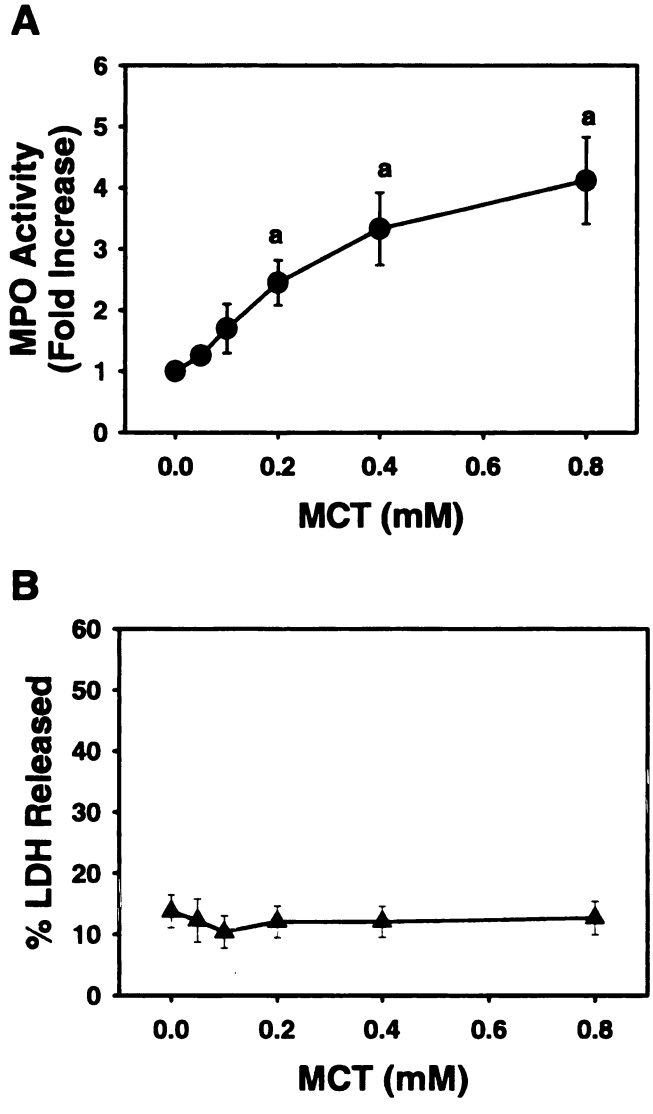

A
Plasma TNF-α Concentration (pg/ml)
1400
1200
1000
800
600
400
200
0
CS
NAS
6 hr post MCT
a
a
Veh/Veh
MCT/LPS
B
Plasma TNF-α Concentration (pg/ml)
60
50
40
30
20
10
0
CS
NAS
18 hr post MCT
a
a
Veh/Veh
MCT/LPS

Figure 6.7. MCT Activates PMNs *In Vitro*. PMNs were incubated with various concentrations of MCT (0 to 0.8 mM) for 30 min at 37°C. Panel A shows MPO activity released into the incubation medium, a marker of PMN degranulation. MPO activity is presented as fold increase over vehicle control (0 mM MCT: 1.2 ± 0.1 mOD/min). Panel B depicts LDH release in the cell-free supernatant. Data are expressed as mean ± SEM. N = 5 – 6 animals.

[a] Significantly different from vehicle control.

[The work in this figure was conducted by Umesh M. Hanumegowda].


A
MPO Activity
(Fold Increase)
6
5
4
3
2
1
0
a
a
a
0.0
0.2
0.4
0.6
0.8
MCT (mM)
B
% LDH Released
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
MCT (mM)

6.E. Discussion

Previously, Yee *et al.* (2000b) reported that synergistic liver injury develops within 18 hours after MCT administration in a model in which a small, noninjurious dose of LPS (7.4 x 10^6 EU/kg) is administered 4 hours 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 hours and continues to be significant until 72 hours after MCT administration (Figures 6.1A and 6.1B). Hepatocellular injury was maximal by 18 hours as marked by increases in plasma ALT and AST activities.

No HPC injury was observed in MCT/LPS-treated animals at 6 hours. 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 (Figures 6.1C, 6.1D and 6.6A, respectively). In animals given LPS alone, the rise in hepatic PMN numbers gradually decreased to vehicle control levels by 48 hours, and liver injury did not develop. In contrast, in MCT/LPS-cotreated animals the increased number of hepatic PMNs was sustained through 72 hours, 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.*, 2000a).

NAS effectiveness was confirmed by the marked reduction of hepatic PMN accumulation in MCT/LPS-cotreated animals 18 hours after MCT administration (Figure 6.3B). NAS pretreatment resulted in pronounced attenuation of the increase in plasma ALT and AST activities (Figures 6.4A and 6.4B), suggesting that PMNs play a causal role in the hepatocellular injury.

The distribution of PMNs at 6 hours was panlobular in livers of LPS-treated animals. No zonal differences in PMN distribution in LPS-treated animals were observed, regardless of MCT administration (Figure 6.2). 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 animals did not affect the qualitative nature of the lesions. However, both centrilobular and midzonal lesion size and frequency were greatly attenuated (Table 6.1). 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 6.2). 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.*, 2002a) 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.

Increased plasma HA concentration was observed by 6 hours, well before the onset of HPC injury, and became more pronounced by 18 hours (Figures 6.5A and 6.5C, respectively). To determine if PMNs were responsible for this early injury to SECs, MCT/LPS-cotreated animals were examined at 6 hours.

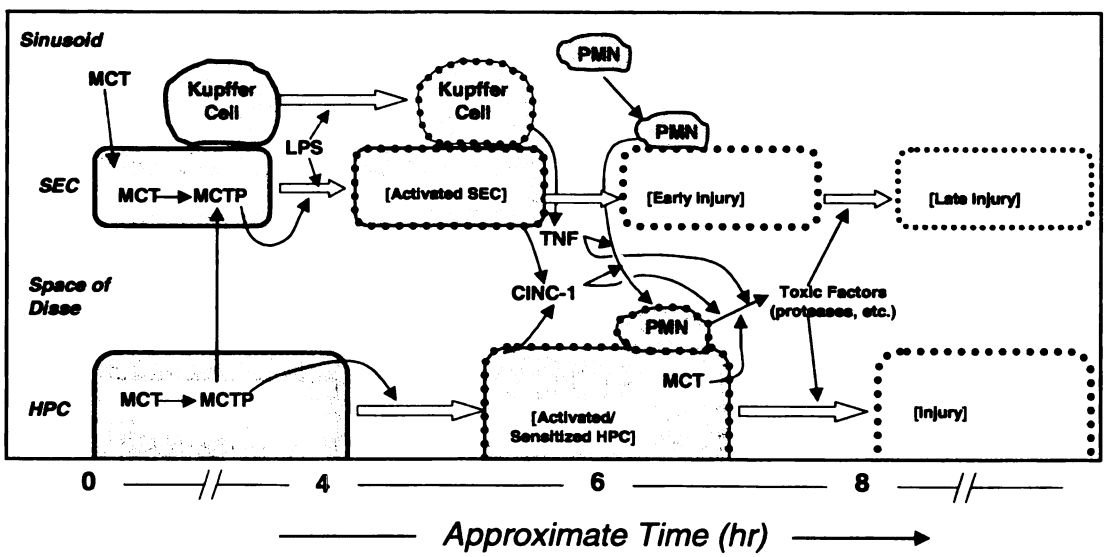
NAS pretreatment of these animals significantly reduced hepatic PMN accumulation (Figure 6.3A) but did not affect the elevation in plasma HA concentration at this time (Figure 6.5A). RECA-1 immunohistochemical staining was not able to detect the subtle changes in SECs 6 hours after MCT/LPS cotreatment (Figure 6.5B). This lesser sensitivity of RECA-1 as a biomarker of SEC injury compared to plasma HA concentration has been observed previously (Copple *et al.*, 2002a). The inability of NAS pretreatment to reduce the increase in plasma HA concentration at 6 hours 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* (Sakamoto *et al.*, 1997; Ohtsuka *et al.*, 2000). NAS pretreatment of MCT/LPS-cotreated animals resulted in a small but significant attenuation of plasma HA concentration at 18 hours (Figure 6.5C). Immunohistochemical staining confirmed endothelial cell injury in MCT/LPS-cotreated animals as a decrease in RECA-1 staining in NAS-treated animals (Figure 6.5D). 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 hours in MCT/LPS-cotreated animals which 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 the pathogenesis occurs independently of PMNs, but that PMNs contribute to the progression of SEC injury at later times (Figure 6.8).

The cause of the early injury to SECs in MCT/LPS-treated rats is not understood. Although SECs express cytochromes P450 (Lester *et al.*, 1993; DeLeve *et al.*, 1997), 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* (unpublished 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 (Figure 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.

Figure 6.8. Putative Sequence of Events in the Pathogenesis of Synergistic Liver Injury from MCT/LPS Coexposure. MCT is bioactivated to its toxic metabolite (MCTP) by HPCs and SECs. At a subthreshold MCT dose, MCTP induces a homeostatic change in HPCs and SECs but not frank injury; however, the change makes these cells more susceptible to injury. LPS acts in concert with MCTP to cause early SEC injury by an unknown mechanism that is independent of PMNs. Circulating LPS also activates KCs to release TNF-α and perhaps CINC-1 (not shown). Activated SECs and HPCs produce CINC-1 and perhaps other factors (not shown) that work in combination with TNF-α to enhance migration of PMNs into parenchyma and promote their activation. MCT itself may contribute to PMN activation, which results in the release of proteases and possibly other cytotoxic factors that damage HPCs and cause additional injury to SECs. [Image in this dissertation is presented in color].


