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Role of Inflammatory Mediators in Endotoxin Hepatotoxicity

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James Alan Hewett

has been accepted towards fulfillment
of the requirements for

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Robert A Roth
Major professor

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ROLE OF INFLAMMATORY MEDIATORS IN ENDOTOXIN HEPATOTOXICITY

By

James Alan Hewett

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ABSTRACT

ROLE OF INFLAMMATORY MEDIATORS IN
ENDOTOXIN HEPATOTOXICITY

By

James Alan Hewett

Endotoxin (lipopolysaccharide, LPS) is an outer cell wall component of gram-negative bacteria. It is thought to be an important contributing factor to the pathophysiologic alterations associated with infections by these bacteria. A myriad of adverse effects are attributed to LPS, including injury to the liver. The overall objective of this dissertation was to examine the role of several endogenous inflammatory mediators in the pathogenesis of LPS hepatotoxicity.

Liver injury occurred between 3 and 6 hr after bolus iv injection of LPS in rats. This was preceded by an increase in hepatic neutrophil (PMN) numbers, an elevation of circulating tumor necrosis factor (TNF)-*alpha* concentration, and activation of the coagulation system. Protection against liver injury was afforded by depletion of circulating PMNs, which attenuated hepatic PMN accumulation, by either TNF-*alpha* antiserum or pentoxifylline, which attenuated the increase in circulating TNF-*alpha* concentration, and by the

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anticoagulants, heparin and warfarin. These results indicate that PMNs, TNF-*alpha*, and the coagulation system contribute to the pathogenesis of LPS hepatotoxicity.

PMNs alone are not sufficient for full manifestation of liver injury since neither TNF-*alpha* antiserum, pentoxifylline, nor heparin pretreatment prevented the accumulation of PMNs in the liver after LPS exposure. Similarly, PMN depletion enhanced circulating TNF-*alpha* concentration by more than 3-fold after LPS exposure suggesting that TNF-*alpha* alone is not sufficient for full manifestation of liver injury. These results are consistent with an interaction between PMNs and TNF-*alpha* in the pathogenesis of LPS-induced liver injury. This interaction may contribute to liver injury by a mechanism which is dependent on the coagulation system since PMN depletion, TNF-*alpha* antiserum and pentoxifylline inhibited activation of the coagulation system after LPS exposure.

The results described in this dissertation provide new insight into the mechanisms of liver injury from bacterial LPS by showing that blood PMNs, circulating TNF-*alpha* and the coagulation system each play important roles in the pathogenesis. While much remains unknown about the specific mechanisms by which each of these inflammatory mediators is involved, the results suggest that complex interactions among them may be necessary for full manifestation of liver injury.

To my family

**Especially to my wife, Sandy,
my grandmother, Mildred, and my
parents, John and Eileen**

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I am especially grateful to my mentor and friend, Dr. Robert Roth, who never seemed to doubt my ability to become an independent researcher. His abiding support has been instrumental in making it attainable. I am also grateful to Dr. Leon Bruner, who played an important role in introducing me to research and to the field of Toxicology.

I also thank members of my thesis committee, Drs. Irshad Chaudry, Lawrence Fischer, and Kenneth Moore, for their guidance, and everyone in B346/7 Life Sciences, including Jim Wagner, Eric Shobe, Simi VanCise, Rob Stachlewitz, Dr. Marc Bailie, Dr. Cindy Hoorn, Dr. Lonnie Dahm, Dr. Eric Schultze and Dr. Patty Ganey who together created a unique and enjoyable working environment.

A number of individuals made important technical contributions for which I am thankful, including Rob Stachlewitz and Simi VanCise, who provided proficient and responsible technical assistance, Dr. Eric Schultze, who's meticulous histopathologic, morphometric, and cytologic analyses contributed greatly to the results presented in Chapter 3, and Dr. Steven L. Kunkel and Pam Lincoln at the University of Michigan, who provided tumor necrosis factor-

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alpha antiserum and who performed measurements of plasma tumor necrosis factor-*alpha*.

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Finally, I am grateful to my wife, Sandy, for her companionship and love, which made the difficult times bearable and the good times even better.

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LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
ANOVA	Analysis of variance
Anti-LC Ig	Immunoglobulin fraction from serum of rabbits immunized with rat lymphocytes
Anti-PMN Ig	Immunoglobulin fraction from serum of rabbits immunized with rat neutrophils
AST	Aspartate aminotransferase
Control Ig	Immunoglobulin fraction from serum of non-immunized rabbits
DDW	Double distilled water
DIC	Disseminated intravascular coagulation
dl	Deciliter
E. coli	*Escherichia coli*
EGTA	Ethylene glycol-bis(*beta*-aminoethyl ether) N,N,N'N'-tetraacetic acid
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hr	Hour
Ig	Immunoglobulin
IL	Interleukin
ip	Intraperitoneal

iv	intravenous
kDa	kilodalton
kg	kilogram
LPS	lipopolysaccharide
mg	milligram
ml	milliliter
ng	nanogram
NPC	nonparenchymal cell
O_2^-	superoxide anion
PMN	polymorphonuclear leukocyte
po	per os (by mouth)
SF U	Sandoz Factor Unit
TNF-alp	tumor necrosis factor-alpha
ul	microliter
WBC	white blood cell

iv	Intravenous
kDa	Kilodalton
kg	Kilogram
LPS	Endotoxin (lipopolysaccharide)
mg	Milligram
ml	Milliliter
ng	Nanogram
NPC	Nonparenchymal cell
O_2^-	Superoxide anion
PMN	Neutrophil (polymorphonuclear leukocyte)
po	Oral (*per os*)
SF U	Sigma-Frankel Units of transaminase activity
TNF-*alpha*	Tumor necrosis factor-*alpha*
ul	Microliter
WBC	White blood cell

CHAPTER 1

GENERAL INTRODUCTION

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1.1 St

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A myriad of pathophysiologic alterations are associated with infection by gram-negative bacteria and many of these alterations can be attributed to gram-negative bacterial endotoxin, or lipolysaccharide (LPS). The purpose of the initial sections of Chapter 1 is to introduce LPS. These sections provide brief descriptions of the structure of LPS, the routes of exposure to and clearance of LPS from the circulation, and the role of LPS in the pathogenesis of alterations associated with infections by gram-negative bacteria. This is followed by a detailed discussion of the response of mammalian cells to LPS. The final sections of this chapter will describe the effects of LPS on extrahepatic tissues. The effects of LPS on the liver are described in detail in Chapter 2. Because the focus of this dissertation is on the role of certain host-derived factors in the pathogenesis of LPS-induced liver injury, the emphasis of these two chapters will be on the role of endogenous mediators in the response of host tissues to LPS.

1.1 Structure of LPS

LPS is an endotoxin derived from gram-negative bacteria. It is a major component of the cell wall of these bacteria and is one of the distinguishing features between

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gram-negative and gram-positive bacteria which do not contain LPS in their cell wall. Although the structure varies slightly among different species and strains of gram-negative bacteria, all LPS molecules are composed of polysaccharide and lipid domains (1). The polysaccharide region consists of a repeating oligosaccharide structure, termed the O-antigen polysaccharide, which extends toward the extracellular environment of the bacterium, and a core polysaccharide which covalently links the O-antigen polysaccharide with the lipid region. Unusual dideoxysugar moieties, such as colitose and paratose, are often constituents of the O-antigen oligosaccharide unit whereas the core polysaccharide is characterized by the presence of the unique sugar, 2-keto-3-deoxyoctonate. In contrast to the structure of the core polysaccharide, which is similar among different strains and species of bacteria, the composition of the O-antigen polysaccharide is usually more variable.

The lipid region of LPS is often refered to as lipid A. This amphipathic structure is a major component of the outer leaflet of the cell wall lipid bilayer. It consists of several long-chain fatty acids linked by amide and ester bonds to two glucosamine residues. These glucosamine residues are joined by a *beta*-1-6 linkage to form the backbone of the lipid A molecule. Charged phosphate or pyrophosphate groups bound to this glucosamine backbone contribute to the amphipathic nature of lipid A along with the hydrophobic fatty acids.

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Many of the biological effects associated with LPS appear to be mediated by lipid A. This is supported by evidence which indicates that exposure to purified lipid A produces many of the same effects as LPS. Furthermore, most of the biological activities of LPS can be neutralized by substances which bind specifically to this region of the LPS molecule such as the antibiotic, polymyxin B, and certain monoclonal antibodies to lipid A (2).

1.2 Exposure to LPS

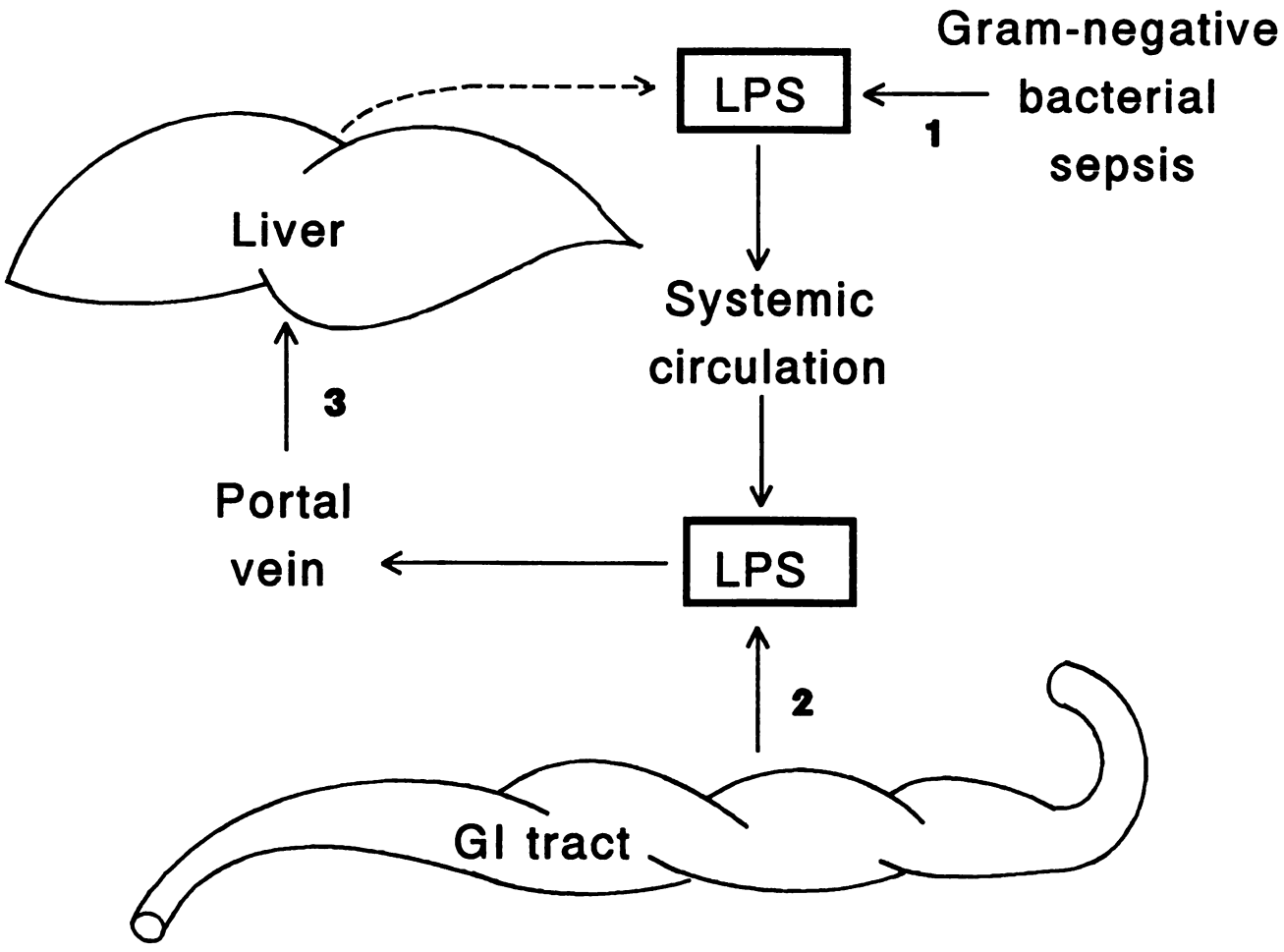
1.2.a Gram-negative bacterial infection

Exposure to LPS may occur by several different routes (Figure 1.1). Perhaps the most obvious route of exposure is during infection by gram-negative bacteria. LPS is an integral component of the cell wall of gram-negative bacteria and is not normally released into the extracellular environment. However, it can be liberated under certain circumstances, particularly during cell division and following death of the bacteria. Evidence from numerous studies indicates that a rise in cell-free LPS accompanies infection by gram-negative bacteria. For example, following abdominal infection of rabbits by *Pasteurella multocida*, cell-free LPS in the plasma increased from non-detectable levels prior to infection to 100 ug/ml by 6 hr after infection (3). Similarly, increases in the concentration of LPS in the cerebrospinal fluid have been associated with

Figure 1.1 Routes of exposure to and clearance of LPS. [1], LPS released from dividing and dead bacteria during infection by gram-negative bacteria; [2], increase in absorption from the gastrointestinal tract of LPS derived from indigenous gram-negative bacterial flora of the gut; [3], once in the circulation, LPS is cleared primarly by the liver.

Figure 1.1

Figure 1.1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gram-negative bacterial meningitis both in animal models of the disease (4) as well as in humans suffering from the disease (5).

Gram-negative bacterial infections are often treated using bactericidal antibiotics. While several of these antimicrobial agents have proven effective in eliminating the bacteria, results from recent studies suggest that they may cause the release of large amounts of cell-free LPS (6,7,8,9,10). Thus, antibiotic therapy may enhance the exposure to LPS during gram-negative bacterial infections.

1.2.b Absorption from the gastrointestinal tract

In contrast to exposure during gram-negative bacterial infection, it has been proposed that LPS exposure could occur in the absence of bacterial infection through an increase in the absorption of LPS from the gastrointestinal tract. The gastrointestinal tract normally contains a large concentration of LPS which is presumably derived from the indigenous gram-negative bacterial flora of the gut (11). Under normal conditions, the intestinal wall acts as a formidable barrier to the passage of LPS from the gastrointestinal tract into the blood stream. However, disruption of this barrier during certain pathophysiologic conditions can lead to endotoxemia. For example, occlusion of the portal vein results in an increase in the LPS concentration in both portal venous and systemic blood (12). Whereas LPS was not detectable in blood from sham operated

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animals, it ranged from 100-300 ng/ml after portal vein occlusion. Because this increase in plasma LPS was not observed in germ-free animals, it was concluded that endotoxemia induced by portal vein occlusion was due to an increase in absorption of LPS from the gastrointestinal tract. In addition to portal vein occlusion, increased absorption of LPS from the gastrointestinal tract has been implicated in certain instances of chemically induced liver injury (13,14), dietary cirrhosis (15), partial hepatectomy (16), and following intestinal ischemia (17). These studies provide strong evidence that exposure to LPS can occur under conditions in which the intestinal barrier to gastrointestinal LPS is compromised.

1.3 Clearance and detoxification of LPS

Following bolus intravenous (iv) administration, LPS is cleared from the circulation in a biphasic manner (18,19). A large fraction of the initial dose disappears from the circulation within minutes after injection. Subsequent to this rapid clearance phase, LPS elimination progresses gradually over a period of hours. Although intravenously administered LPS is found in numerous tissues, including the spleen, lungs, kidneys, and adrenal glands, the majority accumulates in the liver where it is associated primarily with Kupffer cells (20,21,22,23). These fixed hepatic

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macrophages are thought to play an important role in the clearance and detoxification of LPS from the circulation.

Clearance of LPS from the blood stream is influenced by plasma lipoprotein particles. LPS binds predominately to high density lipoprotein particles in plasma. This prolongs the half-life of circulating LPS (24). However, because LPS complexed with high density lipoprotein particles is less biologically active, it has been proposed that this may function as a protective mechanism against LPS toxicity (25). LPS can also be neutralized by an LPS-binding protein contained within specific granules of neutrophils (PMNs) (26). This 50-60 kDa protein, which specifically recognizes the lipid A region of the LPS molecule (27), is referred to as bacterial/permeability increasing protein because it can lyse and kill gram-negative bacteria by binding to LPS on the surface of the bacteria (28,29,30).

1.4 Pathogenic role of LPS in gram-negative bacterial sepsis

1.4.a Lethality

Death resulting from shock and multiple organ failure is often a consequence of overwhelming infection by gram-negative bacteria. Indeed, a large percentage of deaths of hospitalized patients can be attributed to gram-negative bacterial sepsis. It has been proposed that LPS is a major contributing factor to the high mortality associated with

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gram-negative bacterial infection. This is supported by evidence from studies in animal models in which treatment with specific neutralizing antibodies to LPS afforded protection against the lethal effects of gram-negative bacterial sepsis (31,32,33,34). Specific antibodies to LPS have also proven to be effective in reducing the mortality in hospitalized patients suffering from gram-negative bacterial infections (35,36,37). Thus, LPS appears to contribute to the mortality associated with infection by gram-negative bacteria.

1.4.b Tissue injury

In addition to its role in lethality, LPS appears to play a role in the development of tissue injury during local gram-negative bacterial infections. For example, LPS has been implicated in the pathogenesis of tissue injury in gram-negative bacterial meningitis. A large percentage of clinical cases of bacterial meningitis can be attributed to certain gram-negative bacteria, including *Haemophilus influenzae* type b, *Escherichia (E.) coli*, and *Neisseria meningitidis*. Infection of the cerebrospinal fluid by these bacteria is accompanied by inflammation of the meninges, which is characterized by the appearance of large numbers of leukocytes and by alterations in blood-brain barrier permeability (38). The adverse effects associated with gram-negative bacterial meningitis appear to be mediated by LPS since intracisternal administration of LPS produces many of

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these same effects and since neutralization of LPS with polymyxin B afforded protection against experimentally induced gram-negative bacterial meningitis (39,40).

LPS has also been implicated in the pathogenesis of tissue injury following gram-negative bacterial infection of the skin. Lesions in the skin following intradermal injection of *E. coli* are characterized by hyperemia, increased vascular permeability, and hemorrhage and are infiltrated by large numbers of neutrophils (41). Similar lesions occurred following intradermal injection of killed *E. coli* or purified *E. coli* LPS (42). Furthermore, the injury was attenuated by pretreatment of the bacteria with polymyxin B (43). Thus, like gram-negative bacteria-induced injury to the blood-brain barrier, microvascular injury in the skin following intradermal injection of gram-negative bacteria is mediated by LPS.

1.5 Interactions between LPS and mammalian cells *in vitro*

1.5.a Cytotoxicity

Results from several studies indicate that LPS is cytotoxic to cells *in vitro* under certain conditions. For example, marked degenerative morphological changes are observed in cultured vascular endothelial cells after exposure to LPS (44). These morphological changes are accompanied by increased cell detachment and leakage of cytoplasmic contents, and by decreased DNA, RNA, and protein

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synthesis. Injury to cultured endothelial cells is dependent on both the duration of LPS exposure (>4 hr) and the concentration of LPS (>0.1 ug/ml) and is enhanced by the presence of serum (45,46). Oxygen radical production and conjugated diene formation are associated with the cytotoxicity, suggesting that LPS-induced endothelial cell injury is mediated by oxygen radical-dependent lipid peroxidation (47). This is supported by evidence indicating that the cytotoxicity is attenuated by oxygen radical scavengers. The source of the oxygen radicals appears to be xanthine oxidase since the xanthine oxidase inhibitor, allopurinol, attenuated the oxygen radical production and afforded protection against the cytotoxicity. Results from a recent study suggest that a similar mechanism may contribute to LPS-induced injury to vascular endothelial cells *in vivo*. In this study, LPS administered locally to hamster cheek pouches caused an increase in microvascular leakage which was prevented by the antioxidant, dimethyl sulfoxide, and by allopurinol (48).

In addition to its effects on vascular endothelial cells, LPS is also cytotoxic to cultured macrophages (49) and fibroblasts (50). However, whether the mechanisms of cytotoxicity in these cells is dependent on reactive oxygen metabolites is not known.

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1.5.b Stimulation of mediator release

In addition to its cytotoxic effects, LPS stimulates the release of a variety of inflammatory mediators from certain cells in culture. Many of these endogenous substances and their sources are listed in Table 1.1. These potent, biologically active substances have been implicated in the pathophysiologic alterations associated with LPS exposure (see below and Table 1.1).

LPS stimulates the release of mediators from cells in culture by several mechanisms. For example, stimulation of the release of arachidonic acid metabolites from macrophages appears to be mediated at least in part by the direct, LPS-induced activation of phospholipase A_2, which catalyzes the release of arachidonic acid from membrane phospholipids (51). In contrast, LPS stimulates the production of the cytokine, tumor necrosis factor (TNF)-*alpha*, from macrophages by inducing TNF-*alpha* gene transcription (51,52). This is indicated by the increase in TNF-*alpha* mRNA which occurs prior to the onset of TNF-*alpha* release. Induction of gene expression by LPS contributes to the production of numerous other inflammatory mediators by cells. Among these are the increased expression of adhesion molecules for PMNs on vascular endothelial cells (53,54), and production of certain interleukins and nitric oxide by macrophages (55,56) and smooth muscle cells (57,58,59).

The signal transduction pathways involved in the LPS-induced alteration in cell function are not completely

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Table 1.1
Inflammatory mediators released by LPS

Mediator	Possible Sources[a]	Likely Target Tissues[b]
Complement factors	Plasma	Cardiovascular
Clotting factors	Plasma	Kidneys Liver Lungs
Tissue factor	Endothelial cells Macrophages	Plasma
Oxygen radicals	Endothelial cells Macrophages Neutrophils	Cardiovascular Lungs Liver
Lysosomal enzymes	Macrophages	Liver
Nitric oxide	Endothelial cells Smooth muscle cells Macrophages Hepatocytes	Cardiovascular Liver
Arachidonic acid metabolites	Endothelial cells Macrophages Neutrophils Platelets	Cardiovascular Gut Lungs Liver
Platelet Activating factor	Endothelial cells Macrophages Neutrophils	Cardiovascular Gut Lungs
Tumor necrosis factor	Endothelial cells Macrophages	Cardiovascular Liver Lungs
Interleukins	Endothelial cells Smooth muscle cells Macrophages	Cardiovascular

[a], See text of Chapter 1 for description and references
[b], See text of Chapter 2 for description of mediators involved in LPS-induced liver injury and Chapter 1 for mediators involved in alterations in extrahepatic tissues

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defined (59a). However, arachidonic acid metabolites appear to be involved in certain instances. This is supported by evidence which indicates that the induction of TNF gene expression by LPS in the HL-60 macrophage-like tumor cell line can be blocked by inhibitors of phospholipase A_2 or 5-lipoxygenase (51). This suggests that LPS-induced alterations in TNF-*alpha* gene expression are dependent on a 5-lipoxygenase product of arachidonic acid.

Increases in phosphatidylinositol metabolism (60), intracellular Ca^{2+} concentration (60), and protein kinase-C (PK-C) activity (61,62) have also been reported after exposure of cells *in vitro* to either LPS or lipid A. In addition, inhibitors of protein kinase C blocked expression of TNF-*alpha* and interleukin (IL)-1 mRNA in primary cultures of mice macrophages treated with LPS *in vitro* (62a). Thus, activation of Ca^{2+}-dependent PK-C appears to play a role in the response of cells to LPS in certain instances. Calmodulin kinase also appears to contribute to expression of IL-1 mRNA in LPS-treated macrophages (62a). Signal transduction after LPS exposure by these second messenger pathways is probably mediated by alterations in protein phosphorylation (62b). Possible signal transduction pathways contributing to the response of cells to LPS are illustrated in figure 1.2.

Figure 1.2 Interactions of LPS with mammalian cells.

A: [1], Binding of LPS-coated particles (eg. intact gram-negative bacteria) to CD18 antigen on phagocytic cells; [2], Serum LPS-binding protein-mediated binding of LPS-coated particles or cell-free LPS to CD14 antigen on macrophages; [3] and [4], direct binding of cell-free LPS to 95 and 80 kDa proteins, respectively, on the surface of certain cells; [5], nonspecific interaction of cell-free LPS with plasma membrane lipids.

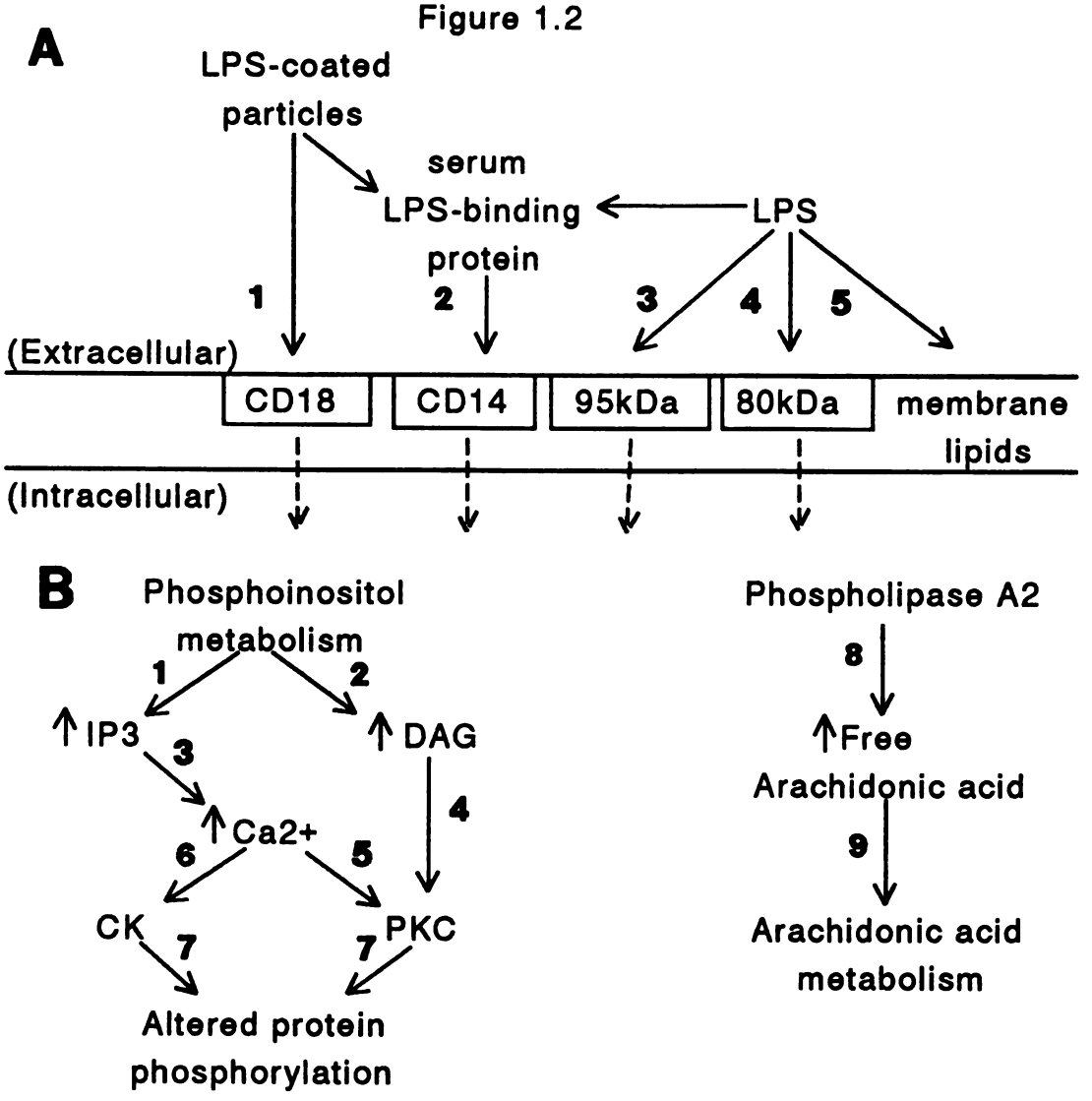
B: Interaction of LPS with plasma membrane receptors initiates transmembrane signal (dashed lines) which may ultimately lead to a response by several intracellular signal transduction pathways:

Stimulation of phosphoinositol metabolism generates inositol trisphosphate (IP3) [1] and diacylglycerol (DAG) [2]; IP3 can mediate an increase in intracellular Ca^{2+} [3]; activation of protein kinase C (PKC) by DAG [4] and Ca^{2+} [5] and/or activation of calmodulin kinases (CK) by Ca^{2+} [6] may contribute to altered protein phosphorylation [7].

Activation of phospholipase A_2 liberates arachidonic acid from membrane phospholipids [8] resulting in the formation of biologically active arachidonic acid metabolites [9].

Figure 1.2

Figure 1.2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LPS can interact with cells by either specific, receptor-mediated binding or by non-specific membrane interactions. Several cell surface receptors have been identified which bind LPS, and the type of receptor-mediated binding is dependent on the nature of LPS. Thus, cell-free LPS (ie, LPS purified from gram-negative bacteria) binds to receptors which are distinct from those that bind cell-associated LPS (ie, LPS associated with gram-negative bacteria). Also, complexes formed between cell-free or cell-associated LPS and a specific serum LPS-binding protein (complexed LPS) bind to different receptors than either form of LPS in the absence of complex formation. The different interactions of LPS with cells are summarized in Figure 1.2.

Binding of LPS to cell surface receptors. At least two apparently different cellular receptors for cell-free LPS have been identified. One receptor, which was first identified in splenocyte isolates, is an 80 kDa protein (63). An analysis of subpopulations of splenocytes showed that this receptor was present on both B and T lymphocytes and on macrophages. However, it was not found on human red blood cells or the undifferentiated murine myeloma cell line, Sp2/0. The second receptor has a molecular weight of 95 kDa and was initially identified in the RAW 264.7 and J774A.1 macrophage-like cell lines (64). Subsequent studies with human blood leukocytes indicated that an identical

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receptor was present on human blood monocytes (65). Human neutrophils (PMNs) as well as the fibroblast cell lines, CHO-K1 and L929, did not have detectable 95 kDa receptor. This indicates that, like the 80 kDa receptor, the 95 kDa receptor exhibited cell specificity. Both receptors recognize the lipid A region of the LPS molecule, are associated with the cell membrane fraction, are sensitive to proteinase K digestion, and exhibit specificity and saturability (64,66).

Several LPS-induced responses appear to be mediated by the binding of cell-free LPS to cell receptors. Among these is the stimulation of tumoricidal activity by macrophages. Macrophages lyse tumor cells after exposure to LPS *in vitro*, and this tumoricidal activity is markedly enhanced by the cytokine, *gamma*-interferon. A monoclonal antibody raised against the 80 kDa receptor blocks binding of LPS to macrophages and stimulates tumoricidal activity by macrophages (67,68). As with LPS, the tumoricidal activity induced by this monoclonal antibody is enhanced by *gamma*-interferon. These results are consistent with the role of the 80 kDa receptor in LPS-induced macrophage tumoricidal activity. Binding to the 80 kDa protein also appears to be required for the induction of B lymphocyte mitogenesis by LPS *in vitro* (66).

The interaction of cell-associated LPS with certain mammalian cells depends on the presence of the CD18 group of adhesion molecules (69,70). These cell surface

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glycoproteins, which are present on phagocytic cells such as macrophages and PMNs, mediate a variety of functions including adherence to surfaces and phagocytosis of particles coated (opsonized) with complement-derived factors and antibodies (71). Interaction of cell-associated LPS with CD18 does not require prior opsonization by complement-derived factors or antibodies and appears to be important for the phagocytosis of gram-negative bacteria by phagocytic cells.

Binding mediated by LPS-binding protein in serum. Although both cell-free and cell-associated LPS can interact directly with specific mammalian cell receptors (80 and 95 kDa and CD18 receptors, respectively), the cellular interaction of both forms of LPS may be modified by complex formation with an LPS-binding protein found in serum (complexed LPS). This 60 kDa acute phase glycoprotein, which is present in low concentrations in serum of normal animals, is synthesized by hepatocytes and increases in concentration during the acute phase response (72,73). It has a high affinity for LPS from a variety of gram-negative bacterial strains and appears to recognize the lipid A region of the LPS molecule (74). Interactions between LPS and macrophages which are mediated by this serum protein are dependent on the macrophage CD14 antigen. This is illustrated by evidence which indicates that binding can be blocked by monoclonal antibodies to CD14 (75).

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Formation of a complex with the 60 kDa acute phase LPS-binding protein markedly enhances the response of isolated macrophages to LPS as indicated by enhanced tumoricidal activity. Monoclonal antibodies to CD14 antigen block the induction of macrophage tumoricidal activity by these complexes, providing further evidence that the interaction of complexed LPS with macrophages is mediated by this cell surface protein (76). Phagocytosis of gram-negative bacteria is also enhanced by 60 kDa LPS-binding protein, which acts as an opsonin apparently by binding to LPS on the surface of the bacteria (77). These studies indicate that, while LPS can affect macrophage function through the direct interaction with cell receptors, these effects are modified by complex formation with the serum LPS-binding protein. This LPS-binding protein also may mediate effects of LPS on other cells, including neutrophils (78).

Nonspecific interactions with membranes. Although the response of cells to LPS in many instances is mediated by the presence of specific cell surface receptors, LPS can bind to cells by nonspecific interactions with the plasma membrane (79). However, it is not clear whether or not this interaction results in a biological response in these cells.

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1.6 Pathophysiologic effects of LPS

An array of pathophysiologic alterations have been attributed to LPS. Among these are circulatory shock, disseminated intravascular coagulation (DIC), and damage to several organs, including the heart, kidneys, gastrointestinal tract, lungs and liver. With the multitude of pathophysiologic effects, it is not surprising that, a high mortality rate accompanies severe endotoxemia, with death often ensuing within 48 hours.

The mechanisms contributing to the manifestation of these alterations are diverse and vary among tissues. Nevertheless, a common motif appears to be the involvement of host-derived, soluble mediators. A variety of endogenous mediators have been implicated in the pathogenesis of LPS-induced alterations. Many of these are listed in Table 1.1 along with their possible sources and example tissues which they may affect. The following section describes briefly the mechanisms contributing to LPS-induced alterations in several tissues with an emphasis on the role of these host-derived mediators.

1.6.a Circulatory shock

Changes in the circulatory system during severe endotoxemia are characterized by marked alterations in cardiac output, mean arterial blood pressure, and organ blood flow.

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Decreased cardiac output. Within 1 hour after bolus iv injection of LPS in pigs, cardiac output falls dramatically (80,81). A brief rebound period is often observed approximately 1 hour after LPS administration. This is followed by a second, sustained decrease in cardiac output which is more gradual in onset in comparison to the initial phase. The early, transient change in cardiac output appears to be mediated by metabolites of arachidonic acid, since it can be blocked by inhibitors of arachidonic acid metabolism (82). In contrast, LPS-induced decrease in myocardial contractility seems to play an important role in the the later stage of altered cardiac output (83). Several other factors have been proposed to be involved as well, including decreased venous return.

Hypotension. A marked decrease in mean arterial blood pressure is also observed during severe endotoxemia (84). LPS-induced hypotension is associated with a decrease in vascular contractility. This alteration in vascular contractility occurs prior to the manifestation of hypotension and is characterized by hyporesponsiveness of vascular preparations to vasoconstrictors such as KCl, norepinephrine, and 5-hydroxytryptamine (84). It is not dependent on vascular endothelium or arachidonic acid metabolism. However, it does depend on protein synthesis (85) and is accompanied by an increase in Ca^{2+}-independent nitric oxide (NO) synthase activity in vascular smooth

muscle cells (59,86). Because NO is a potent vasodilator, it has been proposed that the aberrant production of nitric oxide (NO) by smooth muscle cells contributes to the alterations in vascular contractility after LPS exposure. Indeed, antagonists of NO vasodilation block the inhibitory effects of LPS on vascular contractility *in vitro* (59) and attenuate the hypotension observed during LPS exposure *in vivo* (87). These results support a role for endogenously derived NO in LPS-induced hypotension. Several other endogenous, vasoactive mediators may be involved as well. These include lipid mediators such as platelet activating factor (88) and arachidonic acid metabolites (89,90,91), endothelium-derived reactive oxygen metabolites (92), and cytokines such as tumor necrosis factor (93) and interleukin 1 (94,95).

Other circulatory alterations. In addition to alterations in cardiac output and vascular contractility, LPS exposure is often accompanied by a redistribution of blood flow (96,97). An increase in vascular permeability in several tissues, particularly in the lungs and gastrointestinal tract, also occurs. Several vasoactive agents have been implicated in the pathogenesis of these changes, including arachidonic acid metabolites, such as thromboxane A_2 and sulfidopeptide leukotrienes, and platelet activating factor (see below).

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1.6.b Disseminated intravascular coagulation

LPS can activate the coagulation system *in vitro* by both the extrinsic and intrinsic pathways through the release of tissue factor (98) and the activation of factor XII, respectively (99). Disseminated intravascular coagulation (DIC) resulting from activation of the coagulation system *in vivo* occurs under certain conditions during LPS exposure, indicating that coagulation can be activated by LPS *in vivo* (100). DIC is characterized by a reduction in circulating platelet numbers, depletion of clotting factors and plasma fibrinogen, and deposition of fibrin within the microcirculation of various tissues. Although its role in the pathogenesis of circulatory shock and lethality is not clear (101,102,103), DIC does appear to contribute to alterations in tissues during LPS exposure under certain circumstances, including injury to the lungs (see Section 1.6.c), to the liver (see Chapter 2, Section 2.3.b) and to the kidneys, where it can cause deposition of fibrin in the glomerular capillary bed with subsequent acute tubular necrosis (100).

1.6.c Multiple organ damage

In addition to circulatory effects and DIC, alterations in numerous tissues are often associated with severe, systemic endotoxemia.

Gastrointestinal tract. A spectrum of changes in the gastrointestinal tract are associated with LPS exposure. These changes resemble ischemic bowel necrosis and are characterized by the detachment of the epithelial cells from the mucosal basement membrane, inflammatory cell infiltration, vascular congestion, and an increase in vascular permeability (104). The mechanisms involved in the pathogenesis of tissue injury have not been clearly elucidated. However, results from several studies suggest that injury to the gastrointestinal tract during LPS exposure may be mediated by certain endogenous factors, including leukotrienes. Leukotrienes are synthesized from arachidonic acid via the 5-lipoxygenase pathway. These sulfidopeptide metabolites of arachidonic acid are potent vasoactive substances and promote increased vascular permeability in certain tissues (105). In the gut, leukotriene D_4 and E_4 receptor antagonists attenuated the increase in vascular permeability during LPS exposure, suggesting that this alteration is mediated by sulfidopeptide leukotrienes (106).

PAF also appears to contribute to the pathogenesis of LPS-induced gastrointestinal injury. This is supported by studies showing that PAF concentrations in the gastrointestinal tract are increased during LPS exposure (107), that PAF receptor antagonists afford protection against the tissue injury (104), and that tissue injury resembling LPS-induced injury to the gut is produced by

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exogenously administrated PAF (108). PAF may mediate injury to the gastrointestinal tract in part through the stimulation of production of the arachidonic acid metabolite, thromboxane A_2 (109).

Lungs. Alterations in the lungs during LPS exposure have been well characterized. Pulmonary hemodynamic alterations during LPS exposure are biphasic in nature (80,81,82). The initial changes in the lung occur prior to 1 hour after LPS administration and are characterized by a marked increase in pulmonary artery pressure and pulmonary vascular resistance. This early, pulmonary hypertensive phase is associated with an increase in the lung lymph of breakdown products of thromboxane A_2 (110) suggesting that this vasoactive arachidonic acid metabolite contributes to the early, pulmonary hypertension. The demonstration that inhibitors of arachidonic acid metabolism attenuate the increase in pulmonary artery pressure during this phase is consistent with this view (82). Inhibitors of the coagulation system markedly attenuate the increase in pulmonary vascular resistance, suggesting that clotting factors may contribute to the hemodyamic changes in the lungs following LPS exposure as well (111).

The early changes in pulmonary artery pressure are followed by an increase in vascular permeability which is associated with PMN infiltration, increased plasma protein leakage, and increased lung lymph flow. Although pulmonary

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artery pressure returns toward normal, it remains elevated throughout this second, permeability phase of lung injury. Unlike the gastrointestinal tract, leukotrienes do not appear to mediate the LPS-induced increase in vascular permeability in the lungs in the rat (106). Because catalase and depletion of circulating PMNs attenuate the increase in protein leakage and lung lymph flow, it has been proposed that PMN-derived oxygen radicals play an important role in the pathogenesis of LPS-induced vascular injury in the lungs (81,112). PAF also appears to contribute to the LPS-induced alterations in lung vascular permeability, since the permeability changes are associated with an increase in pulmonary PAF concentration and since PAF receptor antagonists attenuate the increase in pulmonary vascular permeability (113,114,115,116).

In addition to alterations in pulmonary hemodynamics, alterations in pulmonary mechanics are observed during LPS exposure. Among these are a decrease in lung dynamic compliance, an increase in the alveolar/arterial oxygen gradient and an increase in alveolar dead space ventilation. Many of these alterations in pulmonary mechanics are attenuated by the 5-hydroxytryptamine (5-HT) receptor antagonist, ketanserin, suggesting that 5-HT contributes to their development (80). Activation of the clotting system by LPS may also contribute to alterations in pulmonary mechanics (117).

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A number of factors have been shown to influence the response to LPS *in vivo* including species differences (118,119), route of exposure (120), dosing protocol (ie, bolus injection or infusion), and age (121). Also, large differences in potency are observed between LPS obtained from different species and strains of gram-negative bacteria. While the outcome of LPS exposure may vary under different conditions, it is clear that many of the adverse effects of LPS are dependent on endogenous mediators generated in response to LPS exposure (see previous section). Indeed, in many instances it seems that a network of host-derived mediators may be required for the full manifestation of the response to LPS. The relationship between these mediators in the pathogenesis of LPS-induced effects on mammalian tissues, however, remains to be determined.

The overall objective of this dissertation was to examine the mechanisms contributing to the pathogenesis of LPS-induced liver injury. Therefore, the following chapter will describe in somewhat greater detail the effects of LPS on the liver. Because many of the adverse effects of LPS appear to be mediated by endogenous factors, attention will be given to the possible contribution of certain host-derived mediators.

CHAPTER 2

LPS-INDUCED LIVER INJURY

2.1 Normal liver function and structure

The liver performs a number of important functions. Among these are maintenance of blood glucose levels, storage of nutrients, fat metabolism, and synthesis and secretion of bile constituents and plasma proteins. It also plays an important role in the elimination and detoxification of xenobiotic agents. These functions are performed primarily by hepatic parenchymal cells, which in humans comprise approximately 80 % of the cells in the liver (122). The remaining liver cells consist of a heterogeneous population of cells which includes fenestrated vascular endothelial cells, resident vascular macrophages (Kupffer cells), fat storage cells (Ito cells), and bile duct epithelial cells. Collectively, these cells comprise the non-parenchymal cell fraction of the liver (123).

The basic structural unit of the liver, where hepatic cells come in close contact with blood, is the liver lobule (Figure 2.1). The lobule consists of a region of parenchymal cells surrounding a central vein. It is bounded by several portal triads which consist of branches of the portal vein, hepatic artery and bile duct. Parenchymal cells in the central vein and portal triad regions of the liver lobule are referred to as centrilobular or periportal parenchymal

Figure 2.1 The liver lobule. Blood enters the liver lobule from branches of the portal vein (PV) and hepatic artery (HA) and travels between cords of hepatic parenchymal cells, or hepatocytes (HC), through hepatic sinusoids which are lined by fenestrated endothelial cells (EC); numerous stellate-shaped hepatic intravascular macrophages, or Kupffer cells (KC), are found within the sinusoids. Blood empties from the sinusoids into a central vein (CV) and is carried ultimately to the inferior vena cava. Bile synthesized by HCs is secreted into bile cannaliculi (not shown) and empties from the liver lobule into branches of the bile duct (BD).

Figure 2.1

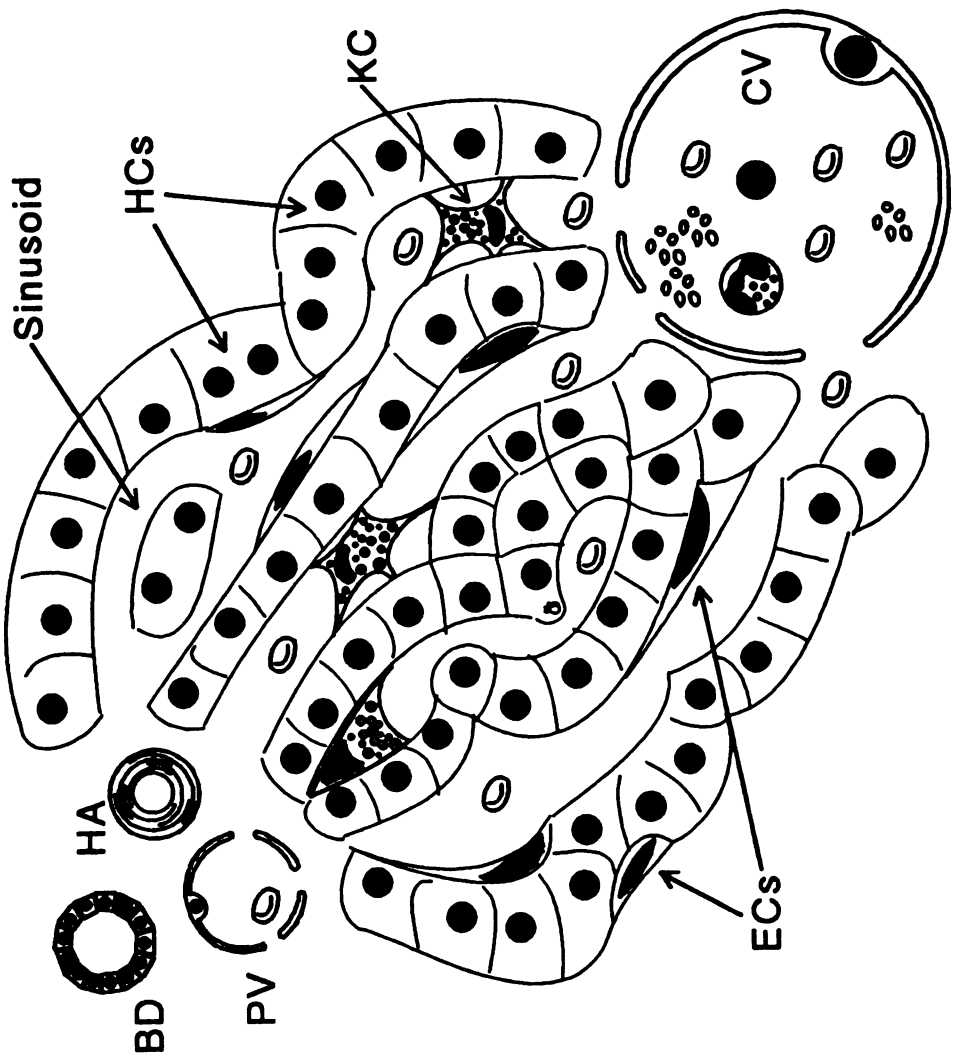

Figure 2.1

cells, respectively. Midzonal parenchymal cells are those cells located between the periportal and centrilobular regions. Blood flows through sinusoids from the periportal to the centrilobular region of the liver lobule between cords of parenchymal cells. In humans, the portal vein and hepatic artery provide approximately 75 and 25 % of the total hepatic blood supply, respectively (124). Fenestrations in endothelial cells lining the sinusoids facilitate contact between parenchymal cells and plasma constituents. Kupffer cells are scattered throughout the sinusoids. These fixed vascular macrophages remove senescent blood cells as well as debri and foreign substances, such as LPS, from the circulation. After flowing through the liver lobule, blood empties into the central vein and eventually into the inferior vena cava. Bile produced by parenchymal cells flows toward the periportal region of the liver lobule in bile canaliculi formed by tight junctions between adjacent parenchymal cells. Canaliculi empty into branches of the bile duct located in the periportal region.

2.2 Morphologic and functional alterations in the liver after LPS

Severe gram-negative bacterial infection results in a variety of changes in the liver (125). Because these changes resemble those produced by purified LPS (121,126,127), and because LPS has been shown to be an important contributing

factor to the manifestation of many of the pathophysiologic effects associated with gram-negative infections (see above), it seems likely that LPS contributes to the pathogenesis of liver injury associated with gram-negative bacterial infections.

2.2.a Morphologic alterations

Marked morphologic changes occur in the liver following exposure to LPS. These include changes in the sinusoids as well as in parenchymal cells (126,127,128).

Sinusoidal changes. Morphologic alterations in Kupffer cells are among the earliest changes in the hepatic sinusoids. Initially, these cells appear moderately swollen and contain an increased number of cytoplasmic lysosomal granules and phagocytic vacuoles. Platelets and PMNs are occasionally observed within phagocytic vacuoles of Kupffer cells. Dilation of Kupffer cell endoplasmic reticulum and damage to the plasma and nuclear membranes are also apparent. The early changes in Kupffer cell morphology are accompanied by injury to endothelial cells and by the appearance of fibrin clumps and platelet thrombi in the sinusoids. The platelets often appear deformed and show signs of degranulation. Large numbers of PMNs also accumulate in the sinusoids during LPS exposure. These morphologic alterations in sinusoids, which are evident as

early as 30 min following LPS administration, become augmented by 4 hr and often persist for at least 24 hr.

Changes in parenchymal cells. Morphologic changes in liver parenchymal cells are associated with the sinusoidal changes. Initially, subtle alterations occur in the mitochondria and endoplasmic reticulum. These changes, which are characterized primarily by moderate dilation of the organelles, occur prior to 1 hr and become progessively worse after 1 hr of LPS exposure. Other early morphologic changes in parenchymal cells include swelling of the microvilli on the sinusoidal border and dilation of bile canaliculi. Subsequent changes in parenchymal cells become apparent betweem 4 and 24 hr after LPS administration and are characterized by signs of hepatocellular degeneration and necrosis. Lesions are multifocal, frequently involve parenchymal cells in the midzonal region of the liver lobule, and are often infiltrated by neutrophils. Increases in plasma activities of liver-specific enzymes, such as AST and ALT, occur between 4 and 8 hrs after LPS exposure, suggesting membrane damage to parenchymal cells (102,129). The morphologic changes in the liver during LPS exposure are more marked in older animals, indicating that the sensitivity of the liver to LPS increases with age (121).

2.2.b Functional alterations

Glycogen content in parenchymal cells was markedly decreased 30 min after LPS exposure and remained reduced for 24 hr (126). This is probably due to both a decrease in gluconeogenesis and to an increase in glycogenolysis (130,131). In contrast, parenchymal cell fat content gradually increased over the 24 hr exposure period. Increased hepatic lipogenesis as well as inhibition of parenchymal cell protein synthesis may contribute to this effect (132,133). These changes indicate that LPS induces functional and metabolic alterations in parenchymal cells that are rapid in onset and persistent.

Alterations in parenchymal cell protein synthesis are observed during LPS exposure. Protein synthesis can be either increased or decreased following LPS administration depending on the specific protein measured. However, the net effect of LPS on hepatic parenchymal cells *in vitro* is to decrease protein synthesis (133).

Liver cytochrome P-450 content is reduced 24 hr after LPS administration in mice and rats (134,135,136). This is accompanied by a decrease in hepatic mixed function oxidase activity. Other functional changes in the liver include cholestasis (23) and circulatory alterations such as decreased hepatic blood flow (137,138) and portal hypertension (139,140).

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2.3 Mechanisms of LPS-induced liver injury

While the hepatic alterations produced by LPS have been well characterized, much remains unknown about the mechanism of LPS hepatotoxicity. Evidence suggests that alterations in the liver during LPS exposure may be due to both direct and indirect (host-mediated) effects of LPS on the liver.

2.3.a Direct effects of LPS on the liver

After intravenous administration of large doses of LPS, the majority of liver-associated LPS is found in Kupffer cells (20,21,22,23). However, small quantities are detected in other liver cells, including endothelial cells and parenchymal cells. Although many of the effects of LPS appear to be mediated indirectly by endogenously derived factors, LPS binds to liver parenchymal cells *in vitro* (79), raising the possibility that, direct, LPS-induced alterations may also contribute to some hepatic alterations.

LPS-induced cholestasis. Among the hepatic alterations which appear to be directly mediated by LPS is cholestasis (23). Perfusion of isolated livers with concentrations of LPS greater than 5 ug/ml results in cholestasis (141). This is characterized by a decrease in bile flow as well as by a decrease in bile clearance of the dyes, sulfobromophthalein (BSP) and indocyanine green (141,142). LPS also caused a decrease in the perfusate flow, suggesting that the

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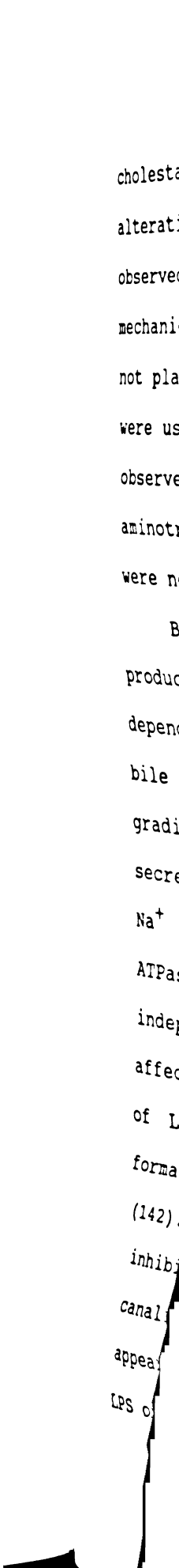

cholestatic effect and decreased dye clearance were due to alterations in tissue perfusion. However, no cholestasis was observed when flow was reduced to a similar degree mechanically in the absence of LPS (141). Blood elements did not play a role in this effect since buffer-perfused livers were used. Furthermore, because decreases in bile flow were observed in the absence of significant changes in perfusate aminotransferase activity, the cholestatic effects of LPS were not due to hepatocellular injury.

Bile is produced by liver parenchymal cells. Bile production can be divided into two fractions, bile salt-dependent and bile salt-independent fractions (143). The bile salt-dependent fraction is formed by an osmotic gradient established in the bile canaliculus by the active secretion of bile acids. In contrast, active transport of Na^+ into the bile canaliculus by parenchymal cell Na^+/K^+-ATPase presumably mediates the formation of the bile salt-independent fraction. Because LPS reduces bile flow without affecting secretion of bile salts, the cholestatic property of LPS is thought to be due to an alteration in the formation of the bile salt-independent fraction of bile (142). This is supported by evidence indicating that LPS inhibits Na+/K+-ATPase activity in partially purified bile canalicular membranes (144). Thus, LPS-induced cholestasis appears to be due, at least in part, to a direct effect of LPS on parenchymal cell bile formation.

LPS-induced hepatic lipid metabolism. Alterations in hepatic lipid metabolism may also result from a direct effect of LPS on the parenchymal cell. Exposure of isolated parenchymal cells to low concentrations of LPS results in an increase in cellular neutral lipid content (132). Increased secretion of neutral lipids is also observed following exposure of parenchymal cells to LPS *in vitro*. These changes are accompanied by an increase in the incorporation of radiolabeled acetate into neutral lipids, suggesting that they are due to an increase in *de novo* lipid synthesis. Thus, direct, LPS-induced alterations in parenchymal cell lipid metabolism may contribute to the accumulation of lipids in the liver as well as to the hyperlipidemia associated with LPS exposure *in vivo*.

2.3.b Indirect (host-mediated) effects of LPS on the liver

Like LPS-induced injury to other tissues (see Chapter 1), several host cells and endogenous mediators may play a role in the pathogenesis of LPS hepatotoxicity. These include circulating PMNs, hepatic fixed macrophages, cytokines, clotting factors, and arachidonic acid metabolites.

Role of PMNs. PMNs originate in the bone marrow from promyelocytic stem cells. After maturation, which occurs primarily in the bone marrow of normal individuals, these

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cells are released into the blood where they join lymphocytes, monocytes, eosinophils and basophils to form the circulating blood leukocyte population. Circulating numbers of PMNs normally range from 20-30% of the total number of white blood cell in rats to 40-75% in humans.

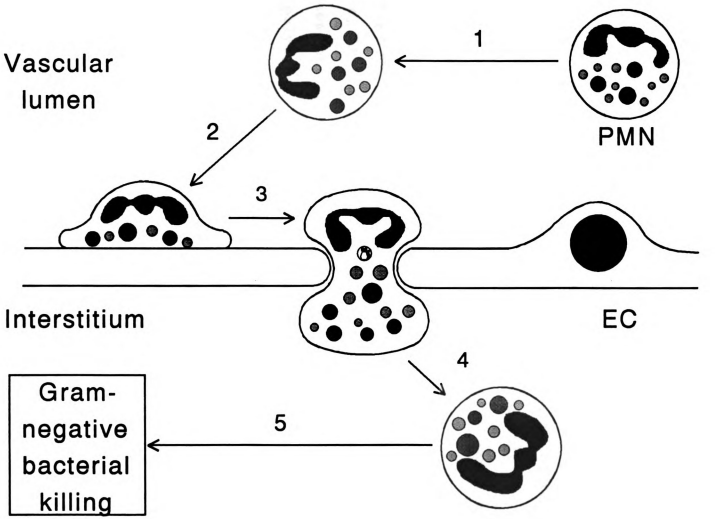
PMNs play an important role in the defense of the host against infection by certain pathogens including gram-negative bacteria. The importance of PMNs in the host's defense against gram-negative bacterial infections is illustrated by studies which show that inhibition of bacterial growth is temporally related to the accumulation of PMNs at the site of infection, and that bacterial growth at these sites is uninhibited in animals previously depleted of circulating PMNs (145). Also, patients with deficiencies in PMN numbers or function exhibit an increased susceptibility to infection by bacteria (146,147,148).

The response of PMNs to gram-negative bacterial infections has been studied extensively, particularly in tissue such as the skin (145). The PMN response involves a complex series of events which include accumulation of PMNs at the site of infection followed by microbial killing. PMN accumulation can be subdivided into chemotaxis, adherence, and diapedesis (Figure 2.2). Chemotaxis is the process of directed cell movement where cells such as PMNs migrate to specific locations along concentration gradients established by specific, soluble chemotactic factors. For example, in the case of gram-negative bacterial infections, chemotactic

Figure 2.2 Neutrophil (PMN) response to infection by gram-negative bacteria. Circulating PMNs are recruited at the site of infection by a series of events which include directed cell migration (chemotaxis) [1], adherence to vascular endothelial cells (EC) [2] and migration from the vascular lumen to the interstitium (diapedesis) [3]. PMNs become activated in the interstitium [4] to engulf (phagocytose) and kill the invading microorganism [5].