Sinusoid
MCT
Kupffer Cell
Kupffer Cell
PMN
PMN
LPS
SEC
MCT→MCTP
[Activated SEC]
[Early Injury]
[Late Injury]
TNF
Space of Disse
CINC-1
Toxic Factors (proteases, etc.)
PMN
MCT
HPC
MCT→MCTP
[Activated/ Sensitized HPC]
[Injury]
0
4
6
8
Approximate Time (hr)

To understand better the interdependence between PMNs and other inflammatory factors, the effect of PMN depletion on plasma TNF-α was studied. 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 hours was unaffected by PMN depletion (Figures 6.6A and 6.6B, 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 $GdCl_3$, a KC inactivator, decreased hepatic PMN accumulation (Table 6.3). This result suggests that TNF-α released by activated KCs contributes to PMN accumulation and/or retention in liver (Figure 6.8). 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 B_1 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, $GdCl_3$ treatment resulted in a decrease in PMN infiltration (Mosher *et al.*, 2001). In the present study, the effects of TNF-α depletors on hepatic PMN accumulation were measured at 18 hours (Table 6.3). 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 hours) in the other models noted above, whereas a prolonged increase in TNF-α occurs in this model (Figure 6.6B). 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 (Figure 6.1D).

TNF-α might be involved in promoting hepatic PMN accumulation/retention in this model by activating SECs (Vasselli, 1992; Bradham *et al.* 1998; Sakamoto *et al.*, 2002). 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 (Figure 6.8). It should be noted, however, that the biologic significance of the prolonged but small increase in plasma TNF-α (Figure 6.6B) 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 (Zhang *et al.,* 1995; Luster, 1998), was significantly elevated in plasma 2 hours after LPS administration, regardless of MCT treatment (Figure 6.1C). This result suggests

that CINC-1 contributes to the recruitment of hepatic PMNs (Figure 6.8). Plasma CINC-1 concentration declined after 2 hours 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 (Deutschman *et al.*, 1996; Ohkubo *et al.*, 1998; Calkins *et al.*, 2002). TNF-α has been shown to induce CINC-1 production by hepatocytes *in vitro* (Thorton *et al.*, 1991; Ohkubo *et al.*, 1998). 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.*, 2003a). In addition to its apparent role in PMN accumulation (Table 6.3), this cytokine might promote injury by priming PMNs to release toxic products that can damage nearby cells (Nagaki *et al.*, 1991; Vasselli, 1992; Kushimoto *et al.,* 1996; Figure 6.8). 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 (Figure 6.1D). Indeed, one action of TNF-α might be to render the HPCs more susceptible to injury (Adamson and Billings, 1992;

Bradham *et al.*, 1998; Xu *et al*, 1998; Jones *et al.*, 2000; Hoek and Pastorino, 2002).

MCT was able to induce PMN degranulation *in vitro* at non-toxic concentrations (Figures 6.7A and 6.7B). 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 (Figure 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 sensitivity to other stimuli (Nagaki *et al.*, 1991; Vasselli, 1992; Kushimoto *et al.*, 1996).

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 6.8 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. Coexposure 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; Yee *et al.*, 2003a), this scenario represents an 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.

CHAPTER 7

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

7.A. Abstract

Coexposure to a small, non-injurious dose of bacterial lipopolysaccharide (LPS; 7.4 x 10^6 EU/kg) and a small, 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 (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.

7.B. Introduction

Histopathological examination revealed pronounced congestion, hemorrhage and fibrin deposition in the hepatic lesions of MCT/LPS-cotreated rats (Yee *et al.*, 2000b, 2003b). These results raise the possibility that the coagulation system has a causal role in hepatocellular necrosis in this model (Yee *et al.*, 2003b).

Activation of the coagulation cascade occurs either intrinsically via surface-mediated reactions or extrinsically through a tissue factor (TF)-derived pathway. At the distal end of the cascade, activated factor X converts prothrombin into active thrombin. Thrombin in turn can convert circulating fibrinogen into insoluble fibrin clots (Bloom, 1990; Schultze and Roth, 1998). It has been postulated that fibrin deposition in the liver leads to local sinusoidal hypoperfusion, which might contribute to HPC injury (Deleve *et al.*, 1996; Ba *et al.*, 2000; Saetre *et al.*, 2000; Copple *et al.*, 2002a).

LPS activates the coagulation system primarily through the expression of TF on endothelial cells, monocytes and PMNs (Bone, 1992; Polack *et al.*, 1997; Todoroki *et al.*, 2000). Thrombin has been implicated in the release of proinflammatory cytokines and other factors from macrophages, monocytes and endothelial cells and is a weak PMN chemoattractant (Bizios *et al.*, 1986; Hoffman and Cooper, 1995; Moulin *et al.*, 1996; Holland *et al.*, 1998). Accordingly, the coagulation system might be involved in the upregulation of

proinflammatory mediators, in addition to forming fibrin clots. Indeed, at acutely toxic doses of LPS, an activated coagulation system is critical for liver pathogenesis, and it appears that interplay between thrombin and inflammatory factors is important (Moulin *et al.*, 2001; Copple *et al.*, 2003).

The degree to which the coagulation system has a role in the synergistic injury from the combination of small doses of MCT and LPS has not been explored. Accordingly, the present study was designed to investigate its role in MCT/LPS-induced liver injury and to explore the interdependence between the coagulation system and inflammatory factors that are critical for the toxic response.

7.C. Materials and Methods

7.C.1. Materials

Dimethyl sulfoxide (DMSO), heparin (Type II, disodium salt; HEP), isopentane, LPS (*Escherichia coli,* serotype 0128:B12, 1.7 x 10^6 endotoxin units (EUs)/mg), pentoxifylline (PTX), phosphate buffered saline (PBS), sodium citrate, and warfarin (3-(α-acetonylbenzyl)-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-rat 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 chemoattractant protein-1 (MCP-1) were purchased from Biosource International, Inc. (Camarillo, CA).

7.C.2. Animals

Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR, Charles River, Portage, MI) weighing 200-300 g were used for all studies. Otherwise, for more information about this section, refer to Chapter 2 Materials and Methods.

7.C.3. Treatment Protocol

For more information about this section, refer to Chapter 2 Materials and Methods.

7.C.4. Treatment with HEP

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

7.C.5. Treatment with WARF

Rats were pretreated with WARF (7.5 mg/kg) or an equivalent volume of DMSO Veh intraperitoneally 34 and 10 hours 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 hours after MCT treatment.

7.C.6. Assessment of Hepatic Injury and Plasma TNF-α and MCP-1 Concentrations

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. Otherwise, for more information about this section refer to Chapter 4 Materials and Methods.

7.C.7. Assessment of Plasma Fibrinogen Concentration

Plasma fibrinogen concentration was evaluated with a BBL fibrometer (Becton, Dickson and Company, Hunt Valley, MD) and a fibrinogen diagnostic kit. Plasma fibrinogen concentration was determined in the HEP and WARF 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, $GdCl_3$ (10 mg/kg) was administered to rats 24 hours before LPS administration. In the TNF-α depletion studies, either PTX (100 mg/kg) or ATS (1 ml/rat) was administered 1 hour 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, 2003c). Administration of $GdCl_3$, PTX, ATS, or NAS did not interfere with MCT bioactivation (Yee *et al.*, 2003a, 2003c).

7.C.8. Histopathologic Evaluation and Morphometry

For more information about histopathologic evaluation, refer to Chapter 4 Materials and Methods. For more information about morphometry, refer to Chapter 5 Materials and Methods.

7.C.9. Enumeration of Hepatic Neutrophils

For more information about this section, refer to Chapter 6 Materials and Methods.

7.C.10. Immunohistochemistry

For more information about fibrin deposition immunohistochemistry, refer to Chapter 4 Materials and Methods.

7.C.11. Quantification of Hepatic Fibrin Deposition

For more information about this section, refer to Chapter 4 Materials and Methods.

7.C.12. Statistical Analysis

Results are expressed as mean ± S.E.M. 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.

7.D. Results

7.D.1. 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.*, 2000a), and this decrease was used as a biomarker for activation of the coagulation system and as a monitor of anticoagulant effectiveness.

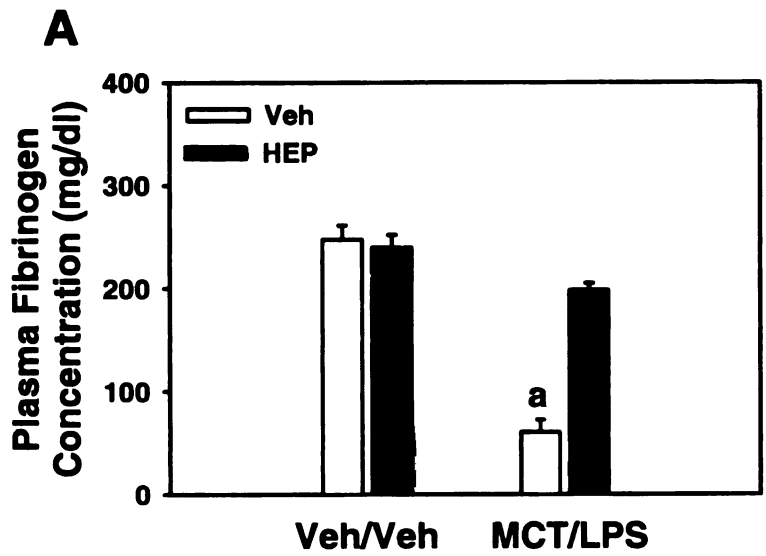
Plasma fibrinogen concentration was significantly decreased in rats treated with MCT/LPS. Both HEP and WARF prevented the activation of the coagulation system in MCT/LPS-coexposed animals (Figures 7.1A and 7.1B, respectively), confirming their effectiveness.

7.D.2. 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 hours after MCT administration (Figures 7.2A and 7.2B, respectively). HEP treatment attenuated the increase in

Figure 7.1. Effect of Anticoagulants on Coagulation System Activation in MCT/LPS-Cotreated Animals. Either HEP or Vehicle (Veh) was administered 1.5 hours after LPS (Panel A), or WARF or Veh was administered 10 and 34 hours before MCT administration (Panel B). LPS (7.4×10^6 EU/kg) or Veh was administered to rats 4 hours after administration of MCT (100 mg/kg) or its Veh. Plasma fibrinogen concentration, a biomarker for coagulation system activation, was determined 18 hours after MCT or its Veh was administered. N = 4 – 14 animals.