Figure 2.2

Figure 2.2


Vascular lumen
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PMN
2
3
Interstitium
EC
4
Gram-negative bacterial killing
5

factor concentration gradients direct PMNs toward the site of infection. Diapedesis is the process by which PMNs migrate from the vascular lumen to the interstitium. This is thought to require adherence of PMNs to the vascular endothelium. The microbicidal activity of PMNs can be subdivided into phagocytosis and cytotoxicity. Upon arrival at sites of infection, PMNs become activated and begin to engulf bacteria by the processes of phagocytosis. Bacteria contained within phagocytic vacuoles are subsequently killed and degraded by cytotoxic factors released into the phagocytic vacuole.

LPS appears to play an important role in eliciting the PMN response to infection by gram-negative bacteria. For example, LPS likely contributes to the accumulation of PMNs at sites of infection. This is supported by observations that polymyxin B and anti-LPS antibodies attenuate PMN accumulation and that PMN accumulation can be induced by injection of purified LPS (41,42,145). LPS is not directly chemotactic for PMNs *in vitro*. However, it can stimulate the production by host tissues of chemotactic factors such as the cytokine, interleukin 8 (149,150) and the complement factor, C5a (1). This suggests that LPS-induced production of endogenous chemotactic factors may contribute to PMN accumulation at the site of infection by gram-negative bacteria.

LPS may also contribute to PMN diapedesis during gram-negative bacterial infections by facilitating PMN adherence

to endothelial cells. This is supported by evidence indicating that LPS exposure induces the expression of PMN adhesion molecules on the surface of endothelial cells *in vitro* and increases the adherence of PMNs to cultured endothelial cell monolayers (53,54). LPS-induced production of the cytokines, IL-1 and TNF-*alpha*, may contribute to the adherence of PMNs to vascular endothelium as well (see below). Thus, LPS appears to mediate the accumulation of PMNs at sites of gram-negative bacterial infections by promoting adherence to the endothelium as well as by inducing production of chemotactic factors.

LPS has been shown to enhance the release of cytotoxic agents from activated PMNs *in vitro* in a serum-dependent manner (151). This raises the possibility that, in addition to its role in PMN accumulation, LPS may promote killing of gram-negative bacteria at sites of infection by inducing the activation of PMNs to produce microbicidal agents.

Following activation, PMNs are capable of releasing a variety of bactericidal agents intracellularly into phagocytic vacuoles or into the extracellular mileu. Among these agents are highly reactive oxygen metabolites, such as superoxide anion (O_2^-). Activation of PMNs is characterized in part by an increase in hexose monophosphate shunt activity and a burst of nonmitochondrial oxygen consumption (152,153). These activities reflect the activation of NADPH oxidase. This plasma membrane-bound enzyme utilizes NADPH derived from metabolism of glucose via the hexose

monophosphate shunt pathway to catalyze a one electron transfer to molecular oxygen to form O_2^-. O_2^- is released into the phagosome where it contributes to bacterial killing or extracellularly, where it may injure host tissue (see below).

While O_2^- may possess direct antimicrobial activities, its cytotoxic effects are thought to be due to the generation of more potent oxidants, such as hydrogen peroxide (H_2O_2) and hydroxyl radical. H_2O_2 can be formed from O_2^- by either a spontaneous or enzyme-catalyzed dismutation process. $\cdot OH$ is thought to be generated from O_2^- and H_2O_2 by an iron-catalyzed pathway. These oxygen metabolites can initiate peroxidation of lipids resulting in membrane damage. They may also cause disruption of cell function by inducing alterations in protein and DNA.

PMN activation is also accompanied by the fusion of lysosomal granules with phagocytic vacuoles. This process is refered to as degranulation and results in the release of lysosomal contents into the phagosome. Lysosomes contain a variety of proteases which may contribute to the microbicidal potential of PMNs. In addition, lysosomal enzymes, such as lysozyme, digest the cell wall of certain bacteria, whereas myeloperoxidase utilizes NADPH oxidase-generated O_2^- and chloride anions to catalyze the production of the powerful oxidant, hypochlorous acid, and chloramines. Other antibacterial agents released from the lysosomes include lactoferin, which chelates iron, and 60 kDa LPS-

binding protein, which can permeabilize gram-negative bacteria.

As indicated above, release of cytotoxic mediators from activated PMNs is not restricted to the phagosome. Extracellular release of these agents has been shown to occur *in vitro* under certain circumstances, and evidence from numerous studies suggests that this can result in damage to host cells *in vitro* and *in vivo* (153,154). Instances in which PMNs have been implicated in the pathogenesis of injury to host tissue include myocardial ischemia/reperfusion injury (155), complement-mediated lung injury (156), injury to the gastrointestinal tract induced by nonsteroidal anti-inflammatory drugs (156), certain instances of chemically induced liver injury (158), and liver injury following hypovolumic shock (159). In addition to these examples, PMNs mediate tissue injury following infection by gram-negative bacteria. For example, PMN depletion affords protection against injury to the microvasculature of the skin following intradermal injection of live *E. coli* (160). Similarly, PMN depletion attenuates the injury to the blood-brain barrier in gram-negative bacterial meningitis (40). This presents a paradox with respect to the role of PMNs at sites of gram-negative bacterial infections in that while they limit the growth of bacteria, they contribute to the pathogenesis of tissue injury associated with gram-negative bacterial infections.

In addition to their role in the pathogenesis of tissue injury at sites of gram-negative bacterial infections, PMNs mediate tissue injury in the lungs after intravenous administration of purified LPS (112, Chapter 1). Thus, exposure to LPS in the absence of gram-negative infection results in tissue injury which is mediated by PMNs. Because PMNs accumulate in the liver and are associated with foci of hepatocellular necrosis during exposure to LPS, it seems possible that these cells contribute to the pathogenesis of LPS hepatotoxicity as well. Results from one study which showed that heat killed *Pseudomonas aeruginosa* injure primary cultures of hepatic parenchymal cells in a PMN-dependent manner support this hypothesis (161). However, the role of PMNs in LPS hepatotoxicity *in vivo* remains to be determined.

Role of Kupffer cells. Kupffer cells are fixed, vascular macrophages which are scattered throughout the hepatic sinusoids. Like other resident tissue macrophages, Kupffer cells originate from promyloblast stems cells in the bone marrow (162). These precursor stem cells differentiate into monocytes in the bone marrow and are released into the circulation. Monocytes may circulate for several days before becoming sequestered in various tissues including the brain, skin, kidneys, lungs, spleen, and liver, where they undergo terminal differentiation to become tissue macrophages. Following differentiation, macrophages may reside in tissues

for extended periods of time. This contrasts with PMNs which are short-lived after emigrating from the vasculature.

Macrophages are phagocytic cells and, like PMNs, they contribute to the nonspecific immune response by phagocytosing and killing invading pathogenic microorganisms (162,163). The macrophage activation process and killing mechanisms are similar to those described for PMNs and include increased nonmitochondrial respiration and hexose monophosphate shunt activity, NADPH oxidase-catalyzed O_2^- production, and lysosomal enzyme release. Nitric oxide and various cytokines, including TNF-*alpha*, are also produced by activated macrophages under certain conditions. These agents possess tumoricidal activities *in vitro* and may contribute to the capacity of macrophages to kill neoplastic cells *in vivo*. In addition to their role in nonspecific immunity, macrophages perform important accessory functions in specific immune responses by processing and presenting antigens to B and T lymphocytes as well as by releasing lymphocyte mitogenic and activating factors, including the cytokine, IL-1.

It has been proposed that an important function of Kupffer cells is to filter the portal circulation of foreign substances and neoplastic cells derived from the gastrointestinal tract. This function is thought to be particularly important in preventing LPS originating from the gut from reaching the systemic circulation.

Although Kupffer cells may protect against systemic endotoxemia, evidence from several studies suggests that these cells also contribute to the manifestation of certain pathophysiologic alterations associated with LPS exposure including lethality. Several strains of mice are exceptionally hyporesponsive to the lethal effects of LPS. Among these is the C3H/HeJ mouse strain (164). Although the mechanism for this has not been clearly elucidated, genetic studies suggest that a defect in a single, autosomal dominant gene locus on chromosome 4, which is referred to as the *lps* locus, may be involved (165). In any case, results from several studies *in vitro* suggest that alterations in macrophage function may contribute to the altered LPS responsiveness exhibited by C3H/HeJ mice. For example, macrophages isolated from C3H/HeJ mice are defective in the release of several mediators following LPS exposure *in vitro*. Because these mediators, which include certain arachidonic acid metabolites (166), TNF-*alpha* (167) and nitric oxide (168), have been implicated in the pathophysiologic alterations associated with LPS exposure *in vivo*, this suggests that defects in macrophage function may contribute to the reduced responsiveness of LPS-resistant mice and that normal macrophage functions may be required for the manifestation of many LPS-induced pathophysiologic alterations.

A defect in phagocytic activity has also been observed by macrophages elicited from C3H/HeJ mice (169). *In vivo*,

fewer numbers of Kupffer cells exhibited phagocytic activity in C3H/HeJ mice compared to LPS-sensitive mice strains (170). This decrease was not due to a reduction in absolute numbers of Kupffer cells since histological stains specific for Kupffer cells revealed that their numbers in liver sections from sensitive and resistant mice were similar. Furthermore, phagocytic activity by Kupffer cells in the resistant mouse strain occured at a lower rate than in LPS sensitive mice strains, indicating that the efficiency of Kupffer cell function in the resistant mice was reduced as well.

Similar differences in Kupffer cell phagocytic activity were observed *in vivo* among animal species (118). A large variation in LPS sensitivity also exists among species. For example, guinea pigs are nearly 10 times more sensitive to the lethal effects of LPS than are rats. This species difference in LPS-induced lethality correlated with the number of Kupffer cells in the liver exhibiting phagocytic activity. Thus, guinea pigs had greater numbers of phagocytic Kupffer cells than rats. Furthermore, phagocytosis by Kupffer cells in less sensitive species occured at lower rates. It was proposed from these studies and from the studies in C3H/HeJ mice that sensitivity to LPS was dependent on Kupffer cell function. Consistent with this is evidence indicating that substances which increase Kupffer cell activity also increase LPS lethality (171,172). Conversely, substances which decrease Kupffer cell activity

protect against LPS-induced lethality. Interestingly, pretreatment with *Bacillus Calmette Guerin*, which markedly increases macrophage activity in normal mice, increased the sensitivity of LPS-resistant mice to LPS (173). These results are consistent with the role of hepatic macrophages in the lethal effects of LPS.

Kupffer cells may play a role in the pathogenesis of LPS hepatotoxicity as well. This is supported by studies showing that the increase in numbers of activated macrophages in the liver following pretreatment with *Corynebacterium parvum* is associated with a dramatic increase in the hepatotoxicity of LPS (174,175). Also, inhibition of Kupffer cell function with methyl palmitate affords protection against LPS hepatotoxicity (175). Because activated Kupffer cells can release several cytotoxic mediators (162), it has been suggested that damage to hepatic parenchymal cells following LPS exposure may be mediated by Kupffer cell-derived substances.

Further support for the role of Kupffer cells in LPS hepatotoxicity has been provided by studies *in vitro* using cocultures of primary liver parenchymal cells and Kupffer cells. Inhibition of protein synthesis by and cytotoxicity to cultured liver parenchymal cells induced by exposure to LPS occurs only in the presence of Kupffer cells (177). These effects are dependent on L-arginine, are associated with an increase in nitric oxide (NO) production in parenchymal cells, and are prevented by inhibitors of NO

synthesis (177). Because similar effects are observed by liver parenchymal cells incubated with supernatant from LPS-treated Kupffer cells, it was concluded that LPS induced the release of soluble factors from Kupffer cells which increased the production of NO by liver parenchymal cells (178,179). The increased production of NO subsequently resulted in decreased protein synthesis in and cytotoxicity to liver parenchymal cells. Although the Kupffer cell-derived factor has not been identified, evidence suggests that these effects may be mediated at least in part by cytokines released from LPS-treated Kupffer cells (179).

Although some studies support the role of Kupffer cells in the pathogenesis of LPS hepatotoxicity, results from other studies argue against this hypothesis. For example, NO synthesis inhibitors do not afford protection against liver injury following exposure to LPS *in vivo* suggesting that, whereas NO mediates injury to hepatic parenchymal cells *in vitro*, NO production does not contribute to parenchymal cell damage *in vivo* (180). Indeed, inhibition of NO synthesis actually enhanced LPS hepatotoxicity. Furthermore, intravenous administration to mice of liposome-encapsulated dichloromethylene diphosphonate, which effectively eliminates the Kupffer cell population of the liver, did not protect against LPS hepatotoxicity (181). Thus, despite evidence supporting the role of Kupffer cells in LPS-induced liver injury, the contribution of Kupffer cells remains unclear.

Role of cytokines. Interleukins, interferons (IFNs), and TNF are examples of soluble mediators comprising cytokines. These proteins are produced and secreted by a wide variety of cell types including monocytes and macrophages, lymphocytes, endothelial and vascular smooth muscle cells, fibroblasts, and hepatocytes (182).

A number of important physiologic functions have been attributed to cytokines (182,183). For example, they form a complex communication network among cells of the hematopoietic system in which they control the differentiation and maturation of blood cells from precusor stem cells in the bone marrow (184). Among the cytokines that have been implicated in the process of hematopoiesis are interleukins such as IL-3, IL-4, IL-5 and IL-7, and colony stimulating factors. Cytokines also contribute to both the specific and nonspecific immune responses by regulating lymphocyte proliferation, differentiation, and activation and by increasing the resistance to viral infection and neoplasia (185, 186, 187,188). Cytokines with immune system functions include various interleukins (most notably IL-1, IL-2, and IL-6), TNF, and the interferons.

Following tissue injury or infection, a shift in protein synthesis by the liver occurs which is characterized by a decrease in synthesis of certain plasma proteins such as albumin, and an increase in synthesis of the class of plasma proteins termed acute phase proteins (182). These proteins contribute to the regulation of the inflammatory

response and their synthesis is mediated by certain cytokines, including IL-1, IL-6 and TNF-*alpha*. This cytokine-induced alteration in plasma protein synthesis by the liver is thought to play a role in the acute phase response to gram-negative bacterial infection and LPS exposure.

Several cytokines, particularly IL-1, IL-6, IL-8, and TNF-*alpha*, appear to play an important regulatory role at sites of inflammation (188). For example, because IL-1 and TNF-*alpha* both induce procoagulant activity in cultured vascular endothelial cells, these cytokines may contribute to the initiation of coagulation at sites of inflammation *in vivo* (189). Furthermore, IL-1 and TNF-*alpha* induce the expression of PMN adhesion molecules on the surface of vascular endothelial cells, thus raising the possiblity that these cytokines contribute to the accumulation of PMNs at sites of inflammation (53,190,191). TNF-*alpha* induces the expression of CD11b/CD18 complex on the surface of PMNs as well (192). In as much as this complex mediates PMN adhesion to surfaces, TNF-*alpha* may mediate PMN accumulation at sites of inflammation by affecting PMNs in addition to affecting vascular endothelium. Finally, these cytokines stimulate the production of the potent cytokine chemotactic factor, IL-8, from host cells suggesting that they may promote PMN accumulation at sites of inflammation by inducing the production of endogenous chemotactic factors (150,193).

While cytokines perform beneficial functions in the hematopoietic and immune systems as well as during inflammation, evidence from numerous studies suggests that they may also mediate tissue injury under certain circumstances. Among the cytokines implicated in the pathogenesis of tissue injury is TNF-*alpha*. A wide variety of pathophysiologic alterations have been attributed to TNF-*alpha*. Exposure to recombinant TNF-*alpha* can produce severe hypotension, shock, and injury to various organs including the lungs and gastrointestinal tract (194,195). Pathophysiologic conditions in which TNF-*alpha* has been implicated include neurologic alterations associated with cerebral malaria (196), injury to the liver and lungs following hepatic ischemia/reperfusion (197) and lung injury following intestinal ischemia/reperfusion (17).

Exposure to LPS is associated with a transient increase in circulating TNF-*alpha* concentration which peaks approximately 1-2 hr after LPS administration (198). Results from several studies suggest that TNF-*alpha* may contribute to the pathophysiologic alterations accompanying LPS exposure. For example, neutralization of TNF-*alpha* with specific TNF antiserum affords protection againsts many LPS-induced alterations, including hypotension (93), lethality (199), and lung injury (17,200). Results from one study suggest that TNF-*alpha* may contribute to the pathogenesis of LPS hepatotoxicity as well (201).

The mechanism by which TNF-*alpha* mediates tissue injury is not known. However, lung injury that follows hepatic ischemia/reperfusion is accompanied by accumulation of PMNs in the lungs, and neutralization of TNF-*alpha* with TNF antiserum attenuates both the pulmonary PMN infiltration as well as lung injury, suggesting that TNF-*alpha*-induced PMN accumulation may contribute to the pathogenesis (197). This view is supported by the observation that PMN depletion attenuates lung injury induced by exposure to recombinant TNF-*alpha* (202). Thus, the ability of TNF-*alpha* to promote PMN accumulation in tissues may predispose the tissue to PMN-dependent injury. Although PMNs accumulate in the liver during LPS exposure, the relationship between hepatic PMN accumulation and TNF-*alpha* in the pathogenesis of LPS hepatotoxicity remains to be elucidated.

In addition to TNF-*alpha*, IL-1 and IL-6 may contribute to certain pathophysiologic alterations that accompany LPS exposure. Circulating levels of IL-1 and IL-6 are markedly elevated 2-6 hr after LPS exposure (198), and IL-1 receptor antagonists (94) and antiserum to IL-6 (202a) attenuated the lethality associated with exposure to purified LPS or infection by gram-negative bacteria, respectively. Thus, these cytokines appear to contribute to the pathogenesis of severe shock induced by LPS or gram-negative bacteria. They have also been implicated in the pathogenesis of tissue injury after LPS exposure in certain instances (202b,

202c,248). However, their role in LPS-induced liver injury, if any, remains to be determined.

Role of the coagulation system. It has been proposed that liver injury during LPS exposure is mediated by circulatory disturbances resulting from activation of the clotting system. Circulating fibrinogen concentration falls more than 90 % 3 hr after LPS administration to rats (203,204). This is accompanied by the appearance of fibrin clumps in the microcirculation of the liver. The role of the coagulation system in the pathogenesis of LPS hepatotoxicity has been implicated by evidence from several studies. For example, infusion of thrombin into the portal vein results in morphologic changes in the liver which resemble those produced by portal venous infusion of LPS. These changes include fibrin deposition and PMN accumulation in the sinusoids and hepatocellular necrosis (129). An evaluation of the time-course of changes in circulating transaminase activity indicated that the onset of liver injury was more rapid following thrombin infusion compared to LPS infusion. This may be due to the time required for generation of endogenous thrombin following activation of the coagulation system by LPS.

The role of thrombin and the coagulation system in the pathogenesis of LPS hepatotoxicity was strengthened by evidence from studies with heparin. Heparin is a naturally occuring anticoagulant which is widely distributed

throughout animal tissues. This complex proteoglycan is synthesized primarily by tissue mast cells and consists of mucopolysaccharide chains of varying length and number which are linked to a core protein (205). The anticoagulant properties of heparin are related to the mucopolysaccharide component of the molecule, which inhibits the thrombin-catalyzed conversion of fibrinogen to fibrin (206). This inhibitory effect is due to a heparin-induced increase in affinity of antithrombin III for thrombin (207). Results from several studies indicate that pretreatment with heparin prevents the fall in circulating fibrinogen concentrations after LPS exposure (203,204) and affords protection against the hepatotoxic effects of LPS (102). This protective effect is not due to an enhanced rate of clearance of LPS from the circulation in heparin-treated animals (208). These results are consistent with the hypothesis that the coagulation system plays a role in the pathogenesis of liver injury following LPS exposure.

Role of arachidonic acid metabolites. A number of products of arachidonic acid are released from cells treated with LPS *in vitro* (Table 1.1). Also, exposure to LPS *in vivo* results in marked increases in the circulating concentrations of several arachidonic acid metabolites, including thromboxane A_2 and several prostaglandins and leukotrienes (209). Arachidonic acid, or *cis*-5,8,11,14-eicosatetraenoic acid, is a 20-carbon, polyunsaturated fatty

acid containing 4 double bonds. It is an essential fatty acid since it can only be obtained from dietary sources or by synthesis from other essential fatty acids such as linoleic acid. Arachidonic acid is incorporated into membranes where it is esterified to phospholipids. Under certain conditions, such as LPS exposure, it is cleaved from membrane phospholipids by phospholipase A_2 and subsequently metabolized by two major pathways. The cyclooxygenase pathway generates prostaglandins and thromboxane whereas the 5-lipoxygenase pathway generates leukotrienes. These arachidonic acid products have potent, biological activities and, although they are important mediators of a variety of physiologic processes, aberrant production can result in pathophysiologic alterations in tissues (210).

Numerous alterations associated with LPS exposure have been attributed to arachidonic acid metabolite (Table 1.1). Evidence from several studies suggests that arachidonic acid metabolites may contribute to the pathogenesis of LPS-induced liver injury. For example, dexamethasone, which inhibits phospholipase A_2, afforded protection against LPS hepatotoxicity in galactosamine-sensitized mice (211). Rats maintained on a diet deficient in essential fatty acids were also resistant to the hepatotoxic effects of LPS (212). These rats were depleted of arachidonic acid, suggesting that the protective effect was due to the absence of arachidonic acid metabolites (213). This view is supported by evidence that essential fatty acid-deficiency prevents

the LPS-induced rise in circulating concentrations of certain metabolites of arachidonic acid (214).

Among the arachidonic acid metabolites that have been implicated in the pathogenesis of LPS hepatotoxicity is the potent vasoconstrictor and platelet aggregator, thromboxane A_2. Pretreatment with the thromboxane synthase inhibitors, imidazole and 7-(1-imidazolyl)-heptanoic acid, markedly attenuated the rise in circulating concentrations of thromboxane B_2 (a stable breakdown product of thromboxane A_2) as well as the increase in transaminase activity that followed LPS administration, suggesting that thromboxane A_2 may contribute to LPS hepatotoxicity (215). In contrast, liver injury following administration of LPS to animals pretreated with galactosamine, which markedly increases the sensitivity of animals to LPS, was not attenuated by either aspirin or ibuprofen (211). Since these cyclooxygenase inhibitors block thromboxane sythesis, this suggests that thromboxane A_2 does not play a role in LPS hepatotoxicity in mice sensitized to LPS by galactosamine. It also raises the concern that different mechanisms may be involved in the pathogenesis of LPS-induced liver injury in nonsensitized and galactosamine-sensitized animals.

Although thromboxane does not appear to play a role in LPS-induced liver injury in galactosamine-sensitized mice, leukotrienes, particularly leukotriene D_4, do (211). This is indicated by studies with leukotriene synthesis inhibitors, which attenuated liver injury in LPS-treated animals, and by

results with exogenously administered leukotriene D, which produced liver injury resembling LPS. The role of leukotrienes in LPS-treated animals in the absence of galactosamine pretreatment remains to be determined.

2.4 Overall aim

It is clear from the evidence presented in this chapter and in the previous chapter that a broad spectrum of pathophysiologic alterations accompany LPS exposure. Although some of these may be mediated by the direct effect of LPS on tissues, many appear to be mediated by host-derived factors. Indeed, in many instances, multiple endogenous mediators appear to be involved (Table 1.1). This seems to be the case with certain LPS-induced alterations in the liver where factors derived from Kupffer cells, arachidonic acid and the coagulation system have been implicated in the pathogenesis. The association of PMNs with foci of hepatocellular necrosis raise the possibility that factors released from these phagocytic cells may contribute to injury to the liver as well.

It is possible that these mediators act by separate, parallel mechanisms. That is, alterations in tissues after LPS exposure result from the sum of the effects of several different mediators. Alternatively, it seems possible that the pathophysiologic alterations associated with LPS exposure are the result of complex interactions among

endogenous mediators. In other words, each mediator by itself is not sufficient to cause the alteration. Such interactions might involve the potentiation of the effects of one mediator by a second mediator. Alternatively, one mediator may stimulate the production or activation of a second mediator. Thus, a series or cascade of interactions among several mediators may be required for the manifestation of LPS-induced alterations *in vivo*.

The overall objective of the following studies was to examine the involvement of several endogenous mediators in LPS hepatotoxicity, including circulating PMNs, TNF-*alpha*, and coagulation factors. Emphasis will be on the possible interactions among these various mediators in the pathogenesis of liver injury.

2.5 Specific aims

2.5.a Specific aim 1

Morphologic changes in the liver are characterized by the infiltration of large numbers of PMNs which are associated with foci of hepatocellular necrosis. Because PMNs have been implicated in the pathogenesis of tissue injury during LPS exposure in certain instances, this raises the possibility that these phagocytic cells contribute to the pathogenesis of LPS hepatotoxicity. Studies were performed to test this hypothesis. The results from these studies are presented in Chapter 3.

2.5.b Specific aim 2

TNF-*alpha* has been implicated in the pathogenesis of LPS hepatotoxicity. However, the mechanism by which this cytokine mediates liver injury is not known. TNF-*alpha* has several effects on PMN function which could contribute to the pathogenesis of LPS-induced liver injury. For example, TNF-*alpha* increases the adherence of PMNs to vascular endothelial cells and enhances the release of cytotoxic substances from activated PMNs *in vitro*. Thus, TNF-*alpha* may contribute to LPS hepatotoxicity by a PMN-dependent mechanism. Studies were performed to test this hypothesis. The results from these studies are presented in Chapter 4.

2.5.c Specific aim 3

Activation of the coagulation system with deposition of fibrin in the microvasculature of the liver occurs after LPS administration, and anticoagulants afford protection against LPS hepatotoxicity. This suggests that clotting factors may contribute to the pathogenesis of LPS hepatotoxicity. The objective of studies presented in Chapter 5 was to test the hypothesis that the coagulation system contributes to LPS-induced liver injury by a mechanism which is dependent on circulating fibrinogen. Interactions between PMNs, TNF-*alpha*, and the coagulation system were examined.

Results from studies in this dissertation contribute to the elucidation of the mechanisms of LPS hepatotoxicity.

Since LPS has been implicated in the pathophysiologic effects associated with gram-negative bacterial infections, and since alterations in the liver may play an important role in the high mortality associated with severe, systemic, gram-negative bacterial infections, an understanding of the mechanism of LPS-induced liver injury may facilitate the development of therapeutic interventions that could improve survival from infections by these bacteria.

CHAPTER 3

ROLE OF NEUTROPHILS IN LPS-INDUCED LIVER INJURY

3.1 Abstract

PMN infiltration is an early occurrence in the liver following exposure to hepatotoxic doses of LPS. The purpose of this study was to test the hypothesis that PMNs contribute to the pathogenesis of LPS hepatotoxicity. The immunoglobulin (Ig) fraction from serum of rabbits immunized with rat PMNs (anti-PMN Ig) was administered iv to rats 18 and 6 hr prior to exposure to an hepatotoxic dose of LPS. This protocol caused a >95 % reduction in circulating PMNs, which was maintained for the duration of the study. The Ig fraction from nonimmunized rabbits was used as a control (control Ig). Rats pretreated with control Ig exhibited a marked increase in the number of PMNs in the liver 1.5 hr after LPS exposure. This increase in hepatic PMNs was significantly reduced by pretreatment with anti-PMN Ig. Marked elevations in both ALT and AST activities were observed in plasma from control Ig-treated rats 6 hr after iv administration of LPS. The response to LPS was greatly attenuated in animals receiving anti-PMN Ig. Pretreatment of rats with Igs to rat lymphocytes (LCs) reduced numbers of circulating LCs but did not afford protection against the hepatotoxic effects of LPS. These results suggest that PMNs contribute to the pathogenesis of LPS hepatotoxicity.

3.2 Introduction

An array of morphologic and functional alterations in the liver have been attributed to LPS (216). Early morphologic changes occur in the sinusoids and include Kupffer cell swelling, formation of platelet thrombi, fibrin clumping and PMN infiltration (126,127). These changes become evident within 1 hr after LPS exposure and precede degenerative changes in parenchymal cells. Increases in serum activities of liver-derived enzymes also occur after LPS exposure, suggesting damage to liver parenchyma (102). Among the hepatic functional alterations associated with LPS exposure are decreased hepatic blood flow (137,138,141), cholestasis (23,141), and alterations in hepatic protein synthesis (217).

Although the effects of LPS have been well characterized, much remains unknown about the mechanisms of LPS hepatotoxicity. The early accumulation of PMNs in the liver after LPS exposure raises the possibility that these phagocytic cells contribute to LPS-induced liver injury. PMNs are an important cellular component of the host's defense system against invading pathogens. However, they have been shown to mediate tissue injury under certain conditions. For example, intradermal injection of *E. coli* LPS produces an acute inflammatory response which is associated with hyperemia, increased vascular permeability, and hemorrhage (41). PMN infiltration occurs concurrently

with the onset of these changes, suggesting that PMNs contribute to their development (40). This is supported by studies which indicate that damage to the skin does not occur in animals depleted of circulating PMNs (160).

Evidence from similar studies suggests that PMNs also contribute to lung injury following iv administration of LPS. The initial response of the lungs to LPS is increased pulmonary artery pressure (82). This hypertensive phase is transient in nature and is followed by injury to the vasculature which is characterized by an increase in vascular permeability (218). PMNs have been implicated in the pathogenesis of this second phase of the response, since PMN infiltration is temporally associated with the increased vascular permeability (219) and since injury to the vasculature is prevented by PMN depletion (112).