[a] Significantly different from all other groups.


A
Plasma Fibrinogen Concentration (mg/dl)
400
300
200
100
0
Veh
HEP
a
Veh/Veh
MCT/LPS

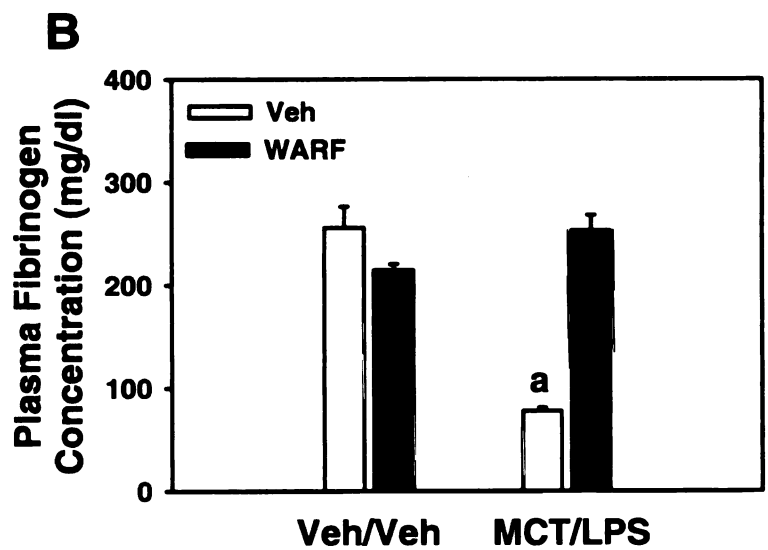
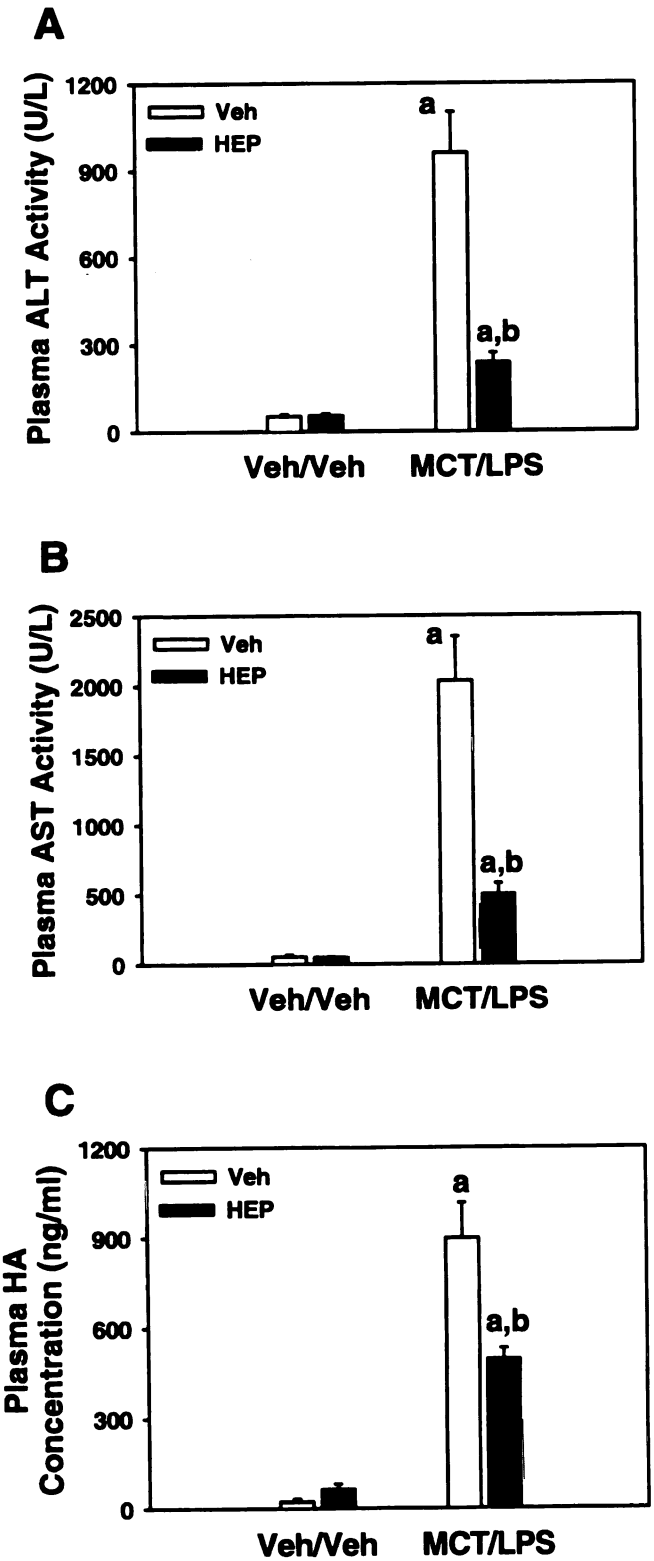

B
Plasma Fibrinogen Concentration (mg/dl)
400
300
200
100
0
Veh
WARF
a
Veh/Veh
MCT/LPS

Figure 7.2. Effect of HEP on MCT/LPS-Induced Liver Injury. MCT/LPS-coexposed rats were treated with HEP anticoagulant as described in Figure 7.1 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 hours after MCT or its Veh. N = 5 – 14 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from MCT/LPS/Veh group.


A
Plasma ALT Activity (U/L)
1200
900
600
300
0
Veh
HEP
a
a,b
Veh/Veh
MCT/LPS
B
Plasma AST Activity (U/L)
2500
2000
1500
1000
500
0
Veh
HEP
a
a,b
Veh/Veh
MCT/LPS
C
Plasma HA Concentration (ng/ml)
1200
900
600
300
0
Veh
HEP
a
a,b
Veh/Veh
MCT/LPS

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 (Deaciuc *et al.*, 1993b, 1994; Copple *et al.*, 2002a). In MCT/LPS-cotreated animals, plasma HA concentration was elevated (Figure 7.2C), and HEP treatment modestly reduced the elevation (48% decrease).

7.D.3. 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 (Figures 7.3A and 7.3B). Plasma HA concentration was also partially attenuated (42% decrease) with WARF treatment (Figure 7.3C).

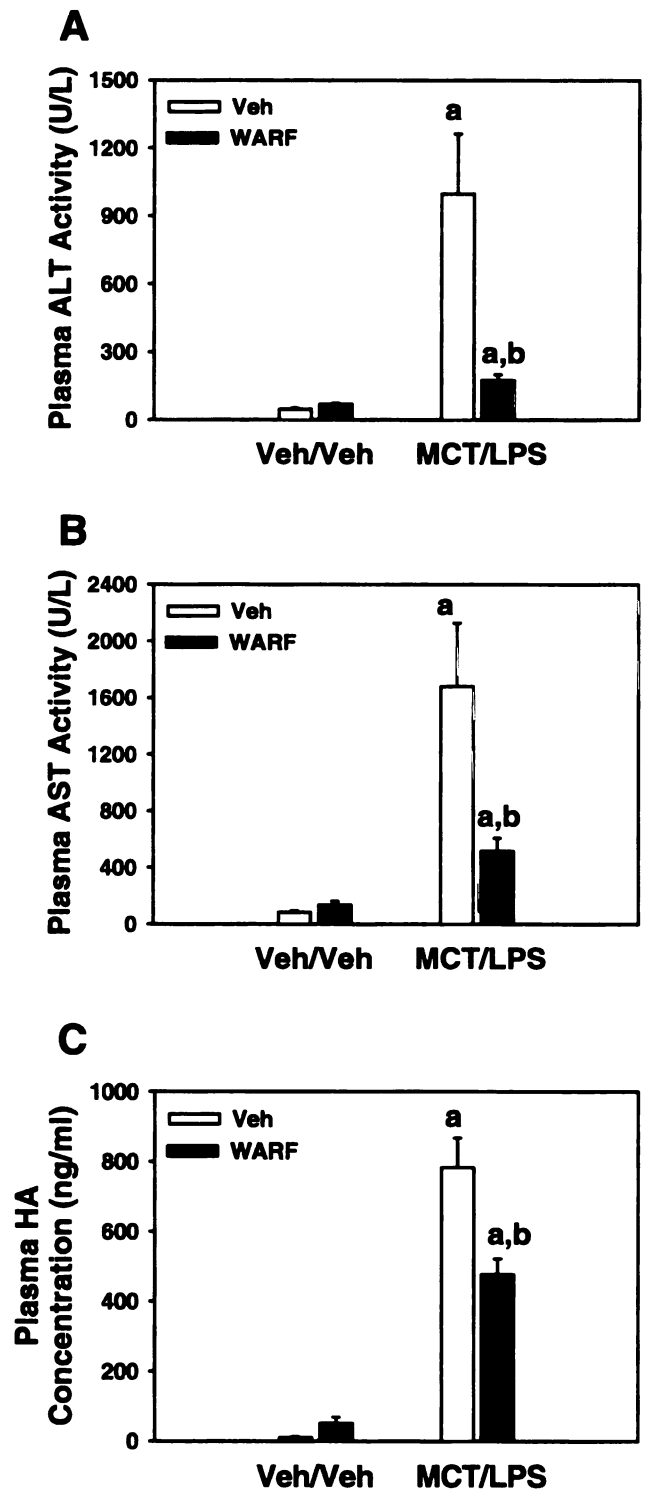
7.D.4. Mortality

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.

Figure 7.3. Effect of WARF on MCT/LPS-Induced Liver Injury. MCT/LPS-coexposed rats were treated with WARF anticoagulant as described in Figure 7.1 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 hours after MCT or its Veh. N = 4 – 8 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from Veh/MCT/LPS group.