Although evidence suggests that PMNs contribute to the pathogenesis of LPS-induced injury in some tissues, their role in LPS hepatotoxicity *in vivo* has not been demonstrated. Results from one study showed that isolated hepatocytes were injured by heat-killed *Pseudomonas aeruginosa* only in the presence of PMNs (161). It was proposed that PMNs may contribute to the liver injury associated with exposure to this gram negative bacterium *in vivo*. Since many of the biological effects associated with gram negative bacterial infection are thought to be caused by LPS, it seemed possible that these effects in the liver were also mediated by LPS. The purpose of this study was to

test the hypothesis that PMNs contribute to the development of liver injury that follows exposure to LPS *in vivo*.

3.3 Materials and methods

3.3.a Animals

Female Sprague-Dawley rats (Charles River, Crl:CD BR (SD) VAF/plus, Portage, MI) weighing 200-250g were maintained on a 12 hr light-dark cycle for at least one week prior to use. Food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and water was allowed *ad libitum* throughout the studies. Female New Zealand white rabbits weighing 2-3 kg were obtained from Bailey's Rabbitry (Alto, MI) and maintained on high fiber Purina Lab Rabbit Chow (Purina Mills, St. Louis, MO).

3.3.b Treatment protocols

LPS (*E. coli* 0128:B12, Sigma Chemical Co, St. Louis, MO) was dissolved in sterile saline immediately prior to administration. It was administered in the tail vein as a single bolus injection in a volume of 5 ml/kg. The response to LPS varied considerably over the course of the studies. As a result, a dose of LPS was chosen for each study which produced consistent liver injury in the absence of significant lethality. The dose of LPS are indicated in the figure legends and ranged from 2 mg/kg in the time-course studies to 8 mg/kg in the lymphocyte (LC)-depletion studies.

A dose of 5 mg/kg LPS produced reproducible liver injury with minimal lethality over a 6 hr exposure period and was used exclusively in subsequent chapters. It should be noted that manipulations prior to LPS exposure which stress the animals (ie. increasing body temperature) may alter the sensitivity of animals to the hepatotoxic and lethal effects of LPS.

In an initial study to assess the effect of PMN depletion on LPS hepatotoxicity, rats were divided into two treatment groups. One group of rats received immunoglobulins (Ig) isolated from serum obtained from untreated rabbits (control Ig). The second group received Ig isolated from rabbits immunized with rat PMNs (anti-PMN Ig). Ig were administered in the tail vein in two separate injections of 0.4 and 0.3 ml, 18 and 6 hr prior to LPS administration, respectively. Both groups received LPS. Rats were anesthetized with diethyl ether 6 hr after administration of LPS (3 mg/kg), and blood samples anticoagulated with sodium citrate (3.8 % w/v sodium citrate in DDW diluted 1/10 with whole blood in the syringe) were obtained from the inferior vena cava for quantification of liver injury and WBC numbers. Liver injury was quantified by changes in plasma ALT and AST activities and total plasma bilirubin concentration. Liver samples were also obtained for morphologic evaluation of liver injury. A similar experimental design was used to examine the effect of PMN depletion on the development of LPS-induced liver injury

over a 24 hr period. In this study, rats were treated with 2 mg/kg LPS to ensure that the animals survived for the duration of the study.

In a separate study, PMN infiltration in the liver was assessed 1.5 hr after LPS exposure. Changes in the cellular composition of the NPC fraction of liver digests, which includes PMNs, were determined after administration of 5 mg/kg LPS or saline vehicle to rats pretreated with either control Ig or anti-PMN Ig. Liver digests were prepared, and NPCs were quantified as described in "Methods".

3.3.c Anti-PMN and LC Ig preparation

Rat PMNs were elicited from the peritoneum of male, Sprague-Dawley, retired breeder rats using a 1 % solution of glycogen in sterile saline as described previously (220). Rabbits were immunized against rat PMNs by injecting PMNs (1.0×10^6 cells/ml suspended in complete Freund's adjuvent) sc into the footpads. This was followed by booster injections of PMNs (1.0×10^6 PMNs suspended in incomplete Freund's adjuvent) in the dorsum area of the rump two and four weeks after the initial immunization. Blood (40-50 ml) was obtained from the central ear artery one week after the second PMN booster injection and allowed to clot. Total Igs were precipitated from serum using ammonium sulfate.

Anti-rat LC serum raised in rabbits was obtained from Accurate Chemical and Scientific Co (Westbury, NY). Total Ig were isolated from the serum by ammonium sulfate

precipitation as described above. Ig fractions from both control Ig and anti-LC Ig serum were incubated on ice for 1 hr with rat peritoneal PMNs (3.5 x 10^6 cells/ml) to remove Ig to PMNs. Following incubation, PMNs were pelleted by centrifugation for 10 min at 1000 x g, and the supernatant was stored at -20^{o}C prior to use.

The total Ig fraction of serum from rabbits was isolated by ammonium sulfate precipitation (221). A saturated ammonium sulfate solution was added drop-wise to serum using a separatory funnel until a 2:1 ratio of serum:ammonium sulfate solution was attained. Serum was mixed thoroughly with a magnetic stir bar during addition of ammonium sulfate. The suspension was stirred for an additional hour at 4^{o}C, spun in a centrifugation at 1000xg for 10 min, and the supernatant was discarded. The pellet was dissolved in sterile saline to the original volume of serum. This solution was precipitated as described above except that a 1:1 ratio of saturated ammonium sulfate solution was used. The precipitate was resuspended in saline to approximately half the orginal volume and dialyzed against saline using Spectrapor 2 dialysis tubing (12-14000 m. w. cutoff, Spectrum Medical Ind., Los Angeles, CA) for 2-3 days at 4^{o}C to remove contaminating ammonium sulfate. After dialysis, the volume was adjusted to its original volume with saline. Flocculent material was removed by spinning in a centrifuge at 1000 x g for 10 min. The supernatant, which contained Ig to rat PMNs (anti-PMN Ig),

was stored at -20°C prior to use. Total Ig from untreated rabbits were isolated in a similar manner and used as a control (control Ig).

3.3.d Evaluation of liver injury

Liver injury was quantified by changes in plasma ALT and AST activities and by changes in total plasma bilirubin concentration. Plasma ALT and AST activities were measured using the spectrophotometric procedure of Reitman and Frankel (222). Total plasma bilirubin concentration was measured spectrophotometrically using an extinction coefficient of 73000 following conjugation with p-diazobenzenesulfonic acid (223).

3.3.e Histopathologic evaluation

Samples of liver were fixed in 10 % buffered formalin and processed for histopathologic examination. Paraffin embedded sections were cut at 6 um and were stained with hematoxylin and eosin (H and E). Slides were randomized, coded and evaluated with light microscopy. A detailed description of the histologic lesions was provided by a pathologist. The severity of each lesion was graded as follows: no lesion (0), mild necrosis (1), moderate necrosis (2), marked necrosis with mild hemorrhage (3), severe necrosis with moderate hemorrhage (4), severe necrosis with marked hemorrhage (5).

3.3.f Morphometric analysis of hepatic lesions

Morphometric assessment of hepatic lesions was performed on coded liver sections. The area of hepatic tissue examined, the area of individual lesions and the number of segmented PMNs per area of tissue were determined with the aid of a Jandel Video Analysis System (Jandel Scientific, Corte Madera, CA) attached to a Nikon Microphot-FX microscope (Nikon Instrument Group, Oak Park, IL). The area of liver with lesions was calculated and expressed as % liver affected, and the number of lesions per area of liver was also determined. The injury index was defined as the product of the mean lesion area (mm^2) and the mean lesion severity score (see "Histopathologic evaluation"). The numbers of PMNs per unaffected and affected area of liver were determined by direct, microscopic examination of tissue and were expressed as cells per area of liver.

3.3.g Quantification of total and individual WBC numbers

Circulating total WBC numbers (cells/uL blood) were determined using a Coulter Counter Model ZM. Differential counts were performed on blood smears stained with buffered, differential Wright-Giemsa stain to obtain the fraction of PMNs and LCs in each blood sample. The total number of WBCs was multiplied by this fraction to obtain numbers of PMNs and LCs (cells/uL blood).

3.3.h Isolation of hepatic NPCs

Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) prior to surgery. The abdominal cavity was opened and a loose ligature was placed around the inferior vena cava proximal to the kidneys. It was severed distal to the ligature, and the liver was cleared of blood by infusing 40 ml of Ca^{2+}-free Hank's balanced salt solution (HBSS, pH 7.4) containing 0.5 M HEPES and 0.5 mM EGTA through a cannula (Intramedic PE-160 polyethylene tubing, Clay Adams, Parsippany, NJ) in the hepatic portal vein. The thoracic cavity was opened, and the superior vena cava was ligated. The liver was flushed with 10 ml HBSS containing Pronase E (0.2 % w/v Type XIV protease, Sigma Chemical Co, St Louis, MO), and the ligature around the inferior vena cava was tightened to isolate the liver from the systemic circulation. An additional 5 ml of HBSS containing Pronase E was infused into the portal vein. The liver was removed and minced with scissors into pieces that could be drawn up into a 5 ml pipette (Pipetman, Rainin Instrument Co, Woburn, MA) without plugging it. Thorough mincing was critical to the complete digestion of the liver. The minced liver was transfered to a 250 ml plastic flask containing 100 ml Ca^{2+}-free HBSS with 0.2 % Pronase E and incubated between 1.0 and 1.5 hr at $37^{o}C$ under 95 % O_2/5 % CO_2 in a shaking water bath. The solution was triturated twice during the incubation period using a 5 ml pipette to facilitate the disruption of the liver. After the incubation period, the

solution was filtered through gauze into 50 ml plastic tubes. The amount of tissue retained by the gauze was routinely small and consisted mostly of connective tissue and portions of the diaphram which were not separated from the liver prior to digestion. The cell suspension was spun in a centrifuge for 20 min at 500 x g, and the pellet was resuspended in 50 ml Ca^{2+}-free HBSS containing 0.5 M HEPES, 0.5 mM EGTA, and 1000 U/L sodium heparin (grade II, Sigma Chemical Co, St Louis, MO). This suspension, which contained hepatic non-parenchymal cells (NPC) and a small number of blood cells, was spun again for 20 min at 500 x g, and the pellet was resuspended in 25 ml Ca^{2+}-free HBSS containing heparin and used for cell enumeration.

3.3.i Cytologic examination of NPCs

Total nucleated NPCs/liver were quantified using a Unopette (test 5859, Becton-Dickinson, Rutherford, NJ) and a hemacytometer. Samples of the NPC isolates were diluted in Ca^{2+}-free HBSS containing heparin and 1 % bovine serum albumin (Sigma chemical Co, St Louis, MO), concentrated on a microscope slide using a cytocentrifuge (Cytospin 2, Shandon Southern Instruments, Sewickley, PA) and stained with a modified Wright's stain. Stained slides were coded, randomized, and a detailed cytologic analysis, including a 300 unit differential count, was performed. Absolute cell numbers were determined by multiplying the relative fraction

of each cell type by the total number of nucleated cells/liver.

3.3.j Data analysis

Results are expressed as means ± SEM. Comparisons between control Ig and anti-PMN Ig treatment groups were made using the Wilcoxon rank-sums test. In the PMN depletion time-course study, multiple comparisons within groups were made with the Wilcoxon rank-sums test utilizing Bonferroni's correction factor. Data from cytologic evaluations of NPCs isolated from liver digests were analyzed following transformation using a completely random 2 x 2 factorial analysis of variance. Between group comparisons were performed using Tukey's omega test. The rank sums test was used when variances were not homogeneous (eg, eosinophils). For histologic and morphometric analyses, data were analyzed by Student's t-test after appropriate transformation to render variances homogeneous or by the rank sums test in cases where variances were not homogenous after transformation. The criterion for significance was $p < 0.05$.

3.4 Results

3.4.a Characterization of control and anti-PMN Ig

The effects of Ig administration on circulating numbers of WBCs, platelets, and red blood cells (hematocrit) in otherwise untreated rats are shown in Table 3.1. In blood

Table 3.1
Effect of control Ig and anti-PMN Ig on circulating blood cell numbers and hematocrit in the absence of LPS

	Treatment	
	Control Ig	Anti-PMN Ig
Total WBC (cells/ul)	12800 ± 1310	7380 ± 1050*
PMNs (cells/ul)	2320 ± 306	92 ± 28*
Lymphocytes (cells/ul)	10500 ± 1230	7140 ± 1070
Monocytes (cells/ul)	46 ± 23	95 ± 22
Eosinophils (cells/ul)	36 ± 16	36 ± 19
Platelets (cells/ul)	790000 ± 38300	681000 ± 15400
Hematocrit (%)	40 ± 0.8	42 ± 0.7

Rats were pretreated with either control or anti-PMN Ig as described in Section 3.3.b. Changes in circulating blood cell numbers and hematocrit were assessed 6 hr after administration of the second dose of Ig.
*, significantly different from respective control Ig treatment value.

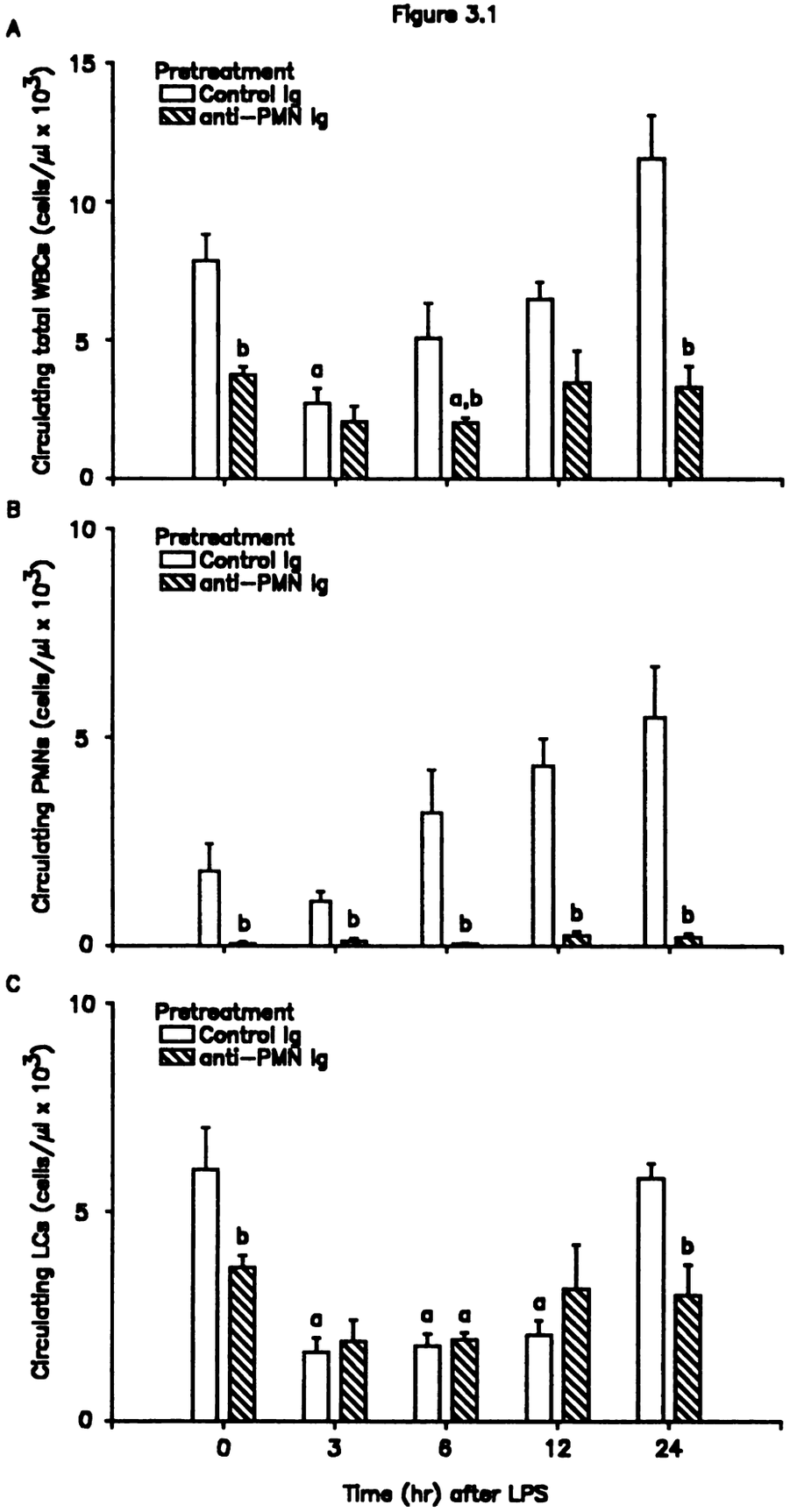
from control Ig-pretreated rats, neither numbers of total WBCs nor numbers of individual WBCs were different from values reported for normal, 8 week old, female, Sprague-Dawley rats (224). In contrast, rats pretreated with anti-PMN Ig exhibited a marked decrease in total WBCs. This appeared to be due to a decrease in both circulating PMNs and LCs, although the decrease in LCs was not statistically significant in this experiment. The reduction in PMNs was pronounced. PMN numbers amounted to <5 % of those from rats pretreated with control Ig. Red blood cells (hematocrit), other WBCs, and platelets were not affected by the anti-PMN Ig. These results are consistent with results obtained by others which showed that antiserum to rat PMNs raised in rabbits using a similar procedure exhibited high antibody titer to PMNs and low antibody titer to LCs, platelets and red blood cells (225,226).

3.4.b Effects of LPS on circulating WBCs

Changes in circulating total and individual WBC numbers in control Ig- and anti-PMN Ig-pretreated rats at various times after exposure to LPS are shown in Figure 3.1. LPS administration to control Ig-pretreated rats caused leukopenia within 3 hr (Figure 3.1A). The LPS-induced decrease in total WBCs was more modest in rats pretreated with anti-PMN Ig and was due primarily to a decrease in circulating LCs (Figure 3.1C). After 3 hr, total WBCs returned to pre-LPS values in both groups. Circulating PMNs

Figure 3.1 Changes in circulating total white blood cells (WBCs), neutrophils (PMNs) and lymphocytes (LCs) after LPS administration to either control Ig- or anti-PMN Ig-pretreated rats. Ig was administered in the tail vein of rats as described in Section 3.3.b. Changes in total WBCs (A), PMN (B), and LCs (C) were measured as described in Section 3.3.g at various times after exposure to LPS (2 mg/kg, iv). Results are expressed as means $\pm$ SEM; N=6.
a, significantly different from respective value at 0 hr.
b, significantly different from respective control Ig pretreatment group.

Figure 3.1


A
Circulating total WBCs (cells/μl x 10³)
15
10
5
0
Pretreatment
Control Ig
anti-PMN Ig
b
a
a,b
b
B
Circulating PMNs (cells/μl x 10³)
10
5
0
Pretreatment
Control Ig
anti-PMN Ig
b
b
b
b
b
C
Circulating LCs (cells/μl x 10³)
10
5
0
Pretreatment
Control Ig
anti-PMN Ig
b
a
a
a
a
b
0
3
6
12
24
Time (hr) after LPS

gradually increased to more than 2-fold the pretreatment value in control Ig-pretreated rats 24 hr after exposure to LPS (Figure 3.1B). In contrast, blood PMN numbers in rats pretreated with anti-PMN Ig remained reduced for 24 hr after LPS exposure. Blood monocyte and eosinophil numbers did not appear to be altered by LPS exposure in these studies (data not shown). However, since circulating numbers of these cells are normally <1 % of the total circulating WBCs number in female, Sprague-Dawley rats (224), alterations in their numbers in the blood were difficult to assess precisely.

3.4.c Effect of PMN depletion on liver PMN number

Table 3.2 summarizes the changes in cellular composition of the NPC fraction of liver digests from control Ig- or anti-PMN Ig-pretreated rats 1.5 hr after administration of either LPS or saline vehicle. The cellular composition of liver digests was similar in saline-treated rats, irrespective of Ig pretreatment (Table 3.2 and Figure 3.2). The primary cell type was a round to polygonal, mononuclear cell with eosinophilic, slightly granular cytoplasm. These cells, which are designated as "other cells", were variable in size and had large, round or oval, basophilic nuclei with homogeneous chromatin. The nucleus was often eccentrically located and had indistinct nucleoli. These morphologic characteristics are consistent with those of endothelial cells (see Section 3.5). The preparations

Table 3.2
Cytologic evaluation of hepatic NPC fraction from liver digests after LPS exposure

Cells/liver ($\times 10^6$)	Control Ig		anti-PMN Ig	
	Saline	LPS	Saline	LPS
Total NPCs	170 ± 10	262 ± 21[a]	150 ± 17	135 ± 8.0[c]
Neutrophils	10.5 ± 3.0	192 ± 21[a]	11.1 ± 7.0	58.4 ± 11[b,c]
Lymphocytes	11.4 ± 3.3	11.6 ± 1.8	11.5 ± 1.5	8.1 ± 1.2
Macrophages	19.6 ± 2.7	16.6 ± 4.0	23.6 ± 1.1	27.2 ± 4.0
Eosinophils	0.60 ± 0.60	0.57 ± 0.57	0.00 ± 0.00	0.45 ± 0.29
Plasma cells	0.60 ± 0.60	1.30 ± 0.59	0.40 ± 0.40	0.63 ± 0.42
Other cells	127 ± 11	40.2 ± 11.2[a]	103 ± 10	40.3 ± 9.3[b]

Rats were pretreated with either control Ig or anti-PMN Ig as described in Section 3.3.b. Cells were quantified in the hepatic NPC fraction 1.5 hr after LPS (5 mg/kg, iv) as decribed in Section 3.3.i. Data are expressed as means ± SEM.

[a] Significantly different from respective control Ig/saline group.

[b] Significantly different from respective anti-PMN Ig/saline group.

[c] Significantly different from respective control Ig/LPS group.

Figure 3.2 Photomicrographs of NPC fractions from rat liver digests after exposure to LPS vehicle. A-B, NPCs from rats pretreated with control Ig; C-D, NPCs from rats pretreated with anti-PMN Ig. Rats were pretreated with Ig as described in Section 3.3.b. Hepatic NPC fractions were isolated as described in Section 3.3.h 1.5 hr after treatment with saline. Magnifications: A and C = 216x; B and D = 540x. Arrows indicate PMNs.

Figure 3.2

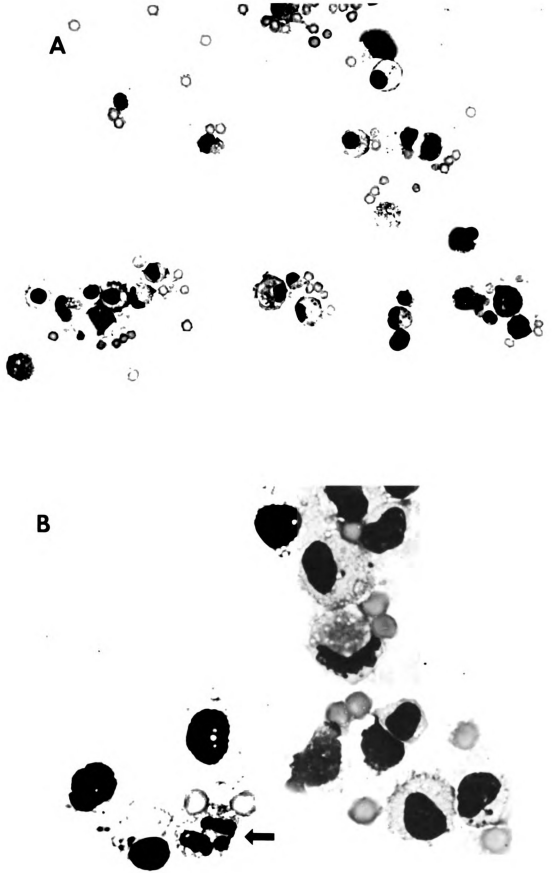
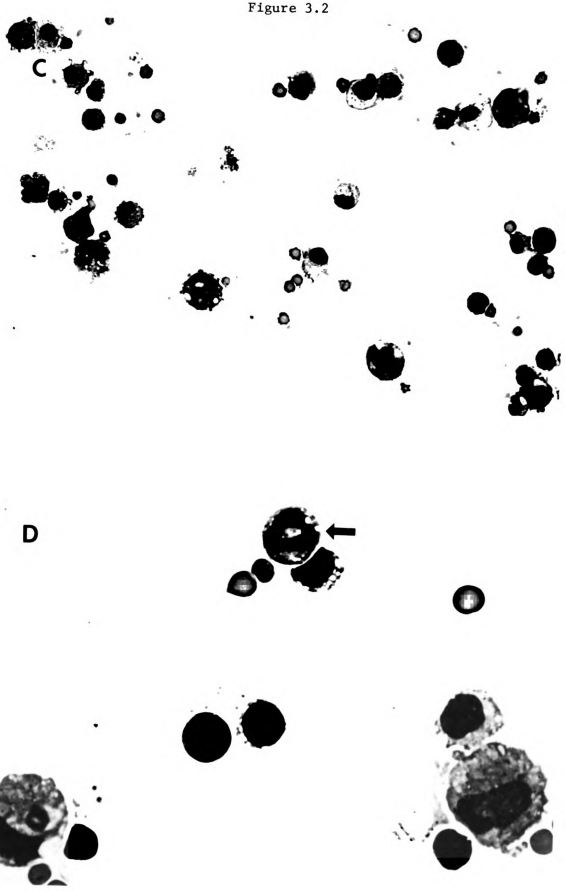


Figure 3.2



contained lesser numbers of macrophages (ie., Kupffer cells), PMNs and small LCs. Plasma cells and eosinophils were seen occasionally and averaged $\leq$ 1 % of the cell population. Occasional mast cells and hepatic parenchymal cells were observed in some digests (< 1 %).

One and a half hr after exposure to LPS, the liver digests from rats pretreated with control Ig had pronounced alterations in cell quantity and cytomorphologic characteristics (Table 3.2, Figure 3.3). These preparations were more cellular than those from rats that received either control Ig and saline or anti-PMN Ig and LPS. This increased cellularity was due to an increase in PMNs (Table 3.2), many of which had degenerate cytomorphologic characteristics including pyknosis, karyolysis and karyorrhexis. The liver digests from these animals also had significantly fewer "other cells" than those from saline controls (Table 3.2).

In livers from LPS-treated rats, the absolute number of PMNs was markedly reduced by pretreatment with anti-PMN Ig (Table 3.2). The degenerate cytomorphology that characterized PMNs from liver digests of rats treated with control Ig and LPS was less common in digests from animals treated with anti-PMN Ig and LPS (Figure 3.3). Although the number and morphology of the macrophages, LCs, eosinophils and plasma cells were unaffected by Ig or LPS treatment (Table 3.2), the number of "other cells" was decreased to a degree similar to that observed in liver digests from rats treated with control Ig and LPS.

Figure 3.3 Photomicrographs of NPC fractions from rat liver digests after exposure to LPS. A-B, NPCs from rats pretreated with control Ig; C-D, NPCs from rats pretreated with anti-PMN Ig. Igs were administered as described in Section 3.3.b. Hepatic NPC fractions were isolated as described in Section 3.3.h 1.5 hr after exposure to LPS (5mg/kg, iv). Magnifications: A and C = 216x; B and D = 540x. Arrows indicate PMNs.

Figure 3.3

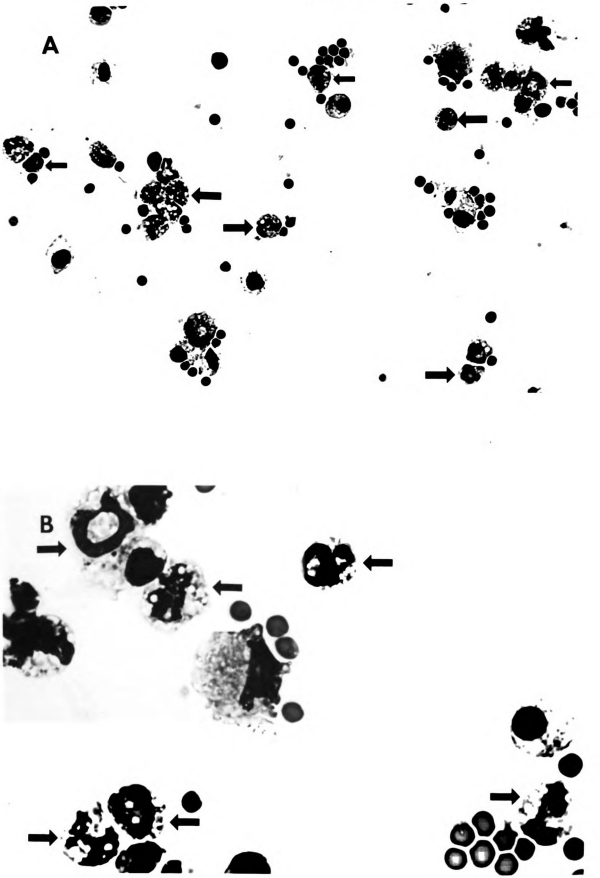
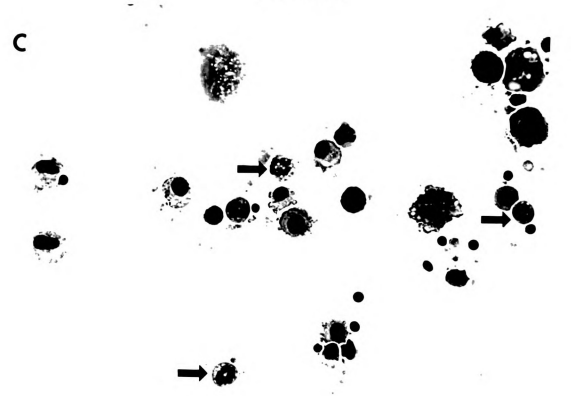
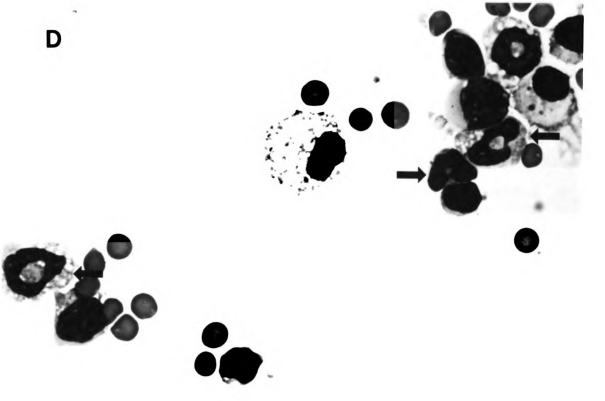


Figure 3.3





3.4.d Effect of PMN depletion on LPS-induced liver injury

To assess the contribution of PMNs to LPS hepatotoxicity, LPS was administered to rats depleted of blood PMNs. In this study, the number of circulating PMNs 6 hr after LPS administration to rats pretreated with control Ig was 2792 ± 576 cells/uL blood compared to 168 ± 50 cells/uL blood in anti-PMN Ig-pretreated rats. Figure 3.4 shows the plasma AST and ALT activities and total plasma biliubin concentration 6 hr after intravenous administration of LPS. In rats pretreated with anti-PMN Ig, LPS exposure resulted in plasma AST and ALT activities which were markedly less than those exhibited by rats treated with control Ig and LPS. Plasma bilirubin concentration in the anti-PMN Ig pretreatment group tended also to be less than in the control Ig pretreatment group after LPS exposure; however, this trend was not statistically significant.