A
Plasma ALT Activity (U/L)
1500
1200
900
600
300
0
Veh
WARF
a
a,b
Veh/Veh
MCT/LPS
B
Plasma AST Activity (U/L)
2400
2000
1600
1200
800
400
0
Veh
WARF
a
a,b
Veh/Veh
MCT/LPS
C
Plasma HA Concentration (ng/ml)
1000
800
600
400
200
0
Veh
WARF
a
a,b
Veh/Veh
MCT/LPS

7.D.5. The 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.* (2000b). CL lesions consisted of moderate to marked hepatocellular degeneration and apoptotic and oncotic 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 7.1). Significant decreases in the areas of CL (77% decrease) and MZ (87% decrease) lesions were found in the livers of MCT/LPS-cotreated 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.

7.D.6. 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

Table 7.1. HEP or WARF Administration Reduces Liver Lesions in MCT/LPS-Cotreated Rats. WARF or Vehicle (Veh) was administered 10 and 34 hours before MCT administration, whereas, in a separate study, HEP or Veh was administered 1.5 hours after LPS administration. LPS (7.4×10^6 EU/kg) or Veh was administered i.v. to rats 4 hours after i.p. administration of MCT (100 mg/kg) or Veh. Livers were taken 18 hours after MCT administration and processed for morphometric analysis. N = 6 – 7 animals.

[a] Significantly different from respective value in the absence of anticoagulant treatment.

.

Treatment	Percent Lesion Area	
	Centrilobular	Midzonal
HEP Study		
MCT/LPS/Veh	3.9 ± 0.7	7.7 ± 1.6
MCT/LPS/HEP	0.9 ± 0.2[a]	1.0 ± 0.3[a]
WARF Study		
Veh/MCT/LPS	4.2 ± 0.9	7.4 ± 0.8
WARF/MCT/LPS	1.6 ± 0.6[a]	1.1 ± 0.3[a]

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 hours, intense fibrin staining was observed in the livers from the MCT/LPS/Veh-cotreated animals (Figure 7.4A). 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 (Figure 7.4B).

7.D.7. 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 (Figure 7.5A) nor WARF treatment (Figure 7.5B) had an effect on plasma TNF-α concentration.

7.D.8. PMN Accumulation after Anticoagulant Administration in MCT/LPS-Coexposed Animals

Figure 7.4. Hepatic Fibrin Deposition in Animals Cotreated with MCT, LPS and HEP. MCT/LPS-coexposed rats were treated with HEP anticoagulant as described in Figure 7.1 legend. Livers were removed from animals 18 hours after MCT or its Veh was administered and processed for immunohistochemistry. Total area (A) and zonal distribution (B) of fibrin immunostaining in liver sections were examined. N = 3 – 5 animals.

[a] Significantly different from all other groups.

[b] Significantly different from all other groups within same region of the liver lobule.

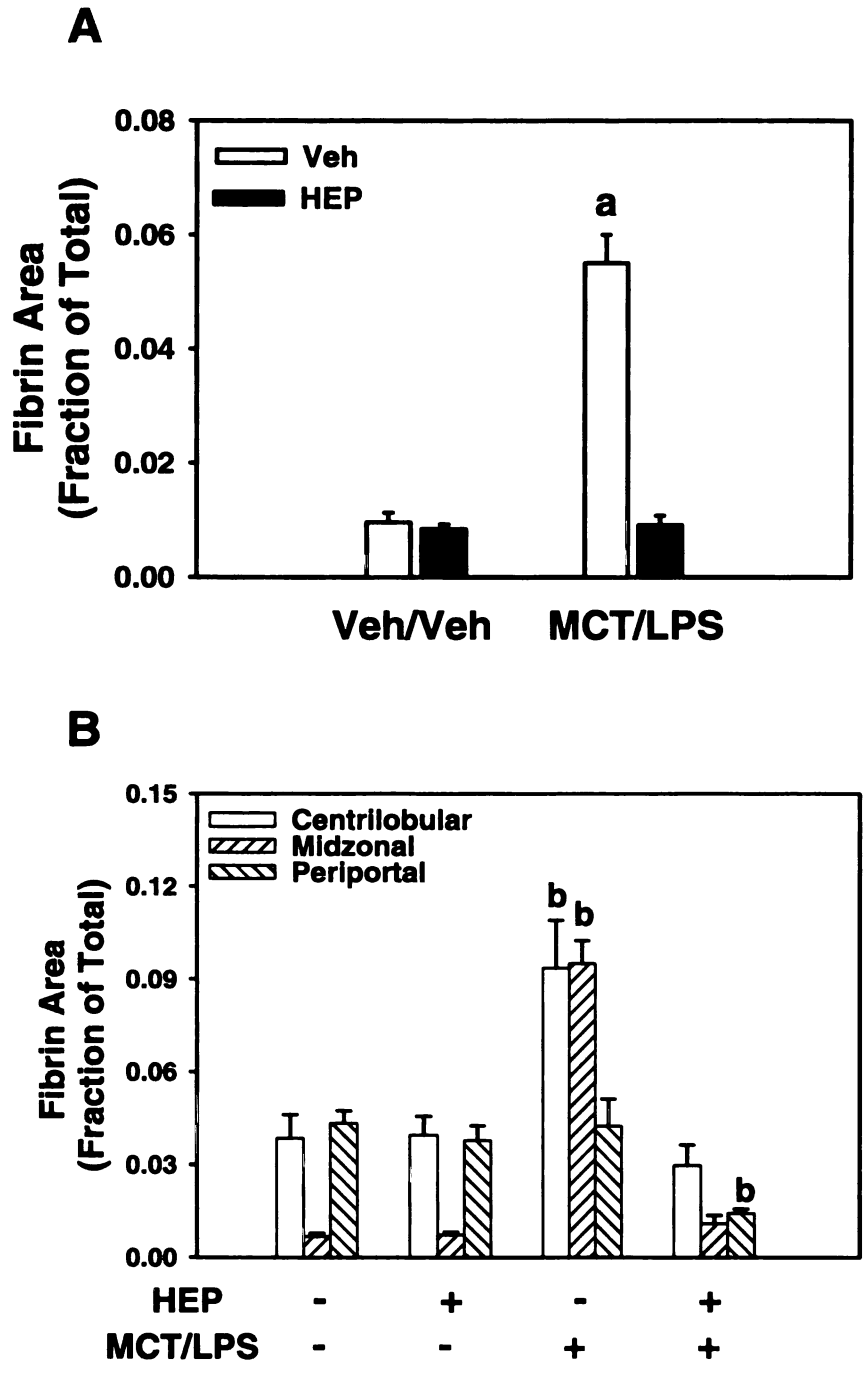
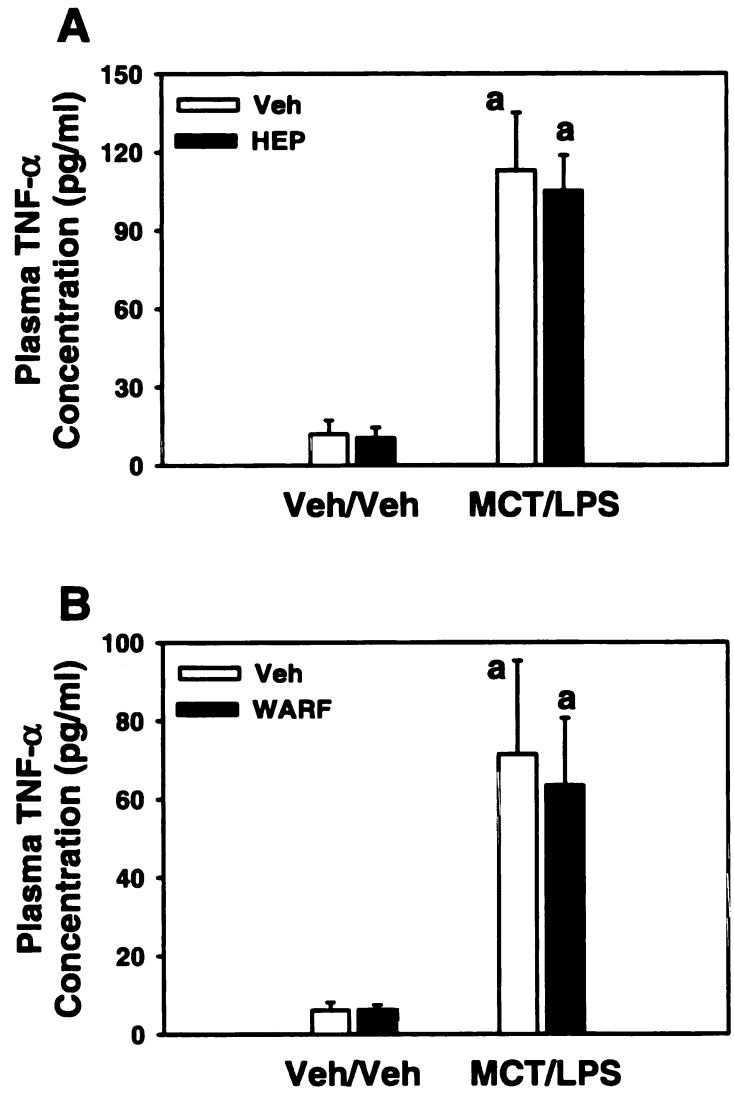

A
0.08
0.06
0.04
0.02
0.00
Fibrin Area
(Fraction of Total)
Veh
HEP
a
Veh/Veh
MCT/LPS
B
0.15
0.12
0.09
0.06
0.03
0.00
Fibrin Area
(Fraction of Total)
Centrilobular
Midzonal
Periportal
b
b
b
HEP - + - +
MCT/LPS - - + +

Figure 7.5. Effect of Anticoagulants on Plasma TNF-α Concentration in MCT/LPS-Cotreated Animals. MCT/LPS-coexposed rats were treated with HEP (A) or WARF (B) anticoagulants as described in Figure 7.1 legend. Plasma TNF-α concentration was determined in MCT/LPS-treated animals 18 hours after MCT or its Veh was given. N = 4 – 14 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.


A
Plasma TNF-α Concentration (pg/ml)
150
120
90
60
30
0
Veh
HEP
a
a
Veh/Veh
MCT/LPS
B
Plasma TNF-α Concentration (pg/ml)
100
80
60
40
20
0
Veh
WARF
a
a
Veh/Veh
MCT/LPS

PMNs are an important component in MCT/LPS-induced liver injury (Yee *et al.*, 2003c). To examine the relationship between PMNs and the coagulation system in this model, hepatic PMN accumulation was assessed in MCT/LPS-cotreated animals given anticoagulants. Treatment with either HEP (Figure 7.6A) or WARF (Figure 7.6B) attenuated hepatic PMN accumulation by approximately 60%.

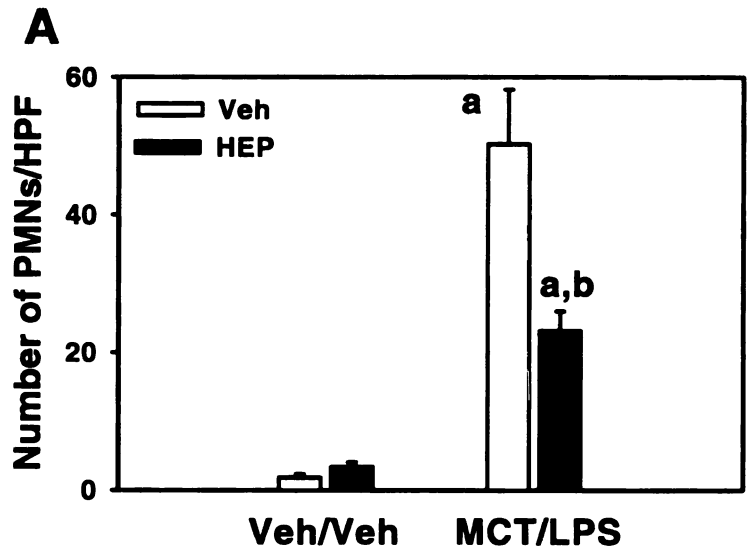
7.D.9. Plasma MCP-1 Concentration in MCT/LPS-Cotreated Animals

The chemokine MCP-1 causes monocyte attraction and activation (Luster, 1998; Gu *et al.*, 1999), 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-cotreated animals (Table 7.2). MCT administration caused no change at any of the times evaluated. By contrast, an elevation in plasma MCP-1 concentration occurred 2 hours after LPS administration, irrespective of MCT-treatment. Plasma MCP-1 remained elevated in Veh/LPS-cotreated animals at all times examined. By 12 hours after MCT administration, plasma MCP-1 was nearly twice as great in MCT/LPS-cotreated animals compared to animals that received LPS alone.

Figure 7.6. 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 7.1 legend. Hepatic PMN accumulation (i.e., number of PMNs per high powered field (HPF), 400X) was determined in MCT/LPS treated animals 18 hours after MCT or its Veh were administered. N = 4 – 14 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from respective MCT/LPS value in the absence of anticoagulant treatment.


A
60
40
20
0
Number of PMNs/HPF
Veh
HEP
a
a,b
Veh/Veh
MCT/LPS

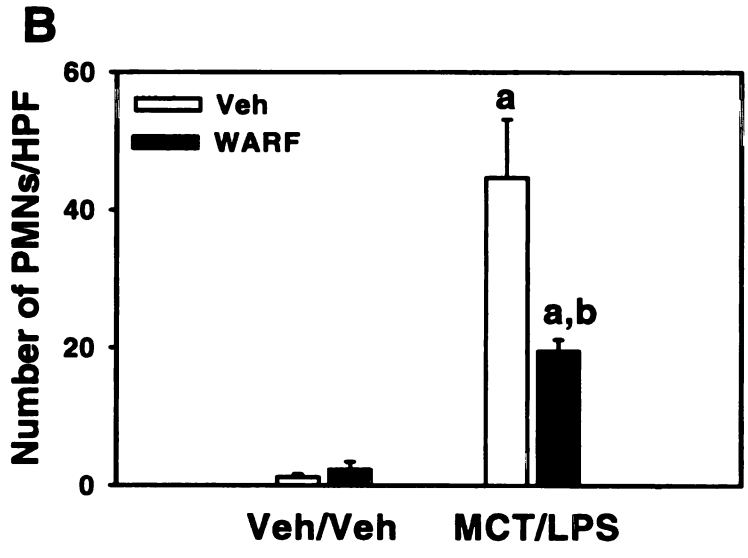

B
60
40
20
0
Number of PMNs/HPF
Veh
WARF
a
a,b
Veh/Veh
MCT/LPS

Table 7.2. Plasma MCP-1 Concentration is Elevated after MCT/LPS-Coexposure. Rats were treated with MCT and/or LPS as described in Table 7.1 legend. N = 3 – 6 animals.

[a] Significantly different from respective values in the absence of LPS at the indicated time.

[b] Significantly different from all other groups at the indicated time.

Time After MCT or MCT Vehicle	Plasma MCP-1 Concentration (pg/ml)
6 Hours	
Veh/Veh	32 ± 7
Veh/LPS	934 ± 108[a]
MCT/Veh	32 ± 6
MCT/LPS	894 ± 34[a]
12 Hours	
Veh/Veh	32 ± 6
Veh/LPS	941 ± 113[a]
MCT/Veh	49 ± 12
MCT/LPS	1552 ± 80[a,b]
18 Hours	
Veh/Veh	38 ± 15
Veh/LPS	798 ± 150[a]
MCT/Veh	40 ± 12
MCT/LPS	1462 ± 137[a,b]

7.D.10 Coagulation System Activation in Animals Depleted of KCs, TNF-α or PMNs

Yee *et al.* (2003a, 2003c) demonstrated that KC depletion by $GdCl_3$, 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 7.3). 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 hours after MCT administration.

7.E. 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 antiinflammatory effects as well. These effects include inhibition of PMN adhesion, chemotaxis and production of superoxide anion and nitric oxide (Matzner *et al.*, 1984; Silvestero *et al.*, 1994; Riesenberg *et al.*, 1995; Shin *et al.*, 1997; Beltran *et al.*, 1999). Because PMNs are critical to the pathogenesis of

Table 7.3. Treatment with NAS but Not with $GdCl_3$, PTX or ATS Prevents Coagulation System Activation in MCT/LPS-Cotreated Animals. Rats were treated with MCT and/or LPS as described in Table 7.1 legend. In addition, rats were given $GdCl_3$ (Kupffer cell inactivator), PTX (TNF-α synthesis inhibitor), ATS (TNF-α depletor) 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 hours after MCT or its Veh administration. N = 3 – 13 animals.

[a] Significantly different from respective value in the absence of MCT/LPS.

[b] Significantly different from all other groups.

Treatment	Plasma Fibrinogen Concentration (mg/dl)
$GdCl_3$ Study	
Veh/Veh/Veh	257 ± 19
$GdCl_3$/Veh/Veh	247 ± 17
Veh/MCT/LPS	115 ± 12[a]
$GdCl_3$/MCT/LPS	129 ± 7[a]
PTX Study	
Veh/Veh/Veh	234 ± 7
Veh/PTX/Veh	227 ± 12
MCT/Veh/LPS	72 ± 10[a]
MCT/PTX/LPS	109 ± 22[a]
ATS Study	
Veh/CS/Veh	325 ± 25
Veh/ATS/Veh	324 ± 26
MCT/CS/LPS	77 ± 10[a]
MCT/ATS/LPS	119 ± 14[a]
NAS Study	
CS/Veh/Veh	301 ± 7
NAS/Veh/Veh	310 ± 28
CS/MCT/LPS	101 ± 10[b]
NAS/MCT/LPS	336 ± 49

MCT/LPS-induced liver injury (Yee *et al.*, 2003c), 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 (Figure 7.1A) or WARF (Figure 7.1B) 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 hours after MCT administration (Figures 7.2 and 7.3). This suggests that the 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 7.1). Accordingly, the coagulation system appears to contribute causally to MCT/LPS-induced liver injury.

MCT is nontoxic and must be bioactivated by the cytrochromes of the P450 3A family to its toxic metabolite, monocrotaline pyrrole (MCTP), in order to produce liver injury (White and Mattocks, 1972; Kasahara *et al.*, 1997; Stegelmeier *et al.*, 1999). This conversion happens rapidly, with peak MCTP production occurring within 2 hours 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 hours 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; DeLeve *et al.*, 1996; Ba *et al.*, 2000; Saetre *et al.*, 2000; Copple *et al.*, 2002a) 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 (Figure 7.4B) 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 an 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 (Wise *et al.*, 1980; Sinha *et al.*, 1983; Kito *et al.*, 1985; Shuman, 1986), 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 (Moulin *et al.*, 2001; Copple *et al.*, 2003). 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 the activation of the coagulation system, PMN depletion did (Table 7.3). This suggests that PMNs promote coagulation system activation in this model. PMNs can affect the coagulation system through a 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; Schultze 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; Plescia and Altieri, 1996; Goel and Diamond, 2001). 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 (Hirata *et al.*, 1989; Seto *et al.*, 1998; Copple *et al.*, 2002a). SEC injury occurs concurrently with activation of the coagulation system (Yee *et al.*, 2003b), 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; Kirchhofer *et al.*, 1994; Esmon, 2000) or through activation of PMNs (Klebanoff *et al.*, 1986; Todoroki *et al.*, 2000; Goel and Diamond, 2001). By contrast, in the MCT/LPS-cotreatment model TNF-α does not appear to be necessary for activation of the coagulation system (Table 7.3). 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 KC 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 hours (Yee *et al.*, 2003a, 2003c). Anticoagulant administration to MCT/LPS-cotreated animals did not affect this later, sustained phase of increased plasma TNF-α concentration (Figures 7.5A and 7.5B), 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.