Liver samples obtained from animals in this study were assessed for histopathologic changes. Liver sections from rats pretreated with control Ig had multifocal to coalescing, moderate, acute, neutrophilic hepatitis 6 hr after LPS exposure (Figure 3.5). The sinusoids contained many PMNs, few plump Kupffer cells and small amounts of an eosinophilic, proteinaceous precipitate. Multifocal, coalescing, irregularly shaped areas of hepatocellular degeneration were observed. These lesions were primarily midzonal and were characterized by slightly swollen, weakly

Figure 3.4 Effect of pretreatment with either control Ig or anti-PMN Ig on LPS-induced liver injury. Rats were pretreated with either control or anti-PMN Ig as described in Section 3.3.b. Plasma AST and ALT activities and total plasma bilirubin concentration were measured as described in Section 3.3.d 6 hr after exposure of both Ig pretreatment groups to LPS (3 mg/kg, iv). Results are expressed as means ± SEM; N=10.

*, significantly different from respective control Ig pretreatment group.

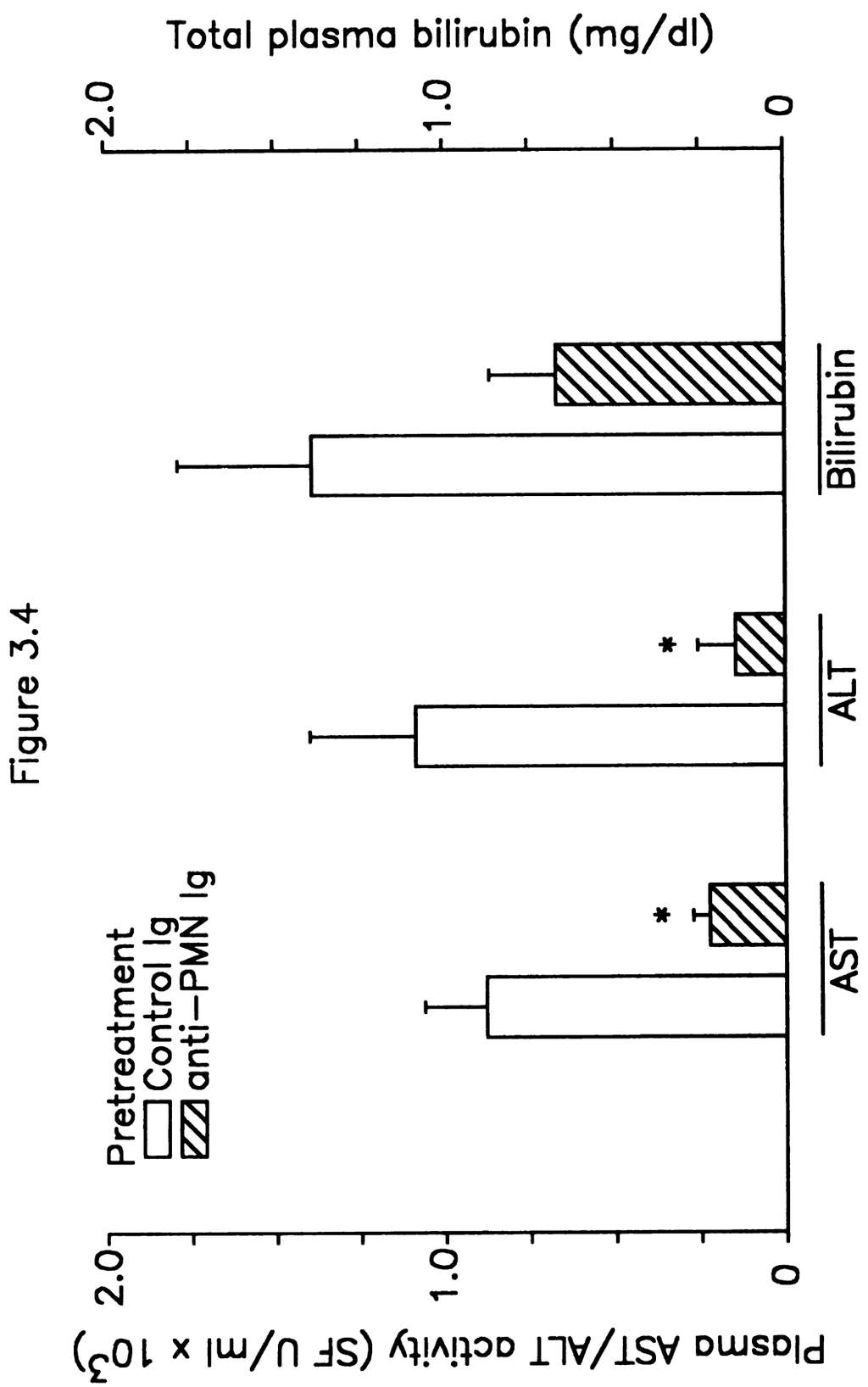
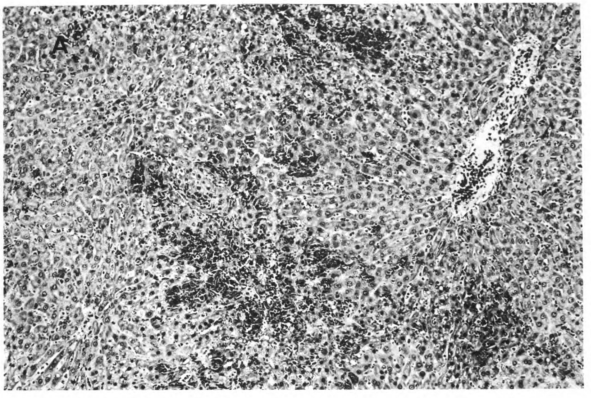
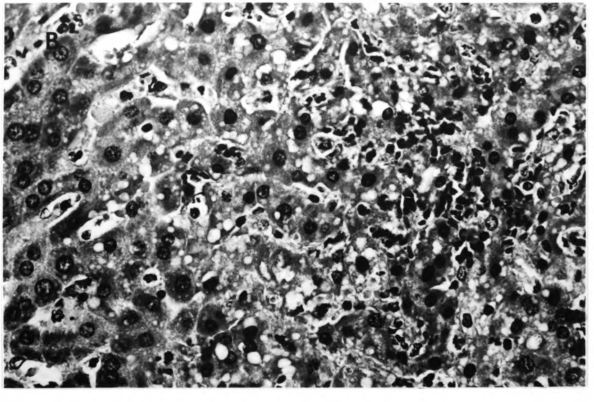

Total plasma bilirubin (mg/dl)
2.0
1.0
0
Plasma AST/ALT activity (SF U/ml x 10³)
2.0
1.0
0
Pretreatment
Control Ig
anti-PMN Ig
*
*
AST
ALT
Bilirubin

Figure 3.4

Figure 3.5 Photomicrographs of liver after administration of LPS to rats pretreated with control Ig. Liver samples from rats in Figure 3.4 were prepared for light microscopy as described in Section 3.3.e. Magnifications: A = 54x; B = 216x.

Figure 3.5





basophilic hepatocytes with swollen, round or oval nuclei. In many areas, the lesions progressed to necrosis. Hepatocytes in necrotic areas were hypereosinophilic with small, pyknotic nuclei or were large and pale with faint nuclei and indistinct cytoplasmic borders. In several affected areas, normal architecture was completely lost. Necrotic foci contained a moderate number of degenerate PMNs. The number of PMNs in these affected areas was greater than that in unaffected areas (Table 3.3, Control Ig/LPS group: PMNs/affected and PMNs/unaffected areas). Hemorrhage was associated with foci in some samples. Few small foci of single cell necrosis were also observed.

Livers from LPS-exposed animals that were pretreated with anti-PMN Ig had similar but significantly fewer lesions per area of liver compared to livers from rats pretreated with control Ig and exposed to LPS (Figure 3.6, Table 3.3). The total area of liver affected with lesions was also significantly less in animals pretreated with anti-PMN Ig. There was a trend for the lesions in rats pretreated with anti-PMN Ig to be smaller and less severe as indicated by a reduction in mean lesion area and individual lesion severity. Rats pretreated with anti-PMN Ig also had a significant reduction in PMNs within both affected and unaffected areas of the liver as well as fewer PMNs per lesion compared to control Ig-pretreated rats.

The development of LPS-induced liver injury is shown in Figure 3.7. Plasma AST activity was less than 50 SF units/ml

Table 3.3[a]
Morphometric analysis of livers from control Ig- or anti-PMN Ig- pretreated rats after exposure to LPS

	Treatment	
	Control Ig/LPS	Anti-PMN Ig/LPS
% liver affected[b]	6.1 ± 2.6	1.0 ± 0.3*
Lesions/area liver (#/cm^2)	51.6 ± 14.2	14.5 ± 3.4*
Mean lesion area (mm^2)	0.094 ± 0.015	0.058 ± 0.012
Lesion severity	2.37 ± 0.30	1.89 ± 0.24
Injury index[c]	0.213 ± 0.043	0.110 ± 0.025*
PMNs/affected area (cells/mm^2)	289 ± 31	94 ± 11*
PMNs/unaffected area (cells/mm^2)	127 ± 14	63 ± 11*
PMNs/lesion	27 ± 4	7 ± 1*

Rats were pretreated with either control Ig or anti-PMN Ig as described in "Materials and Methods". Morphometric analysis was performed as described in Section 3.3.f on liver sections from studies shown in figures 3.4-3.6, 6 hr after LPS exposure (3 mg/kg, iv). Data are expressed as means ± SEM.

*, significantly different from control Ig/LPS treatment group.

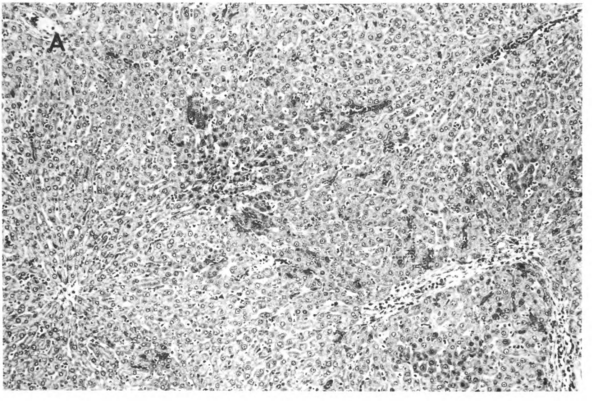
[a], data obtained by Dr. Eric Schultze

[b], determined as lesion area/total area examined for each rat multiplied by 100.

[c], determined as the product of severity score (0-5) and mean lesion area for each rat.

Figure 3.6 Photomicrographs of liver after administration of LPS to rats pretreated with anti-PMN Ig. Liver samples from rats in Figure 3.4 were prepared from light microscopy as described in Section 3.3.e. Magnifications: A = 54x; B = 216x.

Figure 3.6



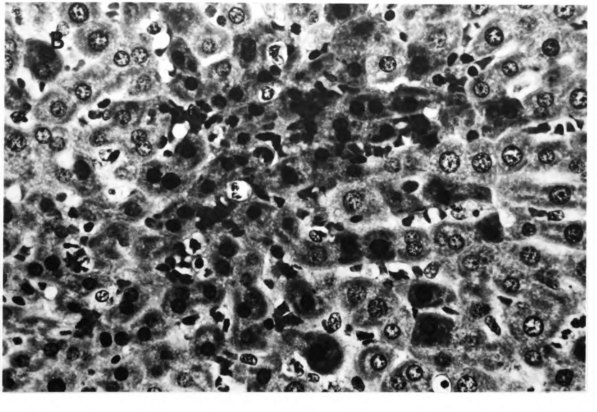
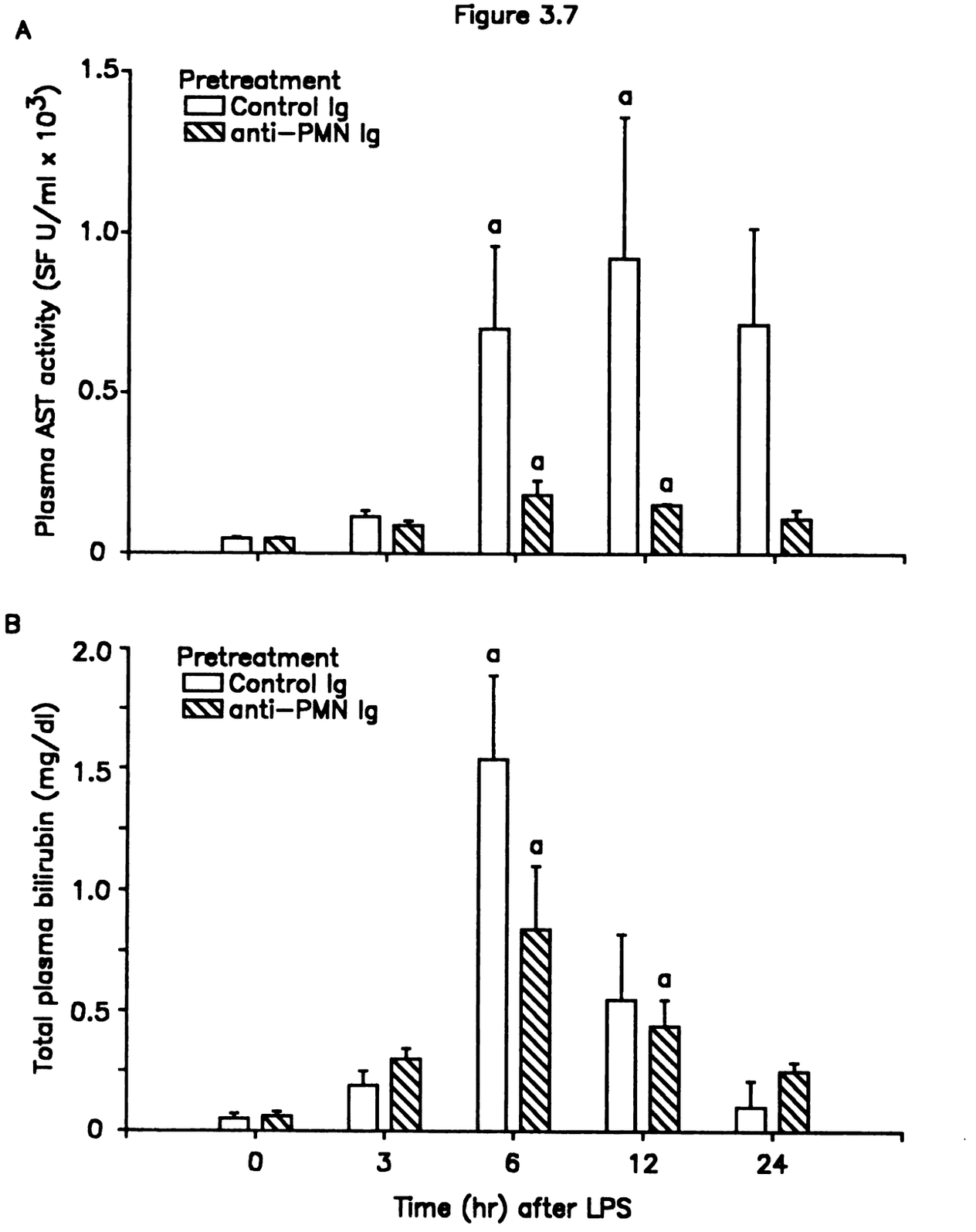


Figure 3.7 Time-course of liver injury after administration of LPS to rats pretreated with either control Ig or anti-PMN Ig. Ig pretreatment was as described in Section 3.3.b. Plasma aspartate aminotransferase (AST) activity (A) and total plasma bilirubin concentration (B) were measured as described in Section 3.3.d at various times after exposure of both Ig pretreatment groups to LPS (2 mg/kg, iv). Results are expressed as means $\pm$ SEM; N=6. **a**, significantly different from respective value at 0 hr.

Figure 3.7


A
Pretreatment
Control Ig
anti-PMN Ig
Plasma AST activity (SF U/ml x 10^3)
1.5
1.0
0.5
0
a
B
Total plasma bilirubin (mg/dl)
2.0
1.5
1.0
0.5
0
0
3
6
12
24
Time (hr) after LPS

in rats treated with either control or anti-PMN Ig prior to the administration LPS (Figure 3.7A, 0 hr). In animals pretreated with control Ig, plasma AST activity increased between 3 and 6 hr after administration of LPS and remained elevated thereafter. In rats pretreated with anti-PMN Ig, a much more modest response occurred in the same time frame. Prior to LPS administration, total plasma bilirubin concentration was less than 0.1 mg/dL in both Ig pretreatment groups (Figure 3.7B, 0 hr). It became maximally elevated 6 hr after LPS administration and returned to preLPS-exposure values by 24 hrs in both pretreatment groups. No lethality occurred at any time in this study.

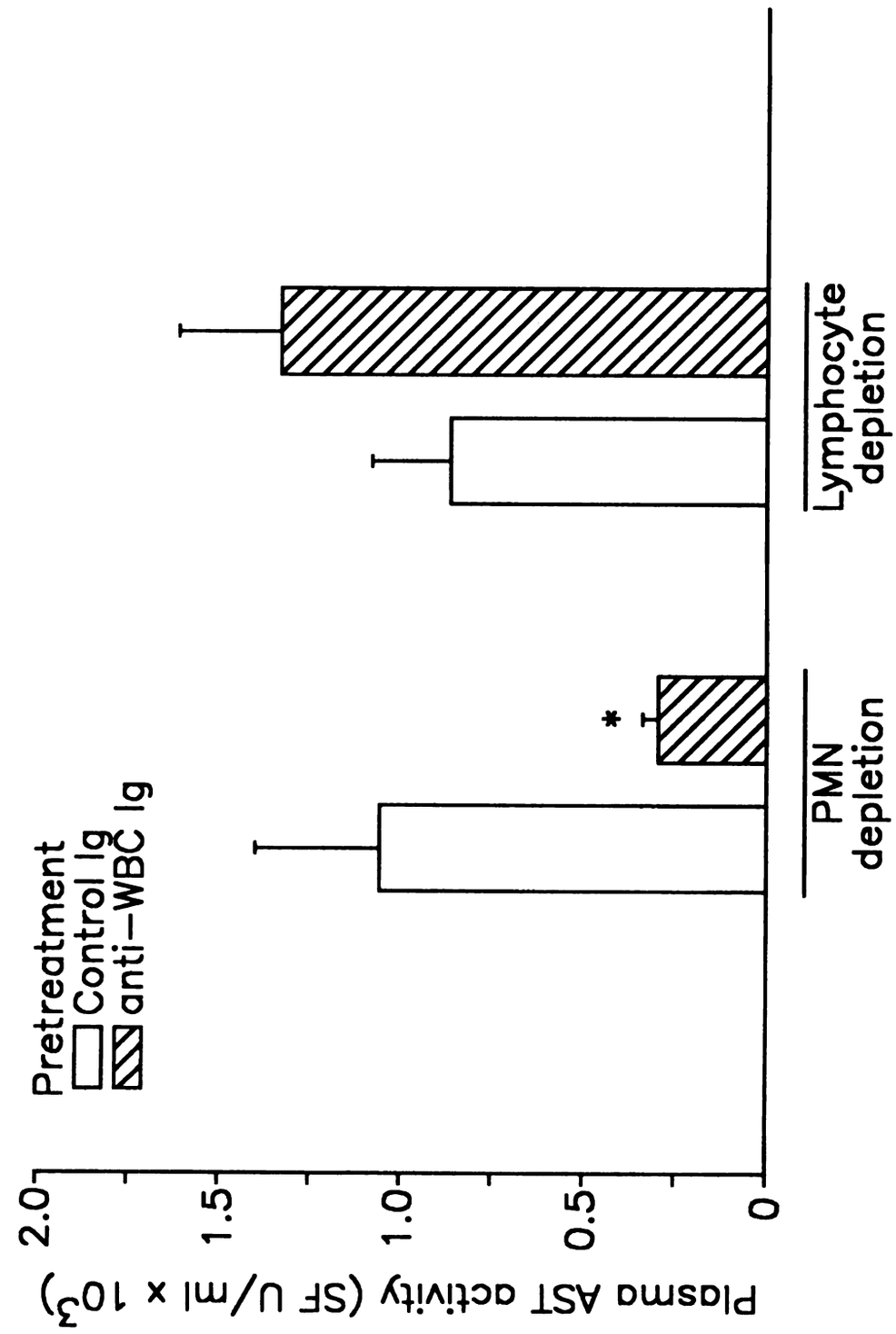
3.4.e Ineffectiveness of LC depletion on LPS hepatotoxicity

The decrease in circulating lymphocytes prior to LPS exposure in rats preteated with anti-PMN Ig (Table 3.1 and Figure 3.1C, 0 hr) indicated that the anti-PMN Ig preparation was highly selective but not completely specific for PMNs. Since it was possible that reduction of lymphocyte numbers contributed to the protection by the anti-PMN Ig preparation, a study was undertaken to test the effect of an anti-lymphocyte Ig preparation (anti-LC Ig) on LPS hepatotoxicity. The results from this study are shown in Figure 3.8 along with the results from a parallel study using anti-PMN Ig. Consistent with earlier findings (above), pretreatment with the anti-PMN Ig preparation largely

Figure 3.8 Comparison of the effect of anti-PMN Ig and anti-LC Ig on plasma AST activity after LPS exposure. Pretreatment with anti-PMN Ig was as described in Section 3.3.b. Anti-LC Ig was administered in the tail vein as a single, 1 ml injection 3 hr prior to LPS administration. Plasma AST activity was measured 6 hr after LPS exposure as described in Section 3.3.d. All groups received LPS (8 mg/kg, iv). Results are expressed as means ± SEM; N=10-12.

*, significantly different from respective control Ig pretreatment group.

Figure 3.8


Plasma AST activity (SF U/ml x 10^3)
2.0
1.5
1.0
0.5
0
Pretreatment
Control Ig
anti-WBC Ig
*
PMN depletion
Lymphocyte depletion

prevented the increase in plasma AST activity 6 hr after LPS administration. In contrast, although pretreatment with anti-LC Ig resulted in >90 % depletion of circulating lymphocytes at the time of LPS administration (7204 ± 738 cells/uL and 561 ± 112 cells/uL in control Ig- and anti-LC Ig-treated rats, respectively; N=4), no protection was observed. The anti-LC Ig did not affect circulating PMN numbers (data not shown).

3.5 Discussion

Evidence from several studies indicates that PMN depletion affords protection against LPS-induced injury to the skin (160) and lungs (112), suggesting that these phagocytic cells contribute to the pathogenesis of injury from LPS in certain tissues. Results from the present study indicate that PMN depletion attenuates liver injury associated with LPS exposure. This suggests that PMNs contribute to the pathogenesis of LPS hepatotoxicity.

PMN depletion markedly attenuated LPS hepatotoxicity. However, it did not afford complete protection. This is indicated by the significant elevation in plasma AST activity and total bilirubin concentration 6 hr following LPS administration in PMN-depleted rats (Figure 3.7). Also, foci of parenchymal cells exhibiting mild degenerative changes were observed in livers from PMN-depleted rats after LPS administration (Figure 3.6). Although pretreatment with

the anti-PMN Ig caused a marked reduction in circulating PMNs, it is possible that enough PMNs remained in the liver after PMN depletion to cause some injury. Alternatively, PMN-independent mechanisms may contribute to LPS hepatotoxicity.

Cholestasis and hyperbilirubinemia are often associated with LPS exposure. LPS has been shown to decrease bile flow from the isolated, buffer-perfused liver (141,142) and evidence suggests that LPS may directly affect bile secretion by inhibiting Na^+,K^+-ATPase on the bile canalicular plasma membrane of hepatocytes (144). Thus, hyperbilirubinemia following LPS exposure may be due to a PMN-independent alteration in bile secretion. This may explain the ability of anti-PMN Ig to attenuate the rise in plasma AST and ALT activities without causing a significant reduction in total plasma bilirubin concentration.

Cytocentrifuge preparations of liver digests contained a large number of cells which were described as "other cells". Bautista *et al.* reported values for hepatic endothelial cell numbers which closely approximate the number of "other cells" observed in the present study, suggesting that these cells are hepatic endothelial cells (227). Additionally, these cells had morphologic characteristics that are similar to cytocentrifuge preparations of cultured rat and bovine vascular endothelial cells (not shown). Exposure to LPS caused a significant decrease in "other cells" which was not prevented by PMN

depletion. Thus, the decrease in "other cells" does not appear to be mediated by PMNs. This finding is consistent with the observation that LPS is directly cytotoxic to endothelial cells grown in culture (45,46). Further studies are necessary to elucidate the mechanism of this decrease in "other cells" following LPS exposure.

LPS is cleared from the circulation primarily by Kupffer cells in the liver (18,20). Because these hepatic macrophages exhibit enhanced activity following LPS exposure *in vivo* (228) and because macrophages exposed to LPS *in vitro* release cytotoxic substances, including reactive oxygen metabolites (228,229,230), it has been proposed that activated macrophages may contribute to the development of LPS-induced liver injury (231). This is supported by evidence indicating that treatments which alter Kupffer cell function alter the response of the liver to LPS (176). Also, studies of cocultures of Kupffer cells and hepatocytes indicate that LPS is cytotoxic to hepatocytes in a Kupffer cell-dependent manner (177). Thus, Kupffer cells might contribute to LPS hepatotoxicity through the extracellular release of cytotoxic mediators. LPS-activated macrophages release chemotactic factors for PMNs *in vitro* (149), and macrophage-derived chemotactic factors have been implicated in the accumulation of PMNs in the pleural cavity (149) and peritoneum (232) after LPS exposure *in vivo*. Thus, it is possible that Kupffer cells contribute to LPS hepatotoxicity by mediating the recruitment of PMNs.

The anti-PMN Ig caused a significant reduction in circulating LCs. Since LCs have been implicated in the pathogenesis of liver injury under certain circumstances (233,234), it could be argued that the protective effect of this Ig preparation was due at least in part to LC depletion. However, administration of an anti-LC Ig preparation resulted in depletion of circulating LC but did not afford protection against LPS hepatotoxicity. Therefore, it is unlikely that the decrease in circulating LCs contributed to the protective effect of the anti-PMN Ig preparation. This result also indicates that the ability of the anti-PMN Ig preparation to protect against LPS hepatotoxicity was not due to the method used for PMN depletion (ie, generation of immune complexes), since depletion of a different blood cell by a similar method did not afford protection. This strengthens the conclusion that the anti-PMN Ig protected against LPS hepatotoxicity by depletion of PMNs and supports the hypothesis that PMNs contribute to the pathogenesis of LPS hepatotoxicity.

Liver injury following LPS exposure is associated with an acute inflammatory response, a prominant feature of which is an early accumulation of PMNs at the site of inflammation. PMN infiltration is usually followed by the appearance of macrophages that are thought to be derived from circulating blood monocytes (235,236). Although macrophages can mediate tissue injury under certain circumstances, it seems unlikely that these infiltrating

macrophages contribute to the early development of liver injury that follows LPS exposure since they usually appear later in the acute inflammatory response (235). This is supported by our results (Table 1.1) and results from a recent study which indicated that the total number of mononuclear phagocytes in the liver was not changed prior to the onset of LPS-induced liver injury (227). By contrast, large numbers of PMNs were observed in the liver as early as 45 min after LPS (data not shown), well before the onset of severe liver injury. Accordingly, the time course of hepatic PMN infiltration after LPS exposure is consistent with their role in the pathogenesis.

Recently, PMNs have been implicated in the pathogenesis of liver injury in other models. For example, depletion of PMNs afforded protection against the hepatotoxicity caused by alpha-naphthylisothiocyanate (158), and liver injury following resuscitation from hypovolemic shock was attenuated by monoclonal antibodies to PMN adhesion molecules (159). The mechanisms by which PMNs mediate injury to the liver in these instances are not known.