Anticoagulant administration reduced hepatic PMN accumulation (Figure. 7.6A and 7.6B). 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., cytokine-induced neutrophil chemoattractant-1; Zhang *et al.*, 1995; Luster *et al.*, 1998). 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 (Luster, 1998; Gu *et al.*, 1999). 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 7.2). By 18 hours, MCP-1 remained elevated in both groups of LPS-treated animals, but the increase was much greater in MCT/LPS-cotreated animals.

Since, the coagulation system is not activated in Veh/LPS animals (Yee *et al.*, 2003b) and since the same level of MCP-1 is seen in MCT/LPS-cotreated animals at 6 hours when coagulation activation occurs, it seems unlikely that MCP-1 alone 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 (Orvim *et al.*, 1994; Shebuski and Kilgore, 2001; Falati *et al.*, 2002). 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 hours 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.

CHAPTER 8

Summary and Conclusions

8.A. Model Development and Characterization

To elucidate further the mechanism through which inflammation augments xenobiotic injury, LPS was used as the inflammagen and MCT, a well-known hepatotoxicant, was used as the model xenobiotic agent. Administration of a small, noninjurious dose of LPS 4 hours after a small, nontoxic dose of MCT resulted in synergistic liver injury in rats 18 hours after MCT exposure. Unlike other models in which LPS enhanced the hepatotoxicant-induced liver lesion, two distinct liver lesions developed. These lesions were CL and MZ, exhibiting characteristics similar to lesions associated with larger, toxic doses of MCT and LPS given separately. Moreover, the nature of the MCT-like, CL lesions and the LPS-like, MZ lesions suggested that each agent enhanced the injury of the other. An *in vitro* study, however, failed to reproduce this synergistic injury in isolated HPCs cotreated with MCT and LPS. This indicated that the enhanced toxicity was not the result of a direct interaction of MCT and LPS on HPCs, but arose rather from an indirect mechanism. Hence, other factors not present in the culture system, such as inflammatory cells and/or soluble mediators, play a role in the synergistic hepatotoxicity observed *in vivo.*

A detailed timecourse evaluation revealed that significant HPC injury (as marked by increases in plasma ALT and AST concentrations) first appeared between 6 and 9 hours after MCT administration, reached maximal injury by 18 hours and continued to 72 hours. Elevated hepatic PMN accumulation and plasma TNF-α and CINC-1 concentrations confirmed that LPS-treated animals were undergoing an inflammatory response starting around 6 hours (i.e., 2 hours

after LPS administration and before the onset of HPC injury). Elevated hepatic PMN accumulation and plasma TNF-α and CINC-1 concentrations decreased toward baseline over time. The decrease was much more rapid in animals treated with LPS alone than in MCT/LPS-cotreated animals. Accordingly, sustained elevation in PMN accumulation and plasma TNF-α and CINC-1 concentrations were characteristics of MCT/LPS-cotreated animals.

Alterations to the sequence of MCT and LPS exposures were explored during model development. The purpose was to determine whether temporal differences in MCT and LPS administrations affected toxicity. Twenty-four hour survival was high (about 85%) when LPS was administered 4 hours before or after MCT. Survival dropped significantly to about 20%, however, when LPS was administered concurrently or an hour before MCT. Some animals became moribund early (i.e., 6 hours), but liver injury proved to be minimal at this time. This result suggested that extrahepatic factors were involved in the decreased survival. LPS is known to cause systemic hypotension through NO-induced shock (Ruetten and Thiemermann, 1996; Wolkow, 1998; Karima *et al.*, 1999), and elevated plasma NO was observed in rats given LPS an hour before MCT. Treatment with an iNOS inhibitor resulted in a decrease in plasma NO as well as a significant reduction in mortality. Hence, the predominant target of toxicity shifted from the liver when LPS and MCT were administered several hours apart to an extrahepatic site(s) when they were administered in close temporal proximity. NO-induced circulatory failure (i.e., hypotension) may be responsible for this LPS/MCT-induced lethality. To avoid complications produced from the

high lethality, the treatment regimen of LPS administered 4 hours after MCT was chosen for further study.

Histopathologic analysis of CL and MZ liver lesions from rats given LPS 4 hours after MCT suggested that injury to the liver vasculature was occurring. In particular, the loss of central vein intima in CL lesions and the disruption of sinusoidal architecture and hemorrhage in both lesions suggested vascular injury. Indeed, plasma HA concentration, a marker for SEC injury, significantly increased in MCT/LPS-cotreated animals before the onset of HPC injury and remained elevated to 18 hours. Further, immunohistochemical analysis of livers from MCT/LPS-cotreated rats revealed a significant decrease in staining with an antibody specific for rat endothelial cells (i.e., RECA-1). This indicated a loss of endothelial cells in CL regions by 6 hours and in MZ regions of the liver lobule by 12 hours.

Congestion and hemorrhage in both lesions along with fibrin deposition in the sinusoids of lesioned areas (observed via electron microscopy) suggested that an activated coagulation system might be another characteristic of this model. Coagulation system activation as marked by a decrease in plasma fibrinogen occurred in MCT/LPS-cotreated rats before the onset of HPC injury. Moreover, immunohistochemical analysis of fibrin deposition in livers from these animals revealed hepatic fibrin deposition in CL and MZ regions of liver lobules by 12 hours. Taken together, these results demonstrated that SEC injury and coagulation system activation are both characteristics of MCT/LPS-induced synergistic hepatotoxicity.

In summary, MCT and LPS coexposure results in synergistic hepatotoxicity, with injury occurring to both HPCs and SECs. Early and sustained activation of the coagulation system, prolonged elevation in PMN accumulation and plasma TNF-α and CINC-1 concentrations are all features of MCT/LPS-synergy and provide clues as to how this liver injury might occur.

8.B. Proposed Mechanism of Injury

Evidence suggested that the synergistic hepatotoxicity from MCT and LPS coexposure arose from the involvement of factors other than a simple MCT-LPS interaction. The presence of an inflammatory response (e.g., PMN accumulation and increased plasma TNF-α and CINC-1 concentrations) before the onset of HPC injury and the characteristics of the LPS-like, MZ lesion in the livers of MCT/LPS-cotreated animals suggested that the synergistic injury resulted, in part, from the ability of LPS to stimulate the inflammatory system. Consequently, the mechanism for LPS-induced liver injury was used comparatively to elucidate the pathogenesis of MCT/LPS-induced hepatotoxicity. LPS-induced liver injury is dependent upon KCs, TNF-α, PMNs, and the coagulation system (i.e., thrombin; Hewett *et al.*, 1992, 1993; Fujita *et al.*, 1995; Hewett and Roth, 1995; Moulin *et al.*, 1996; Brown *et al.*, 1997). Accordingly, each of these factors (Table 8.1) was investigated in the MCT/LPS-cotreatment model.

Inactivation of KCs and depletion of TNF-α prevented HPC injury but only modestly decreased SEC injury in MCT/LPS-cotreated animals. Morphometric

Table 8.1. Summary of the Pharmacological Manipulations Used to Investigate the Pathogenesis of MCT/LPS-induced Liver. LPS (7.4×10^6 EU LPS/kg) was administered intravenously to rats 4 hours after interperitoneal administration of MCT (100 mg/kg). In addition, rats were treated with $GdCl_3$ (KC inactivator), PTX (inhibitor of TNF-α synthesis) ATS (inactivator of TNF-α), NAS (PMN inactivator), HEP (anticoagulant) or WARF (anticoagulant) as described in the Materials and Methods of Chapters 5, 6 and 7. Various markers were observed 18 hours after MCT administration to determine if a protective (PROT) effect occurred with the addition of a pharmacologic agent to MCT/LPS-cotreatment or not (NOT). The markers observed were HPC and SEC injury, plasma TNF-α concentration, coagulation system activation and liver PMN accumulation.

[a] Modest but significant decrease.

[b] Not significant, but trend toward decrease.

Agent	Intended Effect	Significant Marker Change				
		HPC Injury	SEC Injury	TNF-α Conc.	Coag. Activ.	Liver PMN Accum.
NAS	PMN Depletion	PROT	PROT[a]	NOT	PROT	PROT
$GdCl_3$	KC Inactivation	PROT	PROT[a]	PROT	NOT	PROT
PTX	TNF-α Synthesis Inhibition	PROT	NOT[b]	PROT	NOT	PROT
ATS	TNF-α Neutralization	PROT	PROT[a]	PROT	NOT	PROT
HEP	Anticoagulant (Thrombin Inhibition)	PROT	PROT[a]	NOT	PROT	PROT
WARF	Anticoagulant	PROT	PROT[a]	NOT	PROT	PROT

analysis confirmed that KC inactivation and TNF-α depletion decreased both CL and MZ lesion areas. The partial protection from SEC injury in this model suggested that other factors were also involved in this injury. Additionally, a specific inhibitor of the COX-2 enzyme given to MCT/LPS-cotreated animals failed to prevent HPC injury. This indicated that COX-2 products, which are known to mediate liver injury in other LPS augmentation models (Ganey *et al.*, 2001), were not needed for HPC injury in this model. In summary, KCs and TNF-α are critical to MCT/LPS-induced liver injury, but COX-2 products are not.

The elevation in hepatic PMN numbers before the onset of HPC injury suggested that PMNs contributed to MCT/LPS-induced liver injury. PMN depletion prevented HPC injury at 18 hours and modestly decreased SEC injury as well. Morphometric analysis revealed a significant decrease in CL and MZ lesion area following PMN depletion in cotreated animals. Interestingly, PMN depletion failed to prevent early SEC injury (i.e., before the onset of HPC injury). This suggested that PMNs were required for HPC injury, but SEC injury was not entirely dependent on PMNs. That is, early SEC injury was independent of the actions of PMNs, but in later stages of the progression of SEC injury, PMNs contributed.