PMNs might cause tissue injury by several mechanisms. For example, activation of PMNs is associated with the extracellular release of reactive oxygen metabolites, including O_2^- and H_2O_2 (153). These cytotoxic substances can initiate peroxidation of lipids and oxidation of protein thiols which might contribute to tissue injury. Products of lipid peroxidation have been detected in the liver following

LPS exposure (237,238), raising the possibility that oxygen radicals released by activated PMNs may contribute to LPS-induced liver injury. This is supported by the observation that O_2^- production in the liver was increased after LPS exposure (239).

Activation of PMNs may also result in the extracellular release of lysosomal enzymes (240,241,242). These enzymes have been implicated in certain instances of PMN-induced tissue injury (243,244,245). They can also injure isolated hepatocytes under certain circumstances (246). Thus, it is possible that LPS-induced liver injury is mediated by lysosomal enzymes, reactive oxygen metabolites or other agents released from activated PMNs. Further studies are necessary to determine which PMN-derived cytotoxic agents are involved in LPS-induced liver injury.

In summary, the PMN accumulation in liver that follows administration of an hepatotoxic dose of LPS occurs before the onset of liver injury, and prior depletion of circulating PMNs attenuates the accumulation of PMNs in the liver and protects against the hepatotoxic effects of LPS. Although additional mechanisms may be involved in LPS hepatotoxicity, the substantial protective effect of PMN depletion indicates that PMNs play an important role in the pathogenesis of LPS hepatotoxicity in the rat.

CHAPTER 4

ROLE OF TUMOR NECROSIS FACTOR-*ALPHA* IN LPS-INDUCED LIVER INJURY

4.1 Abstract

TNF-alpha and blood PMNs have been implicated in the pathogenesis of LPS hepatotoxicity. However, the mechanism by which these endogenous factors mediate liver injury during LPS exposure is uncertain. The objective of this study was to test the hypothesis that TNF-alpha contributes to LPS hepatotoxicity indirectly by a PMN-dependent mechanism. Pretreatment with pentoxifylline (100 mg/kg, iv) attenuated circulating TNF-alpha concentration 1.5 hr after administration of 5 mg/kg LPS and afforded protection against liver injury. Pretreatment of rats with an antiserum to TNF-alpha also afforded protection against LPS hepatotoxicity. Hepatic PMN accumulation 1.5 hr after exposure to LPS was not significantly reduced by pretreatment with either pentoxifylline or TNF-alpha antiserum. Depletion of circulating PMNs, which protects against LPS hepatotoxicity, enhanced circulating TNF-alpha concentration more than 3-fold compared to control rats 1.5 hr after LPS exposure. These results support the hypothesis that TNF-alpha mediates LPS-induced liver injury in the rat by a PMN-dependent mechanism.

4.2 Introduction

TNF-alpha is one of several cytokines which are thought to play a role in the acute inflammatory response to tissue injury or infection. Among its proinflammatory activities are induction of procoagulant activity in vascular endothelial cells (189) and the stimulation of PMN adherence to surfaces in vitro, including endothelial cell monolayers (247,248,249). This action may be important in the accumulation of these phagocytic cells at sites of inflammation in vivo. TNF-alpha also induces the synthesis of acute phase proteins by the liver (182,250), suggesting that TNF-alpha may contribute to the regulation of the inflammatory response under certain conditions. Although TNF-alpha mediates certain important beneficial functions during inflammation, results from numerous studies suggests that this cytokine contributes to tissue injury associated with certain pathophysiologic conditions, including cerebral malaria (196) and ischemia/reperfusion in the liver (197) and in the gastrointestinal tract (17).

Exposure to LPS is accompanied by a marked rise in circulating concentration of TNF-alpha (251), and evidence from numerous studies suggests that many of the pathophysiologic effects associated with exposure to LPS may be mediated by TNF-alpha, including LPS-induced liver injury. The notion that TNF-alpha mediates the liver injury that occurs after exposure of animals to LPS is supported by

studies showing that neutralization of circulating TNF-alpha with a specific antiserum attenuated liver injury in galactosamine-sensitized mice given LPS (201). Futhermore, administration of recombinant murine TNF-alpha to galactosamine-sensitized mice in the absence of LPS produced alterations in the liver which resembled LPS-induced liver injury (252).

Although TNF-alpha appears to play a role in the pathogenesis of LPS hepatotoxicity, the mechanisms by which it mediates tissue injury remains unclear. Depletion of PMNs afforded protection against lung injury induced by recombinant human TNF-alpha suggesting that TNF-alpha mediates tissue injury in vivo by a PMN-dependent mechanism (202). Because TNF-alpha increases the adherence of PMNs to vascular endothelial cells, it has been proposed that TNF-alpha may mediate tissue injury by promoting accumulation of PMNs in tissues (192). Consistent with this is evidence which indicates that pretreatment with antiserum to TNF-alpha or with pentoxifylline, which inhibits the LPS-induced increase in circulating TNF-alpha (251), attenuated the accumulation of PMNs in lungs as well as the pulmonary injury after exposure to LPS (200,253). Recent evidence indicates that PMNs accumulate in livers of LPS-exposed rats and that PMN depletion attenuates hepatic injury from LPS, indicating that these cells contribute to liver injury induced by LPS (254 and Chapter 3). This raises the

possibility that TNF-alpha may contribute to LPS-induced liver injury by a PMN-dependent mechanism.

In contrast, results from other studies suggest that TNF-alpha may mediate liver injury by mechanisms independent of blood PMNs. For example, LPS is cytotoxic to cocultures of Kupffer cells and hepatic parenchymal cells (255). This cytotoxicity was associated with a rise in TNF-alpha in the culture supernatant and could be attenuated by antiserum to TNF-alpha. Since PMNs were not included in this culture system, these results indicate that TNF-alpha can contributed to cytotoxicity to liver cells in vitro by a PMN-independent mechanism. Whether TNF-alpha contributes to the pathogensis of LPS-induced liver injury by a similar mechanism in vivo remains unknown.

The purpose of this study was to gain insight into the mechanism by which TNF-alpha contributes to liver injury after LPS exposure in vivo. Specifically, the hypothesis that TNF-alpha mediates liver injury after LPS exposure by a PMN-dependent mechanism was tested.

4.3 Materials and methods

4.3.a Animals

Female, Sprague-Dawley rats (Charles River, Crl:CD BR (SD) VAF/plus, Portage, MI) weighing 200-250 kg were used in all studies. Animals were maintained on a 12 hr light-dark cycle for at least one week prior to use. Food (Wayne Lab-

Blox, Allied Mills, Chicago, IL) and water were allowed ad libitum. Female New Zealand White rabbits (Bailey's Rabbitry, Alto, MI) were maintained on high fiber Purina Lab Rabbit Chow (Purina Mills, St. Louis, MO).

4.3.b Treatment protocols

LPS from E. coli, 0128:B12 (Sigma Chemical Co, St Louis, MO) was administered in the tail vein. To test the effect of antiserum to TNF-alpha on liver injury and hepatic PMN accumulation after LPS administration, rats were divided into two treatment groups. One group was pretreated 1 hr prior to LPS exposure with serum from rabbits immunized against recombinant murine TNF-alpha (TNF-alpha antiserum). The second group was pretreated with serum from untreated rabbits (control serum). Rabbit serum was diluted 1:1 with sterile saline and administered in the tail vein in a volume of 2 ml. Both treatment groups received 5 mg/kg LPS in the tail vein. TNF-alpha antiserum was a gift from Dr. Steven L. Kunkel.

A 2 x 2 factorial design was used to test the effect of pentoxifylline (Sigma Chemical Co, St Louis, MO) on LPS hepatotoxicity. Pentoxifylline (100mg/kg) or saline vehicle (0.5 ml/0.1 kg body weight) was administered as a bolus in the tail vein 1 hr prior to administration of either 5 mg/kg LPS or saline vehicle. A similar design was used to examine the effect of pentoxifylline on circulating TNF-alpha

concentration and hepatic PMN infiltration after LPS administration.

To determine the relationship between circulating PMNs and TNF-alpha in the pathogenesis of LPS hepatotoxicity, the effect of PMN depletion on circulating TNF-alpha concentration after LPS exposure was determined. In this study, rats were pretreated 18 and 6 hr prior to LPS (5 mg/kg, iv) exposure with total Ig fractions isolated from rabbit serum. One group of rats received Igs from rabbits immunized with rat PMNs (anti-PMN Ig). A second group of rats received Igs from untreated rabbits (control Ig). This treatment protocol with anti-PMN Ig markedly reduces circulating PMN numbers prior to LPS exposure and affords protection against LPS hepatotoxicity (254 and Chapter 3).

4.3.c Quantification of plasma TNF-alpha concentration

The concentration of TNF-alpha in plasma samples obtained from the inferior vena cava of anesthetized rats (sodium pentobarbital, 50 mg/kg, ip) was determined by the lysis of the fibrosarcoma cell line, WEHI 164 clone 13 (256). Plasma from blood anticoagulated with sodium citrate was used in these studies since heparin can interfere with the assay (257).

4.3.d Enumeration of hepatic PMN numbers

PMN numbers in liver tissue were estimated in the NPC fraction of the liver as described in 3.3.h (254).

4.3.e Evaluation of liver injury

Liver injury was quantified 6 hr after LPS administration except in the TNF-alpha antiserum study in which liver injury was also quantified 1.5 hr after exposure to LPS. Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and anticoagulated blood samples (1/10 dilution in whole blood of 3.8 % w/v sodium citrate in DDW) for quantification of liver injury were obtained from the inferior vena cava. Liver injury was assessed by changes in plasma AST activity and by changes in total plasma bilirubin concentration as described in section 3.3.d.

4.3.f Quantification of circulating WBCs

Total circulating WBC numbers were quantified in blood anticoagulated with sodium citrate as described in 3.3.g. Circulating numbers of PMNs and LCs were obtained by multiplying the total WBC number by the fraction of each cell type, which was determined by differential counts made on blood smears stained with buffered, differential Wright-Giemsa stain.

4.3.g Anti-PMN Ig preparation

Igs to rat PMNs were raised in female rabbits as described in section 3.3.c (254). This polyclonal Ig preparation selectively depleted circulating PMNs and afforded protection against LPS hepatotoxicity (254 and Chapter 3).

4.3.h Data analysis

Results are expressed as means ± SEM. In most studies, comparisons were made using the t-test ($p<0.05$). In studies in which variances were non-homogeneous, analyses were performed on log transformed data.

4.4 Results

4.4.a Effect of antiserum to TNF-alpha on LPS hepatotoxicity

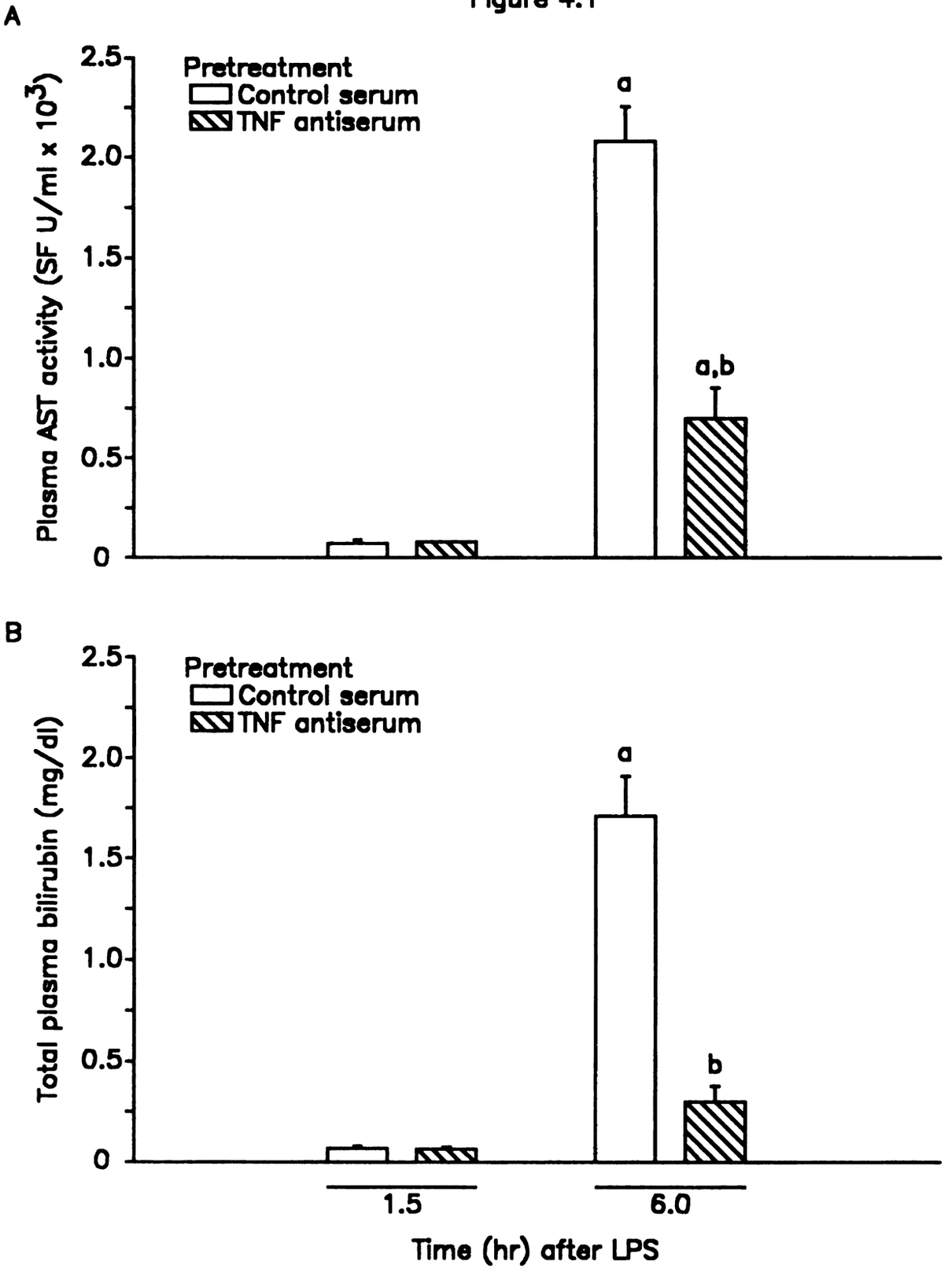
Antiserum to TNF-alpha affords protection against LPS hepatotoxicity in mice sensitized with galactosamine (201). Initial studies were performed to determine if antiserum to this cytokine also affords protection against LPS-induced liver injury in the rat without galactosamine sensitization. The results of these studies are shown in Figure 4.1. Liver injury, as indicated by elevations in plasma AST activity (Figure 4.1A) and total plasma bilirubin concentration (Figure 4.1B), occured by 6 hr after LPS administration in rats pretreated with control serum. This LPS-induced liver injury was markedly attenuated by pretreatment with antiserum to TNF-alpha. Regardless of pretreatment, plasma AST activity and total plasma bilirubin concentration were unchanged 1.5 hr after administration of LPS, indicating that the onset of liver injury occured between 1.5 and 6 hr after LPS administration.

Figure 4.1 Effect of TNF antiserum on LPS hepatotoxicity in rats. Rats were pretreated with either TNF antiserum or control serum in the tail vein 1 hr prior to LPS exposure. LPS (5mg/kg, iv) was administered to all animals. Liver injury was assessed 1.5 and 6 hr later by changes in plasma aspartate aminotransferase (AST) activity (A) and by changes in total plasma bilirubin concentration (B) as described in Section 4.3.e. Results are expressed as means $\pm$ SEM; N=3-5.

a, significantly different from respective saline treatment group at 1.5 hr.

b, significantly different from respective control serum pretreatment groups.

Figure 4.1


A
Pretreatment
Control serum
TNF antiserum
Plasma AST activity (SF U/ml x 10^3)
2.5
2.0
1.5
1.0
0.5
0
a
a,b
B
Pretreatment
Control serum
TNF antiserum
Total plasma bilirubin (mg/dl)
2.5
2.0
1.5
1.0
0.5
0
a
b
1.5
6.0
Time (hr) after LPS

4.4.b Effect of pentoxifylline on circulating TNF-alpha concentration after LPS exposure

A previous study showed that the concentration of circulating TNF-alpha was maximally increased 1.5 hr after administration of LPS to rats and that pretreatment with pentoxifylline attenuated this increase (251). In a confirmatory study (Table 4.1), a pronounced increase in plasma TNF-alpha concentration was observed 1.5 hr after administration of LPS to rats. This increase was markedly attenuated by pretreatment of rats with 100 mg/kg pentoxifylline. The concentration of TNF-alpha in plasma from rats pretreated with either pentoxifylline or saline vehicle was less than 1 ng/ml in the absence of LPS exposure (Table 4.1).

4.4.c Effect of pentoxifylline on LPS hepatotoxicity

To test the possibility that pentoxifylline affords protection against LPS hepatotoxicity, rats were pretreated 1 hr prior to LPS administration with pentoxifylline or saline vehicle. The results from this study are shown in Figure 4.2. Rats pretreated with saline then treated with LPS exhibited marked elevations in plasma AST activity and total plasma bilirubin concentration by 6 hr after exposure to LPS. Both indices of liver injury were markedly attenuated in animals pretreated with pentoxifylline. Pentoxifylline did not affect either plasma AST activity or

Table 4.1
Effect of pentoxifylline on the LPS-induced increase in circulating TNF-alpha concentration.

Pretreatment	Treatment	TNF-alpha (ng/ml)
Saline	Saline	< 1
Pentoxifylline	Saline	< 1
Saline	LPS	$34 \pm 13 \times 10^{3}$ **a**
Pentoxifylline	LPS	$5.4 \pm 2.2 \times 10^{3}$ **a,b**

Rats were pretreated with either pentoxifylline (100 mg/kg, iv) or saline vehicle 1 hr prior to treatment with either LPS (5 mg/kg, iv) or saline vehicle. TNF-alpha was measured in plasma 1.5 hr after LPS exposure as described in Section 4.3.c.

a, significantly different from respective saline treatment group.

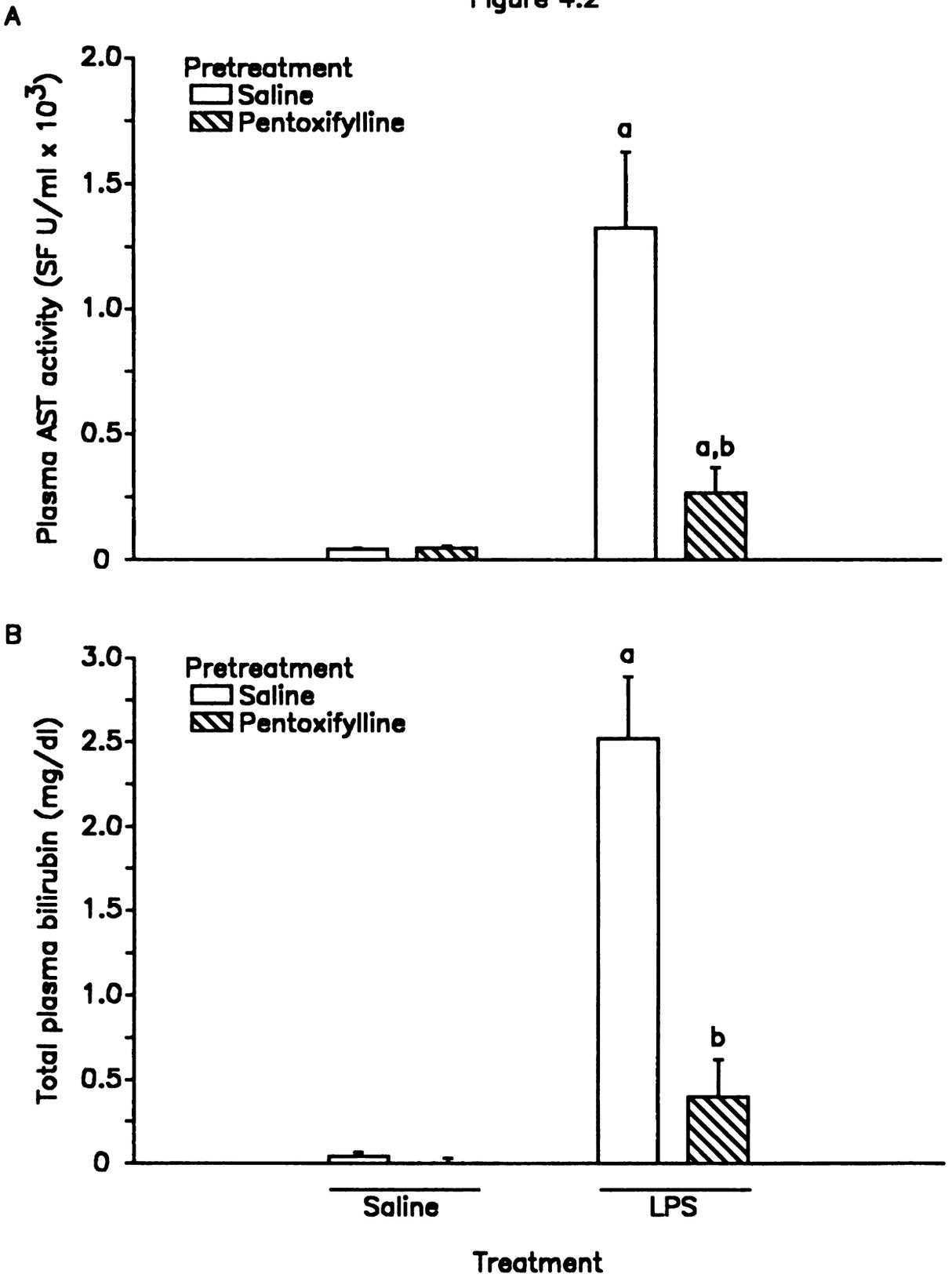
b, significantly different from respective vehicle-pretreated control.

Figure 4.2 Effect of pentoxifylline on LPS hepatotoxicity. Rats were pretreated with either pentoxifylline (100mg/kg, iv) or saline vehicle and then treated 1 hr later with either LPS (5mg/kg, iv) or saline vehicle. Liver injury was assessed by changes in plasma aspartate aminotransferase (AST) activity (A) and by changes in total plasma bilirubin concentration (B) 6 hr after exposure to LPS as described in Section 4.3.e. Results are expressed as means ± SEM; N= 4 and 10 in the saline and LPS treatment groups, respectively.

a, significantly different from respective saline treatment group.

b, significantly different from respective vehicle-pretreated control.

Figure 4.2


A
Plasma AST activity (SF U/ml x 10^3)
2.0
1.5
1.0
0.5
0
Pretreatment
Saline
Pentoxifylline
a
a,b
B
Total plasma bilirubin (mg/dl)
3.0
2.5
2.0
1.5
1.0
0.5
0
Pretreatment
Saline
Pentoxifylline
a
b
Saline
LPS
Treatment

total plasma bilirubin concentration in the absence of LPS exposure.

4.4.d Effect of PMN depletion on circulating TNF-alpha concentration after LPS exposure

Results from Chapter 3 indicate that depletion of circulating PMNs affords protection against LPS hepatotoxicity (254). Accordingly, studies were performed to assess whether PMN depletion modulated the increase in circulating TNF-alpha concentration after LPS exposure. Pretreatment with anti-PMN Ig caused a marked reduction in circulating PMNs compared to control Ig-pretreated animals 1.5 hr after exposure to saline (77 $\pm$ 40 and 1150 $\pm$ 62 PMNs/ul, respectively). As indicated in Table 4.2, administration of LPS to rats pretreated with control Ig was associated with a pronounced increase in plasma TNF-alpha concentration 1.5 hr after LPS exposure. In comparison, this increase was more than 3-fold greater in PMN-depleted rats.

4.4.e Effect of pentoxifylline on hepatic PMN number

Figure 4.3 shows the effect of pentoxifylline pretreatment on LPS-induced alterations in hepatic NPC and PMN numbers. NPC numbers were significantly increased 1.5 hr after administration of LPS to saline-pretreated rats (Figure 4.3A). A trend toward increased NPCs also occurred after exposure of pentoxifylline-pretreated rats to LPS; however, this did not attain statistical significance. The

Table 4.2
Effect of PMN depletion on the LPS-induced increase in circulating TNF-alpha concentration.

Pretreatment	Treatment	TNF-alpha (ng/ml)
Control Ig	Saline	< 1
Anti-PMN Ig	Saline	< 1
Control Ig	LPS	$46 \pm 8.5 \times 10^{3}$ [a]
Anti-PMN Ig	LPS	$177 \pm 40 \times 10^{3}$ [a,b]

Rats were pretreated with either control Ig or anti-PMN Ig 18 and 6 hr prior to treatment with either LPS (5 mg/kg, iv) or saline vehicle. TNF-alpha was measured in plasma 1.5 hr after LPS exposure as described in Section 4.3.c. Values represent the means $\pm$ SEM; N=3-5.

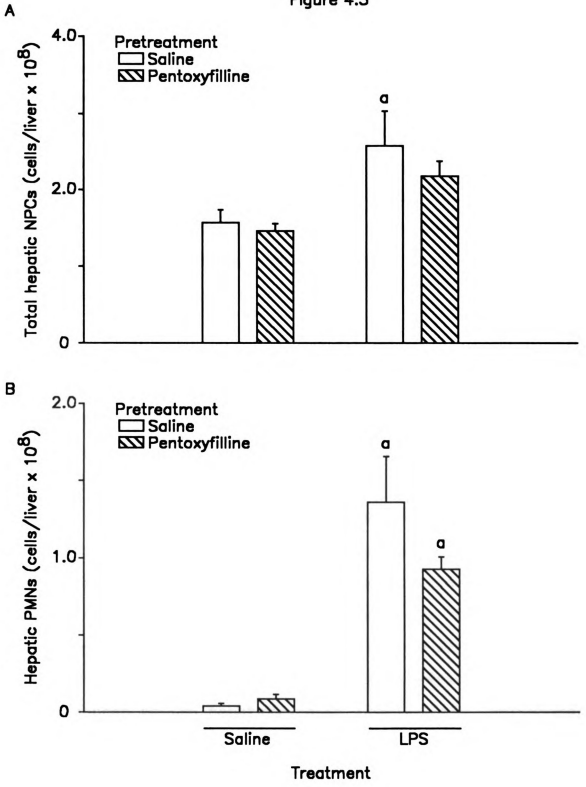
a, significantly different from respective saline treatment group.

b, significantly different from respective control Ig pretreatment group.

Figure 4.3 Effect of pentoxifylline on hepatic nonparenchymal cell (NPC) and neutrophil (PMN) numbers after LPS exposure. Pretreatments with either pentoxifylline (100 mg/kg, iv) or saline vehicle were administered in the tail vein 1 hr prior to treatment with either LPS (5 mg/kg, iv) or saline vehicle. Hepatic NPCs (A) and PMNs (B) were quantified 1.5 hr after treatment with LPS as described in Section 3.3.h. Results are expressed as means ± SEM; N=3.

a, significantly different from respective saline treatment group.

Figure 4.3


A
Pretreatment
Saline
Pentoxyfilline
Total hepatic NPCs (cells/liver x 10^8)
4.0
2.0
0
a
B
Pretreatment
Saline
Pentoxyfilline
Hepatic PMNs (cells/liver x 10^8)
2.0
1.0
0
a
a
Saline
LPS
Treatment

LPS-induced increase in NPCs in both pretreatment groups was due largely to an increase in hepatic PMN numbers (Figure 4.3B). Hepatic PMN numbers in pentoxifylline-pretreated rats were not significantly different from those of rats pretreated with saline either in the presence or absence of LPS. Similarly, pretreatment of rats with antiserum to TNF-alpha did not significantly alter hepatic PMN accumulation 1.5 hr after LPS administration (11 $\pm$ 1.3 x 10^7 and 8.7 $\pm$ 1.7 x 10^7 PMNs/liver in rats pretreated with control serum and TNF-alpha antiserum, respectively).

4.5 Discussion

Exposure to LPS is associated with an elevation in circulating TNF-alpha concentration (251, Table 4.1), and evidence from numerous studies suggests that this cytokine may mediate many of the pathophysiologic alterations accompanying LPS exposure (199,93,258). The observation that antiserum to TNF-alpha afforded protection against LPS hepatotoxicity in galactosamine-sensitized mice (201) suggested that TNF-alpha may play a role in the pathogenesis of LPS-induced liver injury. However, several differences exist between normal and galactosamine-sensitized animals with respect to LPS hepatotoxicity. For example, thromboxane synthase inhibitors afforded protection against LPS hepatotoxicity in rats in the absence of galactosamine sensitization, suggesting that the potent vasoactive

arachidonic acid metabolite, thromboxane A_2, contributes to the pathogenesis of liver injury in these animals (215). In contrast, this arachidonic acid metabolite does not appear to contribute to LPS hepatotoxicity in galactosamine-sensitized animals, since cyclooxygenase inhibitors did not afford protection (211). Although differences in the mechanism of liver injury in nonsensitized and galactosamine-sensitized animals may exist, TNF-alpha appears to be an important mediator of LPS-induced liver injury in both instances. This is supported by results from the present study, which showed that LPS hepatotoxicity in rats was attenuated by pretreatment with TNF-alpha antiserum.

Pretreatment with pentoxifylline also afforded protection against liver injury after exposure of rats to LPS. Pentoxifylline is a methylxanthine derivative which has been used clinically to treat circulatory disorders (259). In addition, it has been shown to protect against certain pathophysiologic alterations associated with exposure to LPS. For example, pretreatment with pentoxifylline improves survival of rats exposed to lethal doses of LPS (251). It was proposed that this protective effect was due to the inhibition of TNF-alpha production since pentoxifylline markedly attenuated the rise in circulating concentration of TNF-alpha after exposure to LPS. Pentoxifylline also inhibited production of TNF-alpha from macrophages exposed to LPS in culture. This inhibitory effect was presumably

mediated by a cAMP-dependent mechanism (284). Results from studies presented in this chapter confirm that pentoxifylline attenuates the increase in circulating TNF-alpha concentration caused by LPS (Table 4.1) and suggest further that TNF-alpha contributes to LPS-induced liver injury in rats.

An argument can be made for both PMN-dependent and PMN-independent mechanisms in the TNF-alpha-mediated liver injury that occurs after LPS exposure. For example, results indicating that TNF-alpha contributes to cytotoxicity after cocultures of Kupffer cells and hepatic parenchymal cells are exposed to LPS raises the possibility that TNF-alpha mediates LPS-hepatotoxicity by a direct, PMN-independent mechanism (255). Conversely, a PMN-dependent mechanism is suggested by the observation that PMN depletion affords protection against LPS hepatotoxicity in vivo (254 and Chapter 3). In the present study, administration of LPS to PMN-depleted rats was associated with a marked increase in circulating TNF-alpha concentration compared to LPS-treated controls (Table 4.2). Inasmuch as the increase occured under conditions that afford protection against LPS hepatotoxicity (ie. PMN depletion), this result indicates that TNF-alpha does not directly mediate LPS-induced liver injury in vivo. Furthermore, it is consistent with the hypothesis that TNF-alpha contributes to liver injury by a PMN-dependent mechanism. Such an interaction between TNF-alpha and blood PMNs has been implicated in the pathogenesis of lung injury

after exposure of guinea pigs to recombinant human TNF-alpha (202).

The mechanism for the marked increase in circulating TNF-alpha concentration caused by PMN depletion in LPS-treated rats is not known. Since circulating TNF-alpha concentration was not increased by PMN depletion in the absence of LPS exposure, PMN depletion does not by itself stimulate TNF-alpha production. Kupffer cells are thought to be a major source of circulating TNF-alpha in rodents exposed to LPS, since LPS stimulates the production of TNF-alpha by rat Kupffer cells in vitro (255) and since TNF-alpha gene expression in Kupffer cells is temporally related to the increase in circulating TNF-alpha after exposure of mice to LPS in vivo (198). Several substances, including interferon-gamma and the yeast cell wall component, glucan, prime macrophages for production of TNF-alpha after exposure to LPS (259a,259b). Kupffer cells are fixed hepatic vascular phagocytic cells and may contribute to the removal of circulating immune complexes formed between PMNs and anti-PMN Ig during the process of PMN depletion. Thus, it seems possible that phagocytosis of immune complexes by Kupffer cells during PMN depletion may prime Kupffer cells for LPS-induced TNF-alpha production. Alternatively, PMN depletion may alter the clearance of TNF-alpha from the circulation. Further studies are required to evaluate these possibilities.

The nature of the interaction between TNF-alpha and blood PMNs in the pathogenesis of LPS hepatotoxicity is not known. Because TNF-alpha increases the adherence of PMNs to vascular endothelial cell monolayers in vitro (248,249,250), it seemed possible that TNF-alpha contributed to LPS-induced liver injury by mediating the accumulation of PMNs in the liver. However, although both TNF-alpha antiserum and pentoxifylline markedly attenuated LPS hepatotoxicity, neither significantly altered the accumulation of PMNs in the liver. This suggests that TNF-alpha does not contribute to LPS hepatotoxicity by recruiting PMNs into the liver. In addition, it is clear from this result that hepatic PMN accumulation alone was not sufficient for the full manifestation of liver injury.

TNF-alpha can stimulate the release of reactive oxygen metabolites and lysosomal enzymes from PMNs in vitro (260). It also enhances the release of these cyototoxic agents from PMNs stimulated with chemotactic peptides and phorbol myristate acetate (261). Accordingly, TNF-alpha might contribute to LPS hepatotoxicity by stimulating or enhancing the release of cytotoxic substances from PMNs.

In conclusion, these results provide evidence for a role for TNF-alpha in the pathogenesis of LPS hepatotoxicity. Furthermore, TNF-alpha appears to mediate liver injury after LPS exposure by an indirect mechanism which may involve interactions between TNF-alpha and PMNs.

CHAPTER 5

ROLE OF THE COAGULATION SYSTEM IN LPS-INDUCED LIVER INJURY

5.1 Abstract

The coagulation system has been implicated in the pathogenesis of liver injury after exposure to LPS. The purpose of this study was to test the hypothesis that the coagulation system contributes to LPS hepatotoxicity by a mechanism which is dependent on circulating fibrinogen. Relationships between PMNs, TNF-alpha and the coagulation system were also examined. A marked reduction in plasma fibrinogen concentration occurred in rats after LPS exposure. This preceded the onset of liver injury. Pretreatment with heparin or warfarin attenuated the decrease in plasma fibrinogen and afforded protection against liver injury in LPS-treated rats. The decrease in circulating fibrinogen after LPS exposure was also attenuated by depletion of circulating PMNs, pretreatment with pentoxifylline, or pretreatment with TNF-alpha antiserum, all of which protect against LPS hepatotoxicity. In contrast, pretreatment with ancrod 4 and 2 hr prior to LPS exposure, which reduced circulating fibrinogen to < 20 mg/dl in rats treated with LPS vehicle, did not afford protection against LPS hepatotoxicity. Heparin did not prevent accumulation of PMNs in the liver after LPS exposure. These results suggest that the coagulation system

contributes to the pathogenesis of LPS-induced liver injury by a mechanism which is independent of circulating fibrinogen and recruitment of PMNs. Also, activation of the coagulation system after LPS is mediated by both PMNs and TNF-alpha.

5.2 Introduction

Activation of the coagulation system is often associated with exposure to LPS. This is characterized in part by a decrease in circulating concentrations of clotting factors (204) and by an increase in the prothrombin and partial thromboplastin times (262,263). These LPS-induced changes are accompanied by a marked decrease in circulating fibrinogen concentration (111,204,262,264) and by the deposition of fibrin in the microvasculature of various tissues, including the kidneys (262,264,265), lungs (264,265) and liver (129,264,265). The decrease in circulating fibrinogen after LPS exposure is thought to reflect the proteolytic action of thrombin, which catalyzes the formation of fibrin monomers from fibrinogen (266).

Evidence suggests that certain pathophysiologic alterations associated with exposure to LPS may be mediated by the coagulation system. Among these is injury to the liver. For example, pretreatment with the anticoagulant, heparin, prevented the reduction in circulating coagulation factors (204) and attenuated liver injury after LPS exposure

(102,203). Also, infusion of thrombin into the portal vein of rats produced morphologic changes in the liver, including fibrin deposition in the hepatic sinusoids and hepatocellular necrosis, which resemble those produced by portal venous infusion of LPS (129).

Although these results point to the coagulation system in the pathogenesis of LPS hepatotoxicity, the mechanisms of liver injury by the coagulation system during LPS exposure have not been elucidated. Results from several studies suggest that the coagulation system may mediate tissue injury in certain instances by a mechanism which is dependent on fibrinogen. For example, pretreatment of sheep with the thrombin-like enzyme, ancrod, which depletes circulating fibrinogen without the concomitant formation of insoluble fibrin clots (267,268), afforded protection against lung injury after intravenous infusion of thrombin (269). Similarly, hirudin, an anticoagulant that binds to and inhibits thrombin (270), attenuated the decrease in plasma fibrinogen concentration, reduced the deposition of fibrin in the pulmonary microvasculature, and protected against lung injury after LPS exposure (111,264). As in the lungs, it seems possible that the coagulation system could contribute to LPS-induced liver injury by a mechanism which is dependent on fibrinogen.

Alternatively, the coagulation system may contribute to LPS hepatotoxicity by a mechanism which is dependent on blood PMNs. Activated PMNs cause tissue injury under certain

circumstances, presumably through the release of cytotoxic substances such as oxygen metabolites and lysosomal enzymes (153). Among the early morphologic changes in the liver after LPS exposure is the accumulation of large numbers of PMNs (126,127), and these phagocytic cells contribute to the pathogenesis of LPS-induced liver (Chapter 3). Because PMN chemotactic factors are generated by activation of the coagulation system (271), it seemed possible that the coagulation system could mediate liver injury after LPS exposure by promoting the recruitment of PMNs.

In addition to PMNs, TNF-alpha, plays an important role in LPS-induced hepatotoxicity (Chapter 4). Indeed, the full manifestation of liver injury after exposure to LPS appears to be dependent on an interaction between this cytokine and PMNs, inasmuch as preventing the rise in plasma TNF-alpha protected the liver but did not decrease hepatic PMN infiltration. Similarly, depletion of circulating PMNs afforded protection against LPS hepatotoxicity (Chapter 3) but did not attenuate the increase in plasma TNF-alpha (Chapter 4). Thus, both PMNs and TNF-alpha have important roles in LPS hepatotoxicity, but neither PMNs nor TNF-alpha alone are sufficient to cause liver injury during LPS exposure. TNF-alpha stimulates PMNs in vitro to release oxygen metabolites (260,261), which appear to activate the coagulation system during exposure of animals to LPS (272). Accordingly, it seems possible that an interaction between

PMNs and TNF-alpha could mediate liver injury after LPS exposure through activation of the coagulation system.

The objective of the present study was to test the hypothesis that the coagulation system contributes to the pathogenesis of LPS-induced liver injury by a mechanism which is dependent on circulating fibrinogen and on recruitment of PMNs into the liver. The possible roles of PMNs and TNF-alpha in activating the coagulation system during exposure to LPS were also examined.

5.3 Materials and methods

5.3.a Animals

Female, Sprague-Dawley rats (Charles River, Crl:CD BR (SD) VAF/plus, Portage, MI) weighing 200-250g were used in all studies. Rats were maintained on a 12 hr light-dark schedule for 1 week prior to use. Food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and water were allowed ad libidum throughout the studies.

5.3.b Treatment protocols

Heparin (Type II), warfarin [3-(alpha-acetonylbenzyl)-4-hydroxycoumarin], ancrod (from Agkistrodon rhodostoma venom), and pentoxifylline were obtained from Sigma Chemical Company (St. Louis, MO).

An initial study was performed to evaluate the temporal relationship between activation of the coagulation system

and the onset of liver injury after exposure to LPS. Activation of the coagulation system and liver injury were quantified 0, 1.5, 3, and 6 hr after exposure to LPS by changes in plasma fibrinogen concentration and by changes in plasma AST activity and total plasma bilirubin concentration, respectively (see below).

Studies were also performed to assess the effects of the anticoagulants, heparin and warfarin, on LPS-induced activation of the coagulation system and liver injury. Heparin (2000 U/kg) or its saline vehicle was administered in the tail vein 0.5 hr prior to LPS exposure. The maximum effects of warfarin on the coagulation system in rats were observed between 16 and 24 hr after po administration of warfarin (273). Thus, warfarin (20 mg/kg, po) or its saline vehicle was administered 16 hr prior to exposure to LPS.

To assess the effects of defibrinogenation on LPS hepatotoxicity, rats were pretreated with ancrod. Ancrod (50 U/kg per administration) or its saline vehicle was administered in the tail vein 4 and 2 hr prior to LPS exposure. This protocol causes an 89 % reduction in plasma fibrinogen concentration in rats 4 hr after the initial exposure to ancrod (274).

In a study to test the effect of depletion of blood PMNs on the LPS-induced activation of the coagulation system, rats were pretreated with the total Igs fraction of serum from rabbits immunized with rat PMNs (anti-PMN Ig) (see below). Total Ig fraction of serum from non-immunized

rabbits was used as a control (control Ig). Igs were administered in the tail vein of rats in a volume of 0.5 ml 18 and 6 hr prior to exposure to LPS. This protocol markedly reduces circulating PMNs and affords protection against LPS hepatotoxicity (Chapter 3).

The effect of pentoxifylline or antiserum to TNF-alpha on the coagulation system was also assessed after LPS exposure. Pentoxifylline (100 mg/kg) or saline vehicle was administered in the tail vein 1 hr prior to LPS administration. Neutralizing antiserum to recombinant mouse TNF-alpha, which was raised in rabbits and which cross-reacts with rat TNF-alpha (275), was a gift from Dr. Steven L. Kunkel. It was diluted 1:1 in sterile saline and administered in a 2 ml volume in the tail vein of rats 1 hr prior to LPS exposure. Serum from non-immunized rabbits was used as a control. These treatments obtund the increase in plasma TNF-alpha that occurs after LPS administration (Chapter 4).

In the above studies, LPS (E. coli 0128:B12, Sigma Chemical Co, St. Louis, MO) or saline vehicle were administered in the tail vein of rats in a volume of 5 ml/kg. Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) 6 hr after exposure to LPS unless stated otherwise. Blood samples anticoagulated with sodium citrate (1/10 dilution in whole blood of 3.8 % w/v sodium citrate in DDW) were obtained from the inferior vena cava for the

quantification of plasma fibrinogen concentration and liver injury.

5.3.c Quantification of plasma fibrinogen concentration and liver injury

Changes in plasma fibrinogen concentration were measured to quantify activation of the coagulation system after LPS exposure and to verify defibrinogenation in studies with ancrod. Plasma fibrinogen concentration was determined from the thrombin clotting time of diluted samples using a fibrometer and a commercially available kit (Data-Fi, B4233-15, American Dade, Aguada, Puerto Rico). The procedure is based on that described by Clauss (276).

Liver injury was quantified by changes in plasma AST activity and total plasma bilirubin concentration as described in Section 3.3.d.

5.3.d Anti-PMN Ig preparation

Igs to rat peritoneal PMNs were raised in New Zealand white rabbits as detailed in Chapter 3 (254). The total Ig fraction of serum from immunized (anti-PMN Ig) and non-immunized (control Ig) rabbits was isolated by ammonium sulfate precipitation (221 and Chapter 3).

5.3.e Quantification of hepatic NPC and PMN number

To assess the role of the coagulation system in hepatic PMN infiltration after LPS exposure, rats were pretreated

with heparin or saline vehicle and treated with LPS as described above. Hepatic PMNs were quantified 1.5 hr after LPS exposure as detailed in Chapter 3 (254). Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and 40 ml of Ca^{2+}-free Hank's balanced salt solution (HBSS, pH 7.4) containing EGTA (0.5 mM) and HEPES (0.5 M) was infused in situ through a cannula placed in the portal vein to flush the liver of blood. The liver was removed, minced thoroughly with scissors and incubated for 1-1.5 hr at 37°C in Ca^{2+}-free HBSS containing heparin (1000 U/ml, grade II, Sigma Chemical Co, St. Louis, MO) and Pronase E (0.2 % w/v, Type XIV protease, Sigma Chemical Co, St. Louis, MO) to lyse parenchymal cells. The remaining cells, which include hepatic NPCs and PMNs, were pelleted in a centrifuge (500xg, 20 min), washed twice with HBSS, and resuspended in 25 ml Ca^{2+}-free HBSS containing heparin.

Total NPCs were quantified using a Unopette (test 5859, Becton-Dickinson, Rutherford, NJ) and a hemocytometer. The NPC isolates were diluted in Ca^{2+}-free HBSS containing 1 % BSA (Sigma Chemical Co, St. Louis, MO) and concentrated on a microscope slide using a cytocentrifuge (Cytospin 2, Shandon Southern Inc, Sewickley, PA). The fraction of PMNs was quantified by differential count after the slides were stained with a modified Wright's stain. The total number of NPCs was multiplied by the fraction of PMNs to obtain the number of PMNs/liver.

5.3.f Data analysis

Data from studies to determine changes in circulating fibrinogen and total plasma bilirubin concentration with time after LPS exposure were anaylzed using the rank sums test and Bonferroni's correction factor. A one-way, completely random ANOVA was used to analyze time-dependent changes in plasma AST activity after LPS exposure. Comparisons among group means were made using the least significant difference test (277).

Data from studies to test the effects of heparin and warfarin on LPS-induced changes in plasma AST activity and total plasma bilirubin concentration were analyzed using the rank sums test (277). Data from studies of the effect of ancrod on changes in plasma AST activity and total plasma bilirubin as well as from studies of the effects of heparin, warfarin, ancrod and pentoxifylline on circulating fibrinongen concentration were analyzed using a 2 x 2 factorial, completely random ANOVA. Changes in hepatic PMN number after administration of LPS to rats pretreated with heparin were also assessed using a 2 x 2 factorial, completely random ANOVA. ANOVAs were performed on log transformed data in instances in which variances were non-homogeneous. Comparisons among group means were made using the least significant difference test.

Results are expressed as means $\pm$ SEM. The criterion for significance was $p < 0.05$ for all studies.

5.4 Results

5.4.a Role of the coagulation system in LPS hepatotoxicity

The temporal relationship between liver injury and activation of the coagulation system is shown in Figure 5.1. Liver injury, as measured by elevations in plasma AST activity and total plasma bilirubin concentration, occurred between 3 and 6 hr after LPS exposure (Figure 5.1A and B). Activation of the coagulation system preceded the onset of liver injury as indicated by the marked decrease in plasma fibrinogen concentration that occurred between 1.5 and 3 hr after LPS administration (Figure 5.1C). Circulating fibrinogen concentration was reduced > 85 % 6 hr after exposure to LPS.

The effects of anticoagulants on LPS-induced liver injury and activation of the coagulation system are shown in Figures 5.2 and 5.3 and in Table 5.1, respectively. Pretreatment with heparin attenuated the elevation in plasma AST activity and total bilirubin concentration 6 hr after administration of LPS (Figure 5.2). This was associated with a complete inhibition of the LPS-induced reduction of circulating fibrinogen concentration (Table 5.1). Like heparin, warfarin afforded protection against liver injury (Figure 5.3) and attenuated the LPS-induced reduction in circulating fibrinogen concentration (Table 5.1). In contrast to heparin, a significant decrease in circulating

Figure 5.1 Development of liver injury and activation of the coagulation system after LPS exposure. LPS (5 mg/kg, iv) or saline vehicle was administered at 0 hr. Liver injury and activation of the coagulation system were quantified by changes in plasma aspartate aminotransferase (AST) activity (A) and total plasma bilirubin concentration (B) and by changes in plasma fibrinogen concentration (C), respectively, as described in Section 5.3.c. Results are expressed as the means ± SEM;
N=3-6.
a, significantly different from the respective value at 0 hr.
b, significantly different from the respective vehicle-treated control.

Figure 5.1

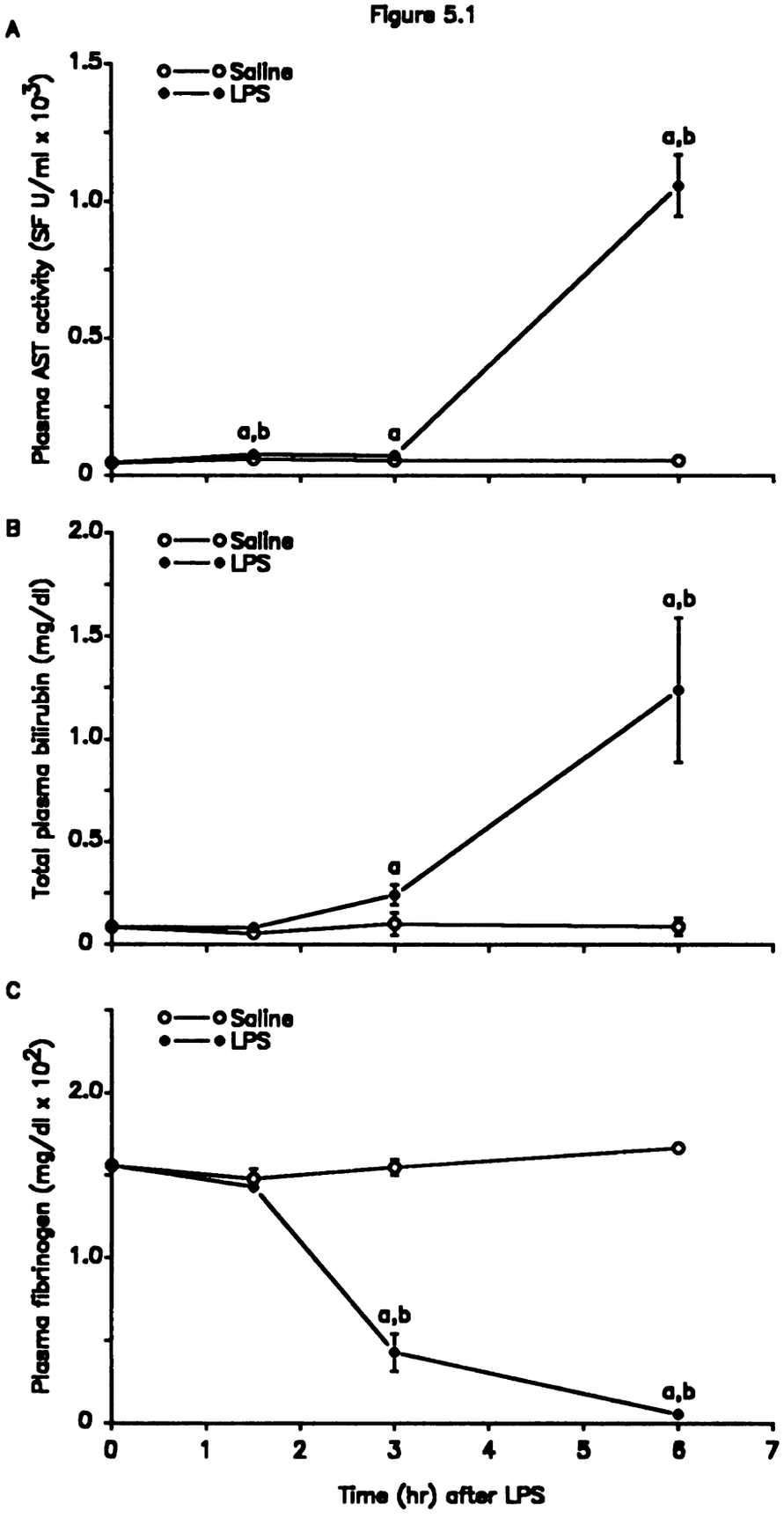

A
Saline
LPS
Plasma AST activity (SF U/ml x 10³)
1.5
1.0
0.5
0
a,b
a
a,b
B
Saline
LPS
Total plasma bilirubin (mg/dl)
2.0
1.5
1.0
0.5
0
a
a,b
C
Saline
LPS
Plasma fibrinogen (mg/dl x 10²)
2.0
1.0
0
a,b
a,b
0
1
2
3
4
5
6
7
Time (hr) after LPS

Figure 5.2 Effect of heparin on LPS-induced liver injury. Rats were pretreated with either heparin (2000 U/kg, iv) or saline vehicle 30 min prior to treatment with either LPS (5 mg/kg, iv) or saline vehicle. Liver injury was quantified 6 hr after LPS exposure by changes in plasma aspartate aminotransferase (AST) activity (A) and total plasma bilirubin concentration (B) as described in Section 5.3.c. Results are expressed as means ± SEM; N=5-10.

a, significantly different from the respective saline treatment group.

b, significantly different from the respective vehicle-pretreated control.

Figure 5.2

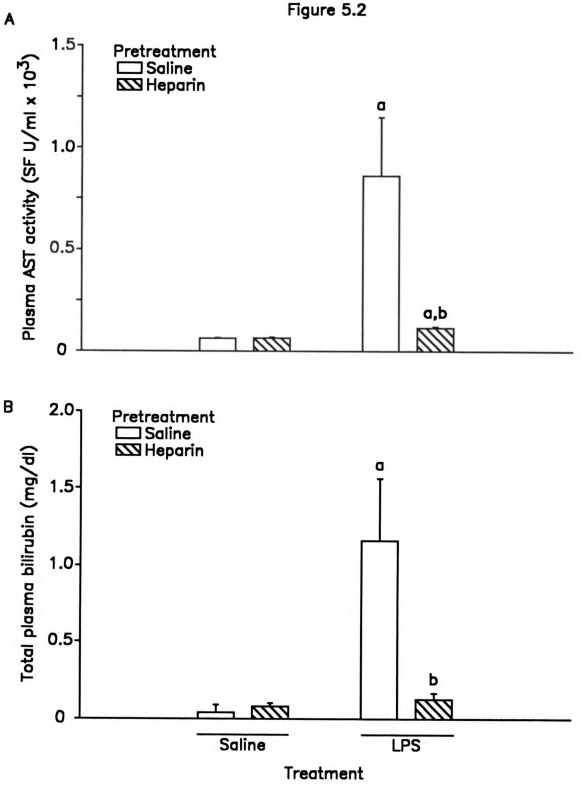

A
Pretreatment
Saline
Heparin
Plasma AST activity (SF U/ml x 10^3)
1.5
1.0
0.5
0
a
a,b
B
Pretreatment
Saline
Heparin
Total plasma bilirubin (mg/dl)
2.0
1.5
1.0
0.5
0
a
b
Saline
LPS
Treatment

Figure 5.3 Effect of warfarin on LPS-induced liver injury. Rats were pretreated with either warfarin (20 mg/kg, po) or distilled water (DDW) vehicle 16 hr prior to treatment with either LPS (5 mg/kg, iv) or saline vehicle. Liver injury was assessed 6 hr after LPS exposure by changes in plasma aspartate aminotransferase (AST) activity (A) and total plasma bilirubin concentration (B) as described in Section 5.3.c. Results are expressed as means ± SEM; N=6-11.

a, significantly different from the respective saline treatment group.

b, significantly different from the respective vehicle-pretreated control.

Figure 5.3

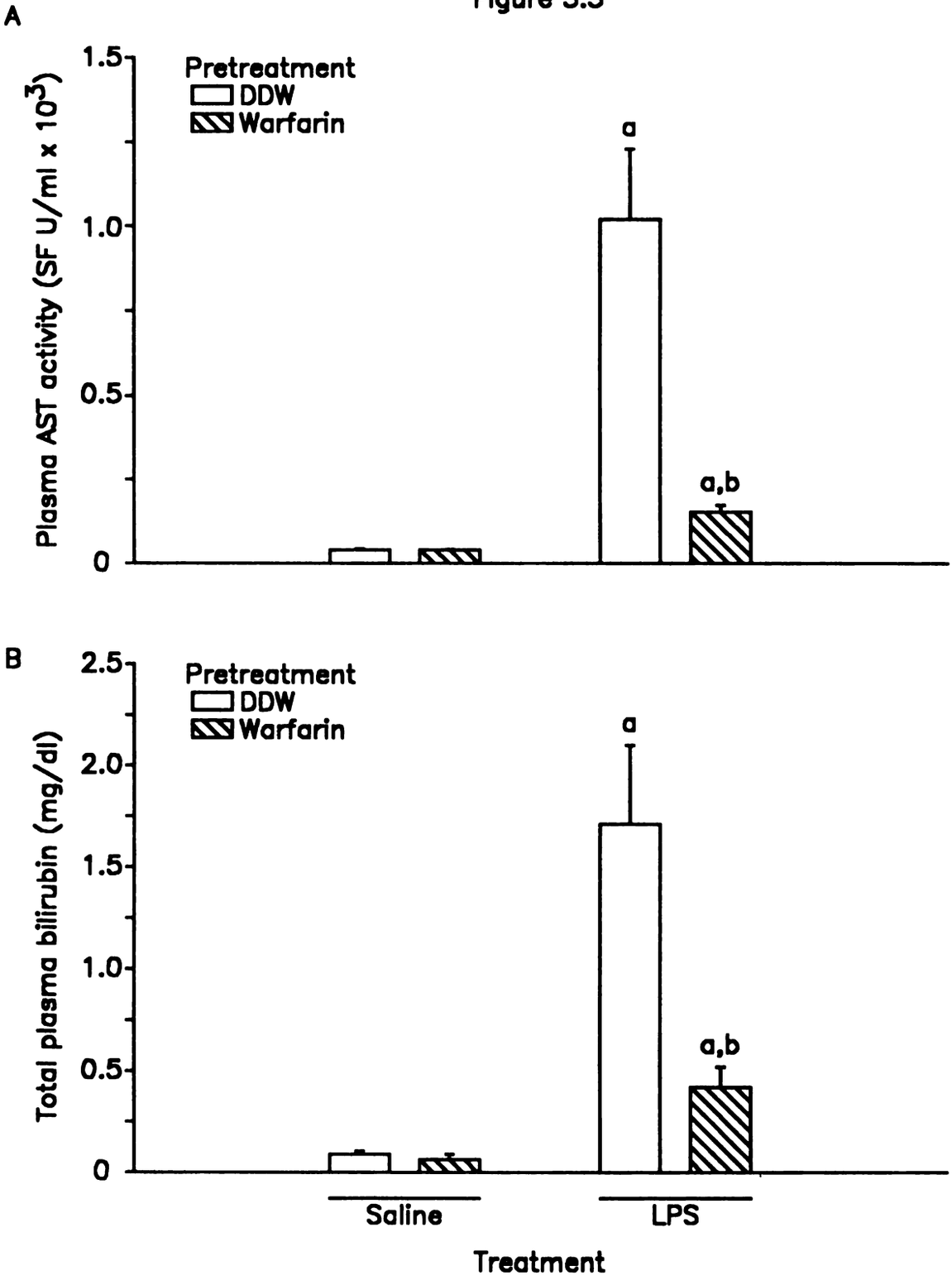

A
Pretreatment
DDW
Warfarin
Plasma AST activity (SF U/ml x 10^3)
1.5
1.0
0.5
0
a
a,b
B
Pretreatment
DDW
Warfarin
Total plasma bilirubin (mg/dl)
2.5
2.0
1.5
1.0
0.5
0
a
a,b
Saline
LPS
Treatment

Table 5.1
Effect of anticoagulants on circulating fibrinogen concentration in the presence and absence of LPS exposure.