The cause of this early SEC injury is unclear. However, both isolated SECs *in vitro* and a rat SEC cell line were able to metabolize MCT to toxic pyrrolic metabolites. This suggested that MCT may injury SECs directly through the formation of its toxic metabolite, MCTP. Moreover, an isozyme of the CYP 3A subfamily was detected in the rat SEC cell line. Isozyme(s) of the CYP 3A

subfamily are responsible for bioactivating MCT. Taken together, this raised the possibility that the early SEC injury observed in MCT/LPS cotreated animals was due to an interaction of MCTP produced by SECs themselves with LPS or an LPS-derived mediator (e.g., TNF-α). It is tempting to speculate that MCTP produced in SECs lowers GSH concentration levels in these cells, and the effects of LPS and/or TNF-α further decrease GSH, resulting in overt SEC injury (Jaeschke, 1992; DeLeve *et al.*, 1996).

As previously described, sustained elevations in plasma TNF-α and CINC-1 were observed in MCT/LPS-cotreated animals. It is possible that both mediators modulate the prolonged accumulation and perhaps activation of PMNs in this injury model. To understand further the interdependence of TNF-α and hepatic PMN accumulation, the effect of PMN depletion on plasma TNF-α concentration and the effect of KC inactivation and TNF-α depletion on hepatic PMN accumulation were studied. PMN depletion did not result in a significant increase plasma TNF-α concentration, which suggested that PMNs were not needed to provoke or maintain plasma TNF-α release in this model. KC inactivation or TNF-α depletion decreased hepatic PMN accumulation, which suggested that KC-derived TNF-α contributed to PMN accumulation/retention in the liver. Accordingly, PMNs play a critical role in MCT/LPS-induced liver injury, and TNF-α may be involved in PMN accumulation/retention in this cotreatment model.

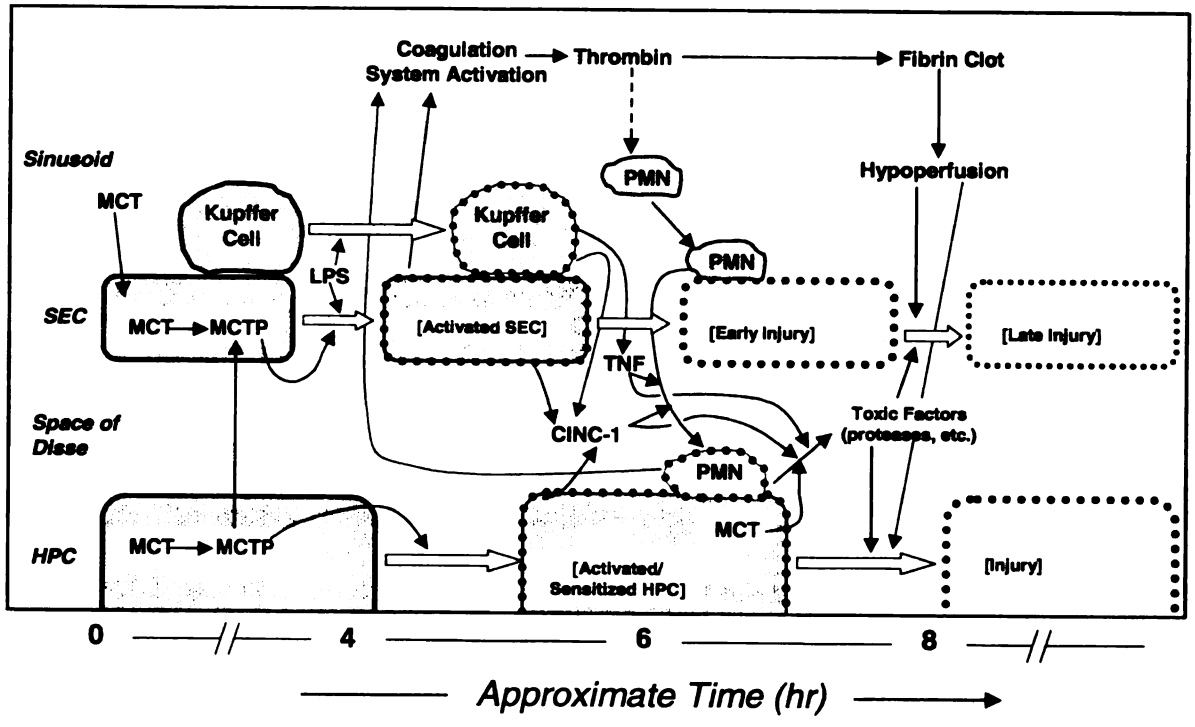
Finally, the role of the coagulation system was explored. Activation of the coagulation system occurred before the onset of HPC injury.

Immunohistochemical analysis of livers from MCT/LPS-cotreated animals revealed hepatic fibrin deposition, which further suggested a contribution of the coagulation system in this liver injury model. Indeed, inactivation of the coagulation system with anticoagulants prevented HPC injury but only modestly decreased SEC injury. Morphometric analysis of lesion size revealed that anticoagulants significantly decreased the CL and MZ lesions in MCT/LPS-cotreated animals. Hence, the coagulation system is another critical component of this liver injury model.

Neither KC inactivation nor TNF-α depletion prevented coagulation system activation, but PMN depletion did. This suggested that PMNs promote coagulation system activation (e.g., perhaps through the induction of TF) and/or, perhaps, modulate other cells (i.e., SECs) to become procoagulant in this model. Moreover, prevention of coagulation system activation did not affect the sustained increase in plasma TNF-α, which indicated that activation of the coagulation system was not needed for TNF-α release. Anticoagulant administration did, however, decrease PMN accumulation, further suggesting interplay between the coagulation system and PMNs in contributing to HPC injury. Overall, the results of this dissertation research suggest that KCs, TNF-α, PMNs and the coagulation system are interdependent components in the pathogenesis of MCT/LPS-induced liver injury.

A simplified mechanism of injury for this model is proposed in Figure 8.1. To summarize, coexposure to MCT and LPS results in early activation of and

Figure 8.1. Pathogenesis of MCT/LPS-induced Liver Injury. At a subthreshold MCT dose, MCTP induces a homeostatic change in HPCs and SECs but not overt injury, although the change makes these cells more susceptible to other insults. LPS or an unknown LPS-derived mediator(s) works in conjunction with MCTP produced in HPCs and/or SECs to cause early SEC injury that is independent of PMNs. Activation of/injury to SECs results in the activation of procoagulant surface and in the expression of adhesion molecules. PMNs are thereby arrested in the sinusoids. Concurrent activation of KCs results in the release of TNF-α, CINC-1, and other mediators. Activated SECs and HPCs produce CINC-1 and possibly other factors that work in combination with TNF-α to enhance migration of PMNs into the parenchyma and promote their activation. One such factor may be thrombin (dashed arrow) arising from the activation of the coagulation system by procoagulant SECs and PMNs. Thrombin formation may lead to fibrin clot formation possibly resulting in local hypoperfusion causing ischemic/hypoxic injury to nearby HPCs and SECs. This may work in conjunction with injury caused by activated PMNs, which release proteases and other cytotoxic factors to damage HPCs and results in further progression of SEC injury. [Image in this dissertation is presented in color].


Coagulation System Activation
Thrombin
Fibrin Clot
Sinusoid
MCT
Kupffer Cell
Kupffer Cell
PMN
PMN
Hypoperfusion
LPS
SEC
MCT→MCTP
[Activated SEC]
[Early Injury]
[Late Injury]
TNF
CINC-1
Space of Disse
Toxic Factors (proteases, etc.)
PMN
MCT
HPC
MCT→MCTP
[Activated/ Sensitized HPC]
[Injury]
0
4
6
8
Approximate Time (hr)

injury to SECs, possibly by the direct interaction of LPS and MCTP generated from SECs and/or HPCs. Activation of SECs results in increased expression of TF (i.e., SECs become procoagulant) and adhesion molecules, thereby leading to the arrest of PMNs in the sinusoids. Concurrent activation of KCs and possibly other cells results in the release of inflammatory factors such as TNF-α and CINC-1, which prompts PMN migration and activation in parenchyma. Activation of the coagulation system results from SEC and/or PMN activation (via induction of TF) and is responsible for the formation of thrombin. Thrombin causes fibrin clot formation, possibly leading to hypoperfusion and ischemic/hypoxic injury to SECs and HPCs. In addition, thrombin can have other roles besides fibrin clot formation, including serving as a PMN chemoattractant (Bizios *et al.*, 1986; Hoffman and Cooper, 1995; Holland *et al.*, 1998). Activated PMNs release toxic factors (i.e., ROS and proteases) that damage HPCs sensitized to injury by MCT exposure and further the progression of SEC injury. Although not every mechanistic question involved in MCT/LPS-induced liver injury was answered, in essence, the hypothesis of this thesis has been supported. That is, inflammatory events (i.e., KCs, TNF-α and PMNs; and the coagulation system) participate causally in the synergistic hepatotoxicity from coexposure to MCT and LPS. A final qualifier may be necessary however, as these findings only apply to a cotreatment model of LPS administered 4 hours after MCT. It is possible that different combinations of inflammatory cells and/or mediators are necessary to induce liver injury depending on the temporal relationship between MCT and LPS administration. Further investigation will be

needed to determine if KCs, TNF-α, PMNs and the coagulation system are also critical in other dosing paradigms for MCT/LPS cotreatment (e.g., LPS administered an hour before MCT).

8.C. Comparison to Other Models

The mechanism for MCT/LPS-induced liver injury is similar to that of LPS-induced liver injury, though some differences exist. In the LPS-induced liver injury model, KCs, PMNs, TNF-α and the coagulation system (i.e., thrombin) are involved in HPC injury (Hewett *et al.*, 1992, 1993; Fujita *et al.*, 1995; Hewett and Roth, 1995; Moulin *et al.*, 1996; Brown *et al.*, 1997). Although SEC injury is known to occur, it is unclear what inflammatory factors cause this injury (Yachida *et al.*, 1998). Similarly, in the MCT/LPS-induced liver injury model, PMNs, KCs, TNF-α and the coagulation system are involved in HPC injury and late-stage SEC injury. The role of platelets and the specific mechanism of the coagulation system have been investigated in the LPS-induced liver injury model but not in the MCT/LPS-cotreatment model (Hewett and Roth, 1995; Pearson *et al.*, 1995). In the LPS-induced liver injury model, platelets and thrombin activation (i.e., PAR-1 activation) but not fibrin clot formation were found to be critical for the development of liver injury (Hewett and Roth, 1995; Pearson *et al.*, 1995; Moulin *et al.*, 1996; Copple *et al*, 2003). Moreover, neither KC inactivation nor TNF-α depletion decrease hepatic PMN accumulation (Hewett *et al.*, 1992), indicating that KCs and TNF-α are not involved in PMN accumulation/retention in the LPS-

induced liver injury model as they are in the MCT/LPS-cotreatment model. Another difference between these two models exists in the role of TNF-α in coagulation system activation. In the LPS-induced liver injury model, TNF-α depletion prevented activation of the coagulation system (Hewett and Roth, 1995), but KC inactivation had no effect on coagulation system activation (Pearson *et al.*, 1996a). This result indicates that TNF-α is involved in coagulation system activation. Conversely, in the MCT/LPS cotreatment model neither TNF-α depletion nor KC inactivation prevents activation of the coagulation system. Finally, in the LPS-induced liver injury model, the anticoagulant heparin did not reduce hepatic PMN accumulation (Hewett and Roth, 1995), but in the MCT/LPS-cotreatment model anticoagulant administration did. Hence, this suggested that the coagulation system was involved in PMN recruitment in the MCT/LPS-coexposure model but not necessarily in the LPS-induced liver injury model. Accordingly, though a similar mechanism of injury occurred in these two models, it is likely that differences in the MCT/LPS-induced liver injury model arose from the contribution of MCT to the injury.

Mechanistically, MCT-induced liver injury is different from LPS-induced liver injury. KCs, PMNs and TNF-α are not required for HPC injury in the MCT-induced liver injury model. However, activation of the coagulation system is important for the development of hepatocellular injury. PMN depletion and inactivation of the coagulation system, on the other hand, failed to prevent SEC injury (Copple *et al.*, 2002b). It is not known what effects KCs or TNF-α have on SEC injury. Hence, based on the present evidence, it does not appear that

elements of the inflammatory system are required for injury in the high dose MCT model. As a related aside, it should be noted that there have been no direct studies to demonstrate that MCTP alters the homeostasis of SECs and HPCs to make these cells more susceptible to LPS. Future studies may be warranted to confirm the presumption that MCTP enhances the susceptibility of these liver cells to LPS or its mediators.

Taken together, it is tempting to speculate that in the MCT/LPS-induced liver injury model initial exposure to a small dose of MCT results in the homeostatic alteration of HPCs and SECs in CL regions of the liver lobule. This alteration in HPCs and/or SECs predisposes these cells to injury and causes the release of mediator(s) which, upon interacting with a modestly stimulated inflammatory system (i.e., after exposure to LPS), generates a profound inflammatory response. The inflammatory response causes HPC and SEC necrosis in CL and MZ regions of the liver lobule. CL lesions likely form as HPCs and SECs progress from altered homeostasis to frank injury due to the molecular effects of LPS or LPS-derived mediators on these sensitized cells. MZ lesions may form directly as a result of the inflammatory response inducing degeneration and necrosis in HPCs and SECs. It is tempting to speculate further that MCT exposure also altered the homeostasis of HPCs and SECs in the MZ region of the liver lobule, albeit to a lesser degree than in the CL region, and this sensitized these cells to inflammation-induced injury. Since the histopathology of the MZ lesion showed an unusual amount of congestion and hemorrhage in MCT/LPS-cotreated animals, it is possible that SECs within this region were

particularly sensitized to destruction by LPS. It is interesting to note that pharmacologic manipulation of this model to inactivate or deplete KCs, PMNs, TNF-α or the coagulation system resulted in a reduction in both lesion areas, indicating that the inflammation-induced injury was critical to both lesions. One intriguing question that arises from this supposition is: what is the role of SEC injury in the MCT/LPS-cotreatment model? Is SEC activation/injury an epiphenomenon as it relates to HPC destruction, or is it indirectly responsible (i.e., through the release of mediators) for modulation of effects in HPCs and inflammatory cells? Further study will be needed to address this question.

Finally, the mechanism of MCT/LPS-induced liver injury is similar to other models of LPS-induced augmentation of hepatotoxicant injury. In the allyl alcohol/LPS model, KCs, PMNs, COX-2 products and the coagulation system are critical to injury but TNF-α is not (Sneed *et al.*, 1997; Sneed *et al.*, 2000; Kinser *et al.*, 2000, 2002; Ganey *et al.*, 2001). Likewise, in the aflatoxin B_1/LPS model, PMNs, TNF-α, and the coagulation system, but not COX-2 products, are involved in HPC injury (Barton *et al.*, 2000a; 2001; Luyendyk *et al.*, 2003). Overall, while similarities in the mechanism with other LPS-potentiation models exist, especially in the role of PMNs and the coagulation system, there are also differences. Accordingly, the mechanism for LPS augmentation of hepatotoxicant-induced injury is not universal across toxicants but does depend on components of inflammation. Differences in the critical inflammatory components may vary depending on the particular hepatotoxicant and how that hepatotoxicant mechanistically contributes to injury.

8.D. Significance and Future Studies

The results of this dissertation, while addressing the original hypothesis, have generated a number of questions and in the process have laid down the foundation for additional research into mechanisms of injury. Answering these questions may be beneficial in elucidating the mechanism by which inflammation acts as a determinant of susceptibility to chemical intoxication. Moreover, such answers may have clinical application in the treatment of individuals suffering from chemical insult as well as contribute to more accurate prediction of conditions that could cause the enhancement of xenobiotic toxicity.

One question arising from this dissertation concerns the role of SEC injury in contributing to HPC injury in this cotreatment model. Although this was addressed in the previous section, it is tempting to speculate that SECs may be an important but overlooked component in models involving LPS augmentation of hepatotoxicants. Another question concerns the causal role of the coagulation system in the MCT/LPS-cotreatment model. That is, what is the role of thrombin and particularly fibrin clot formation in the development of liver injury? In the LPS-induced liver injury model, thrombin's effect is independent of fibrin clot formation (Hewett and Roth, 1995). Hence, in the MCT/LPS-cotreatment model, is thrombin involved in fibrin clot formation, PMN chemotaxis, proinflammatory response and/or activation of PAR-1 receptor and how do these ultimately apply to HPC and SEC injury? In particular, is fibrin clot formation necessary for liver

injury? In the MCT/LPS cotreatment model, based on the formation of hepatic fibrin deposits, suggests that clot formation may lead to local hypoperfusion and subsequent injury. Fibrin clot formation may thus be an important contributing component to the synergistic liver injury that occurs in this model (Figure 8.1). Finally, what is the role of platelets and TF in MCT/LPS-induced liver injury? In the LPS-induced liver injury model, it was postulated that platelets were critical for liver injury due to the release of mediators rather than through clot formation (Pearson *et al.*, 1996). One such mediator released is PAF. If platelet activation is important in the MCT/LPS cotreatment model, then PAF could be a critical mediator in this liver injury. Although the role of PAF in LPS-induced injury is controversial, PAF can promote PMN chemotaxis, aggregation, endothelial cell adhesion, granular secretion and superoxide production (Imura *et al.*, 1986; Lorant, *et al.*, 1991; Coughlan *et al.*, 1994; Zimmerman *et al.*, 1994; Pearson *et al.*, 1997). Since PMNs are critical to the pathogenesis of MCT/LPS-induced liver injury, it is possible that PAF is an important modulator of PMN activity in this model. Furthermore, induction of TF is important for the activation of the coagulation system in the LPS model (Karima *et al.*, 1999). It is known that TF is induced on the surface of SEC and PMNs, and this may have a role in activating the coagulation system (Stern *et al.*, 1985; Bray *et al.*, 1987; Schultze and Roth, 1998; Todoroki *et al.*, 2002). Accordingly, does TF have a role in activating the coagulation system in the MCT/LPS-induced liver injury model? Other possible activators of the coagulation system include monocytes and platelets (Osterud,

1995; Pearson *et al.*, 1995; Polack *et al.*, 1997). Are these factors involved as well?

In conclusion, coexposure of rats to small, noninjurious doses of MCT and LPS resulted in synergistic hepatotoxicity. KCs, TNF-α, PMNs and the coagulation system all play critical roles in the development of MCT/LPS-induced liver injury. By discerning the factors involved and noting the similarities between the mechanism of injury in this model and other models of LPS potentiation of xenobiotic toxicity, a better understanding of the enhancement phenomenon can be achieved. For example, based on some model systems examined (e.g., allyl alcohol/LPS, aflatoxin B_1/LPS, galactosamine/LPS and other LPS potentiation models), the involvement of PMNs and the coagulation system frequently appear to be critical components in LPS potentiation of hepatotoxicants (reviewed in Ganey and Roth, 2001; Kinser *et al.*, 2000, 2002; Barton *et al.*, 2000a; Luyendyke *et al.*, 2003).

This knowledge may be useful clinically in treating individuals suffering from chemical toxicities that may be enhanced by inflammation. In the example noted above, it is tempting to speculate that inhibitors of PMNs or anticoagulants may be useful in treating such injury. Moreover, this work emphasizes the importance of considering the effects of underlying inflammation in formulating risk and safety assessments for hepatotoxicants. Results of this study suggest that individuals experiencing a mild inflammatory response may be at particular risk for illness from foods or alternative medicines containing PAs or other potentially hepatotoxic compounds.

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