Pretreatment	LPS	Plasma fibrinogen (mg/dl)
Heparin		
-	-	196 ± 14(4)
+	-	195 ± 23(4)
-	+	46 ± 17(7)[a]
+	+	207 ± 13(8)
Warfarin		
-	-	182 ± 26(6)
+	-	194 ± 10(6)
-	+	40 ± 10(12)[a]
+	+	97 ± 10(10)[a,b]
Ancrod		
-	-	147 ± 12(3)
+	-	18 ± 3(3)[b]
-	+	38 ± 13(7)[a]
+	+	12 ± 1(8)

Rats were pretreated with either heparin (2000 U/kg, iv), warfarin (20 mg/kg, po), ancrod (50 U/kg x 2, iv) or their respective vehicles (-) and then treated with either LPS (+) or saline vehicle (-). Plasma fibrinogen was quantified 6 hr after exposure to LPS (5 mg/kg, iv) as described in Section 5.3.c. Results are expressed as means ± SEM. Number of measurements are in parentheses.

a, Significantly different from the respective saline treatment group.

b, Significantly different from respective vehicle-pretreated control.

fibrinogen concentration occurred in warfarin-pretreated rats after LPS exposure. However, this decrease was less than that which occurred in LPS-exposed rats that received no warfarin.

5.4.b Mechanism of liver injury by the coagulation system after LPS exposure

Ancrod causes a marked reduction in circulating fibrinogen concentration without significantly altering circulating concentrations of other clotting factors (278). Rats were pretreated with ancrod to assess the role of circulating fibrinogen in the pathogenesis of liver injury after LPS exposure. Ancrod pretreatment reduced circulating fibrinogen concentration by more than 85 % in the absence of LPS exposure (Table 5.1) but did not cause hepatotoxicity (Figure 5.4). Elevations in plasma AST activity and total plasma bilirubin concentration produced by administration of LPS were not significantly attenuated by pretreatment of rats with ancrod.

In an earlier report, we presented evidence indicating that PMNs mediate liver injury from LPS (254 and Chapter 3). It seemed possible that activation of the coagulation system by LPS exposure might contribute to recruitment of PMNs into the liver. This possibility was tested by examining hepatic PMN accumulation in LPS-exposed rats after pretreatment with heparin anticoagulant. The results from this study are shown in Figure 5.5. A significant increase in hepatic PMNs was

Figure 5.4 Effect of ancrod on LPS-induced liver injury. Rats were pretreated with ancrod (50 U/kg, iv) or saline vehicle 2 and 4 hr prior to treatment with either LPS (5 mg/kg, iv) or saline vehicle. Plasma aspartate aminotransferase (AST) activity (A) and total plasma bilirubin concentration (B) were quantified 6 hr after LPS exposure as described in Section 5.3.c. Results are expressed as the means ± SEM; N=3-8.

a, significantly different from the respective saline treatment group.

Figure 5.4

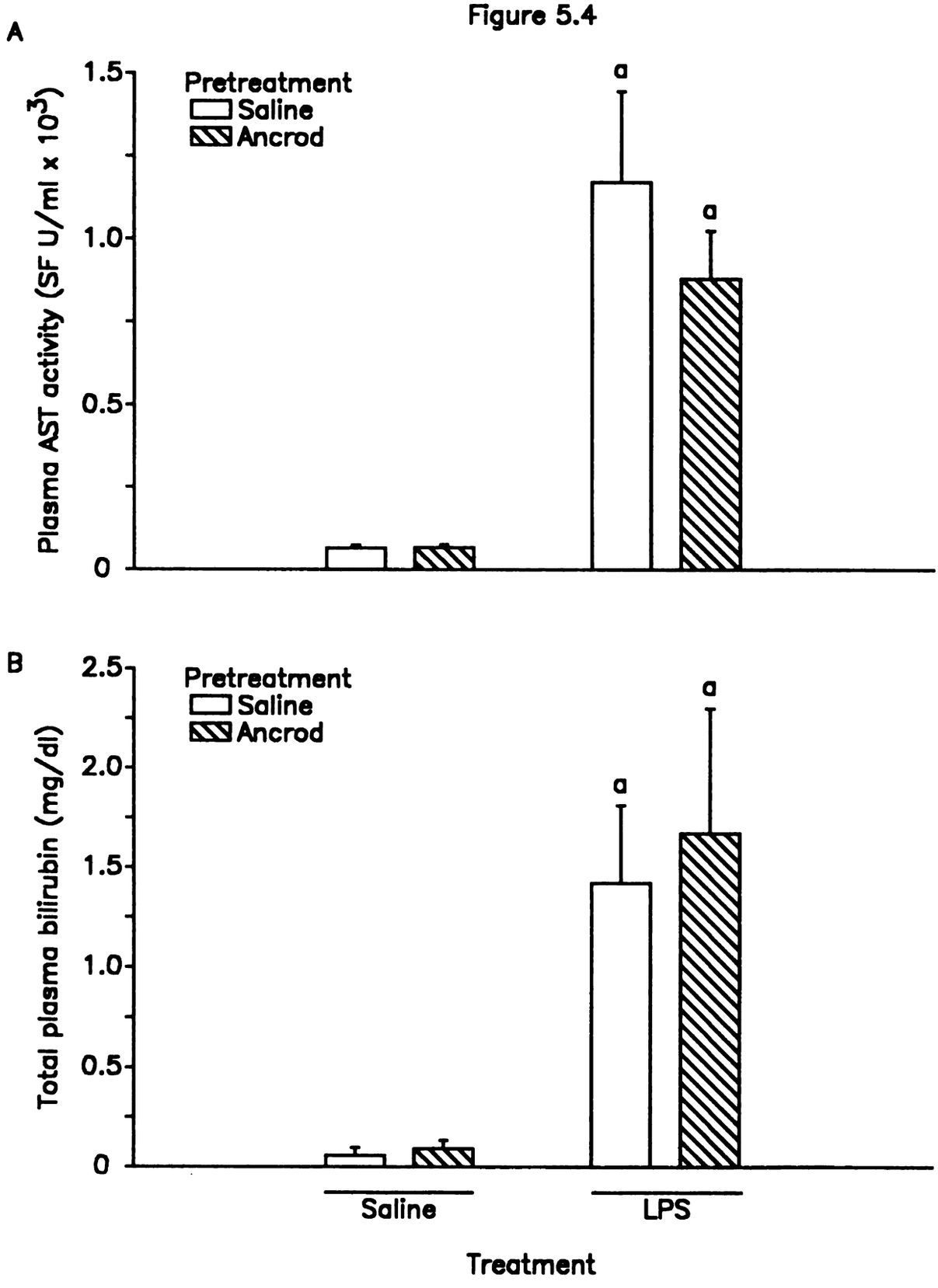
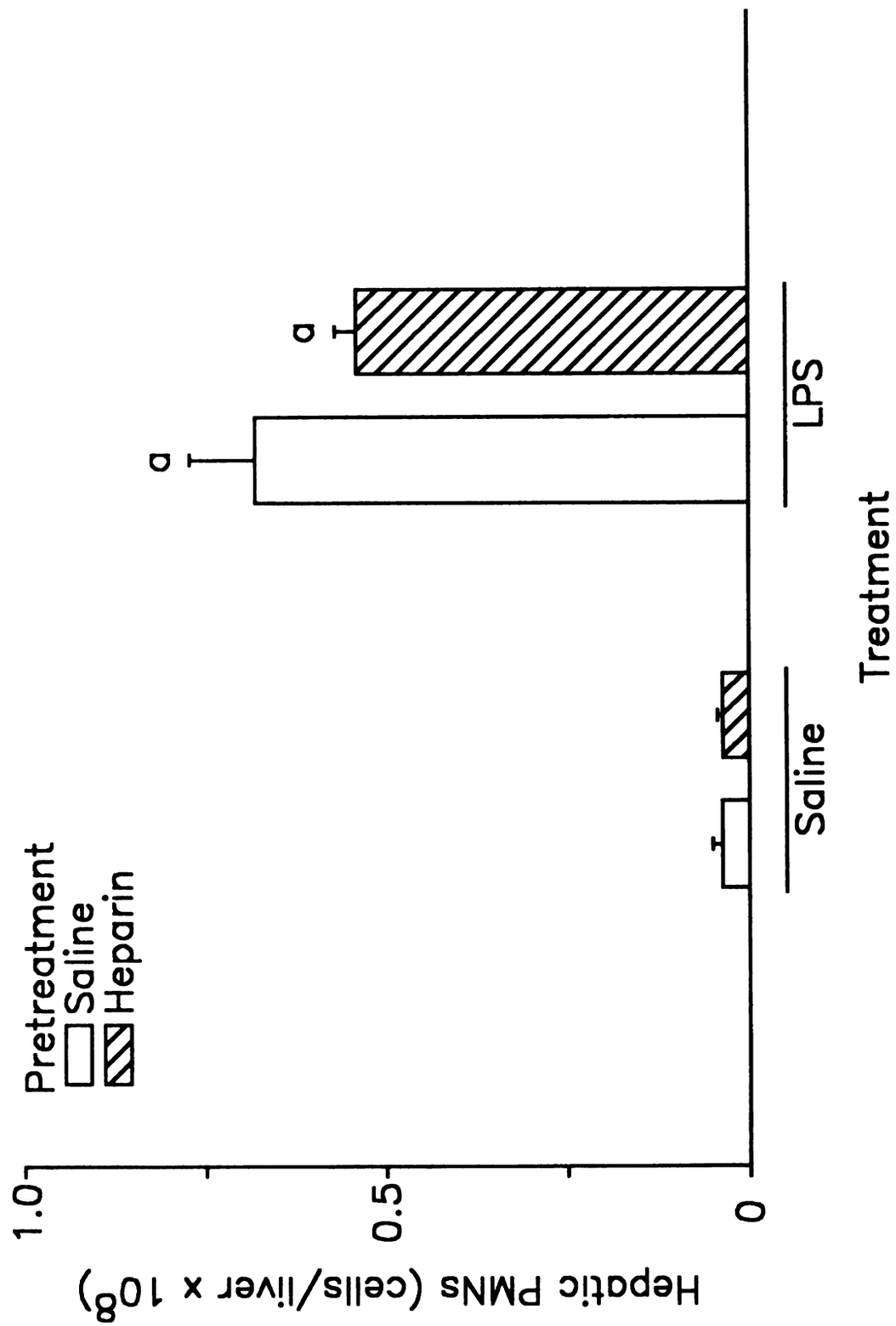

A
Plasma AST activity (SF U/ml x 10^3)
1.5
1.0
0.5
0
Pretreatment
Saline
Ancrod
a
a
B
Total plasma bilirubin (mg/dl)
2.5
2.0
1.5
1.0
0.5
0
Pretreatment
Saline
Ancrod
a
a
Saline
LPS
Treatment

Figure 5.5 Effect of heparin on hepatic neutrophil (PMN) numbers after LPS administration. Rats were pretreated with either heparin or saline vehicle and then treated with either LPS or saline vehicle as described in Figure 5.2. Hepatic PMN numbers were quantified 1.5 hr after LPS exposure as described in Sections 3.3.h-i and 5.3.e. Results are expressed as means ± SEM; N=4-9.
a, significantly different from respective saline treatment group.

Figure 5.5


Hepatic PMNs (cells/liver x 10^8)
1.0
0.5
0
a
a
Pretreatment
Saline
Heparin
Saline
LPS
Treatment

observed after administration of LPS alone. The accumulation of PMNs was not significantly attenuated by pretreatment with heparin. Thus, heparin affords protection from LPS hepatotoxicity but does not prevent influx of PMNs into the liver.

5.4.c Mechanism of activation of the coagulation system after LPS

To test the possibility that activation of the coagulation system during LPS exposure is dependent on PMNs, the effects of PMN depletion on the LPS-induced reduction in plasma fibrinogen concentration was examined. The results from this study are shown in Figure 5.6. A time-dependent decrease in plasma fibrinogen concentration was observed in LPS-treated rats that were pretreated with control Ig. Plasma fibrinogen concentration was maximally decreased 6 hr after LPS exposure and returned to pre-exposure values thereafter. Pretreatment of rats with anti-PMN Ig resulted in a modest but statistically significant elevation in plasma fibrinogen concentration prior to LPS exposure and attenuated the decrease in plasma fibrinogen concentration 6 hr after administration of LPS.

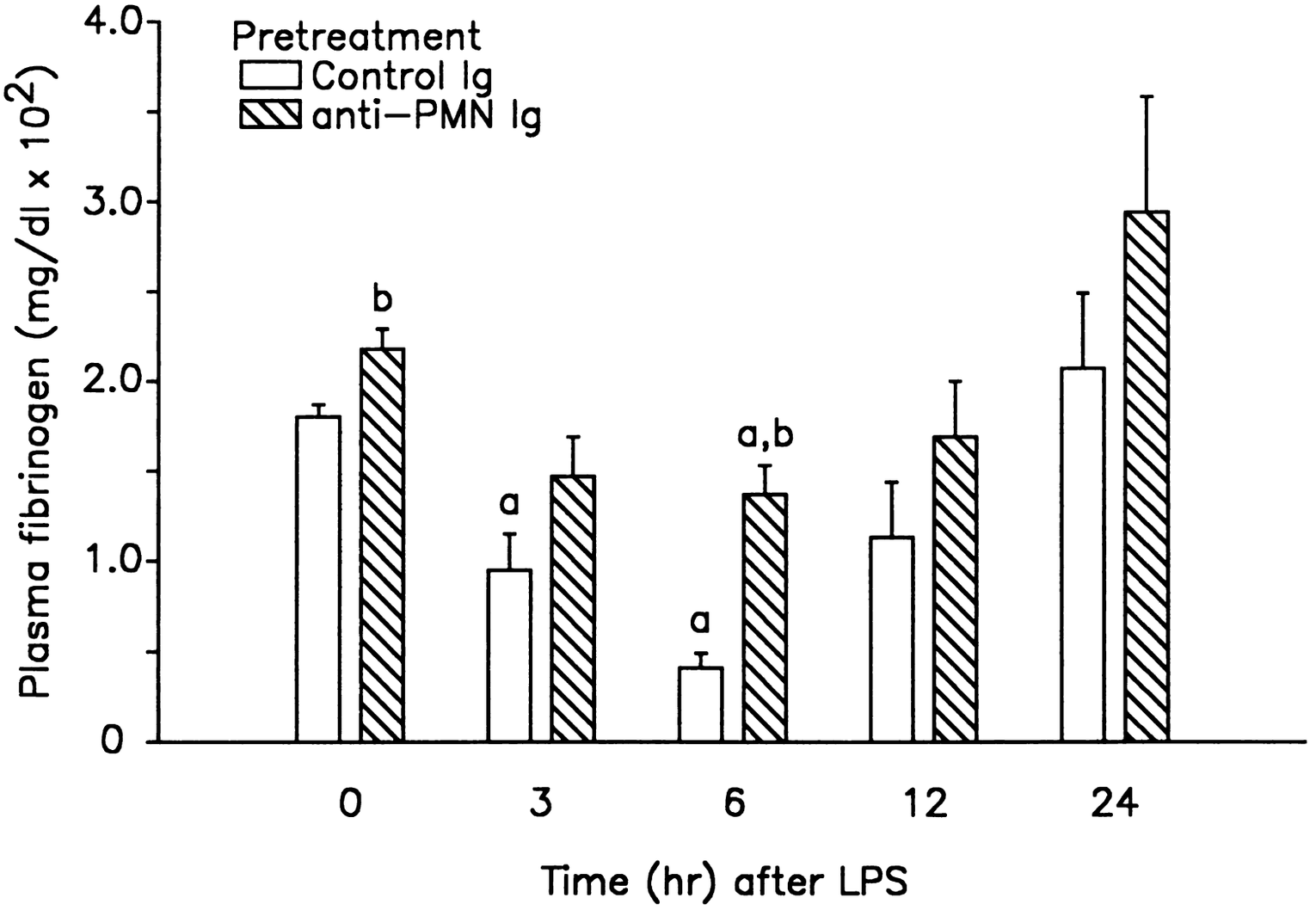
Pretreatment of rats with either pentoxifylline or antiserum to TNF-alpha obtunds the LPS-induced increase in circulating TNF-alpha concentration and affords protection against LPS hepatotoxicity (Chapter 4). Rats were pretreated with either pentoxifylline or antiserum to TNF-alpha to

Figure 5.6 Effect of depletion of circulating neutrophils (PMNs) on activation of the coagulation system after LPS exposure. Rats were pretreated with either control Ig or anti-PMN Ig as described in Section 5.3.b. Activation of the coagulation system at indicated times after treatment with LPS (2 mg/kg, iv) was quantified by changes in plasma fibrinogen concentration (see Section 5.3.c). Results are expressed as means $\pm$ SEM; N=6.

a, significantly different from the respective value at 0 hr.

b, significantly different from the respective control Ig pretreatment group.

Figure 5.6


Pretreatment
Control Ig
anti–PMN Ig
Plasma fibrinogen (mg/dl x 10^2)
4.0
3.0
2.0
1.0
0
b
a
a
a,b
0
3
6
12
24
Time (hr) after LPS

examine the possibility that TNF-*alpha* contributes to activation of the coagulation system after LPS exposure. Pentoxifylline pretreatment attenuated the decrease in circulating fibrinogen concentration 6 hr after LPS administration (Figure 5.7). Similarly, pretreatment with antiserum to TNF-*alpha* significantly attenuated the LPS-induced decrease in circulating fibrinogen (19 $\pm$ 5 and 60 $\pm$ 3 mg/dl plasma in LPS-treated rats pretreated with control serum or TNF-*alpha* antiserum, respectively).

5.5 Discussion

Results from the this study indicate that activation of the coagulation system after LPS exposure occurs prior to the onset of liver injury. Furthermore, inhibition of the coagulation system by pretreatment with either heparin or warfarin afforded protection against LPS-induced liver injury. These results support the hypothesis that the coagulation system is important in the pathogenesis of LPS hepatotoxicity.

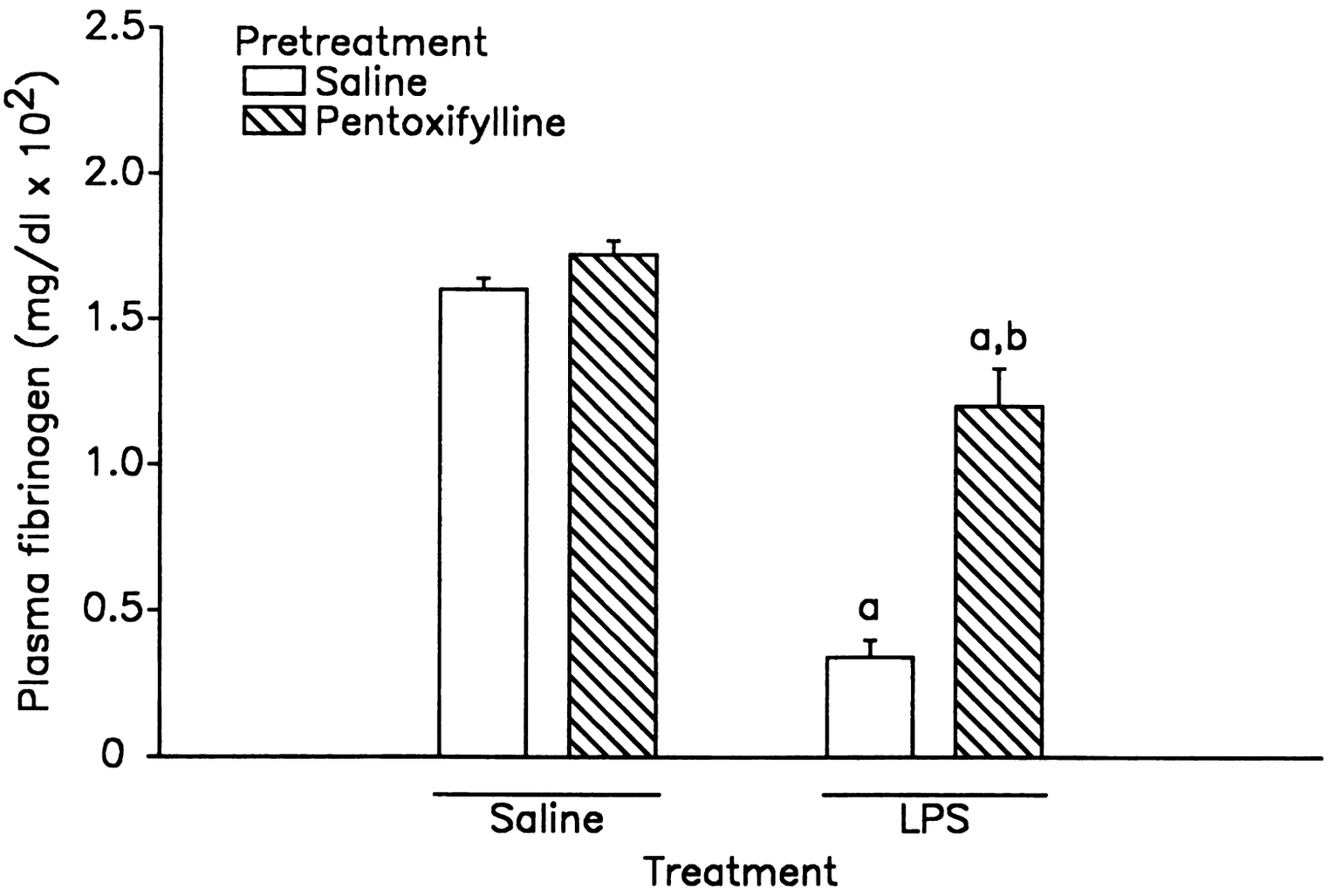
It has been suggested that circulatory disturbances from occlusive fibrin thrombi in the hepatic microvasculature contribute to the pathogenesis of LPS-induced liver injury (129). However, pretreatment with ancrod, which markedly reduced circulating fibrinogen concentration, did not afford protection against liver injury after LPS exposure. Since ancrod acts by converting

Figure 5.7 Effect of pentoxifylline on LPS-induced activation of the coagulation system. Rats were pretreated with either pentoxifylline (100 mg/kg, iv) or saline vehicle 1 hr prior to treatment with either LPS (5 mg/kg, iv) or saline vehicle. Activation of the coagulation system was assessed by changes in plasma fibrinogen 6 hr after administration of LPS as described in Section 5.3.c. Results are expressed as means ± SEM; N=4-10.

a, significantly different from the respective saline treatment group.

b, significantly different from the respective vehicle-pretreated control.

Figure 5.7


Pretreatment
Saline
Pentoxifylline
Plasma fibrinogen (mg/dl x 10²)
2.5
2.0
1.5
1.0
0.5
0
a
a,b
Saline
LPS
Treatment

fibrinogen to soluble fibrin monomers which do not form clots (267,268), this result argues against the role of fibrin thrombi-induced circulatory disturbances in LPS-induced liver injury and strongly suggests that the coagulation system contributes to LPS hepatotoxicity by a mechanism which is independent of fibrinogen.

Taken together, the results with ancrod, heparin and warfarin suggest that components of the coagulation pathway proximal to fibrinogen are important in the pathogenesis of liver injury. Because intraportal infusion of thrombin produces liver injury which resembles that caused by LPS (129), it seems possible that thrombin, independent of its action on fibrinogen, may contribute to LPS hepatotoxicity. Thrombin has a number of biologic activities that are independent of its proteolytic action on circulating fibrinogen. Among these is the stimulation of aggregation of and thromboxane A_2 release from platelets (279,280,281,282). Circulating concentrations of thromboxane are markedly increased within 2 hr after exposure to LPS (214), and this vasoactive arachidonic acid metabolite may contribute to the pathogenesis of LPS-induced liver injury (212,215). Thus, thrombin might play an important role in LPS hepatotoxicity by stimulating release of thromboxane A_2 from platelets. Thrombin also binds to specific receptors on the surface of hepatic parenchymal cells (283). However, the pathophysiologic consequenses of this binding remain to be determined.

Exposure to LPS is associated with the accumulation of large numbers of PMNs in the liver (Chapter 3 and Figure 5). Because PMNs have been implicated in the pathogenesis of liver injury after LPS exposure (Chapter 3), it seemed possible that the coagulation system could contribute to LPS hepatotoxicity by promoting the accumulation of PMNs in the liver. However, pretreatment with heparin, which inhibited the reduction in circulating fibrinogen after LPS exposure and afforded protection against liver injury, did not significantly reduce accumulation of PMNs in the liver. Thus, the coagulation system does not appear to contribute to the pathogenesis of LPS hepatotoxicity solely by mediating the recruitment of PMNs.

It has been suggested that leukocytes may be involved in the activation of the coagulation system during LPS exposure (263). In the present study, depletion of circulating PMNs markedly attenuated the decrease in circulating fibrinogen concentration associated with LPS exposure. This suggests that activation of the coagulation system after exposure to LPS is mediated by PMNs. The mechanism of activation of the coagulation system by PMNs after LPS administration is not known. The prolongation of prothrombin and partial thromboplastin times, reduction in circulating fibrinogen concentration, and accumulation of fibrin in tissues after LPS exposure are attenuated by administration of superoxide dismutase and catalase (272). This suggests that oxygen metabolites contribute to the

activation of the coagulation system after LPS exposure. Because extracellular release of reactive oxygen metabolites can accompany activation of PMNs (153), it is possible that PMNs mediate activation of the coagulation system during LPS exposure by an oxygen radical-dependent mechanism. Additional studies are necessary to determine the role of PMN-derived oxygen metabolites in activation of the coagulation system after LPS exposure.

Results from several studies suggest that TNF-*alpha* contributes to the pathogenesis of liver injury during LPS exposure. For example, neutralization of circulating TNF-*alpha* with antiserum to this cytokine afforded protection against LPS-induced liver injury (252,254). Pentoxifylline, which inhibits TNF-*alpha* production from LPS-stimulated macrophages *in vitro* and attenuates the rise in circulating TNF-*alpha* *in vivo* (251,284), also afforded protection against LPS hepatotoxicity (Chapter 4). Both pentoxifylline and TNF-*alpha* antiserum attenuated the decrease in circulating fibrinogen concentration after LPS exposure. This result suggests that TNF-*alpha* may contribute to the pathogenesis of liver injury by mediating the activation of the coagulation system.

TNF-*alpha* directly induces procoagulant activity from vascular endothelial cells in culture (189). Perhaps TNF-*alpha* contributes to activation of the coagulation system after LPS exposure *in vivo* by a similar mechanism. However, PMN depletion enhanced circulating TNF-*alpha* concentration

by more than 3-fold during LPS exposure (Chapter 4) but attenuated the decrease in circulating fibrinogen concentration. This indicates that TNF-*alpha* by itself is not sufficient to activate the coagulation system during LPS exposure. Furthermore, this result suggests that TNF-*alpha* promotes activation of the coagulation system by a PMN-dependent mechanism.

In summary, these results indicate that the coagulation system contributes to the pathogenesis of liver injury after LPS exposure by a mechanism which is not dependent on circulating fibrinogen or the recruitment of PMNs. In addition, results from PMN depletion studies and studies with pentoxifylline and TNF-*alpha* antiserum suggest that activation of the coagulation system after exposure to LPS is dependent on both PMNs and TNF-*alpha*.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The overall aim of this dissertation was to examine the mechanisms of LPS-induced liver injury. Specifically, the role of host-derived inflammatory mediators in the pathogenesis was assessed. Liver injury was quantified in most studies by changes in liver specific enzymes activities in the plasma, which are indicative of hepatic parenchymal cell injury, and by changes in total plasma bilirubin concentation, which is consistent with alterations in parenchymal cell function and cholestasis. Evidence from the studies presented in this dissertation suggests that 1) liver injury after LPS exposure is mediated largely by certain host-derived factors including circulating PMNs, TNF-*alpha* and the coagulation system, and 2) that full manifestation of LPS-induced liver injury is dependent on complex interactions between these endogenous factors.

6.1 Host-derived mediators in the pathogenesis of liver injury after exposure to LPS

Liver injury, as measured by changes in total plasma bilirubin concentration and liver-specific enzyme activities in the plasma, occurred between 3 and 6 hr after administration of LPS in the tail vein of rats (Figures 3.7 and 5.1).

6.1.a Role of blood PMNs in LPS-induced liver injury

The results from studies presented in Chapter 3 indicated that large numbers of PMNs accumulate in the liver prior to the onset of liver injury and that depletion of circulating PMNs afforded protection against liver injury after LPS exposure. These results support the hypothesis proposed in Specific Aim 1 (Section 2.4.a) which stated that LPS-induced liver injury was dependent on circulating PMNs.

6.1.b Role of TNF-*alpha* in LPS-induced liver injury

Circulating TNF-*alpha* concentration was markedly increased after exposure to LPS (Chapter 4). This increase preceded the onset of liver injury suggesting that TNF-*alpha* contributes to the pathogenesis. Consistent with this are results from studies with pentoxifylline which attenuated the rise in circulating TNF-*alpha* concentration after LPS exposure and protected against LPS hepatotoxicity (Chapter 4). Neutralization of circulating TNF-*alpha* using a specific antiserum also afforded protection against LPS hepatotoxicity.

6.1.c Role of the coagulation system in LPS-induced liver injury

In addition to PMNs and TNF-*alpha*, results from studies presented in chapter 5 indicated that the coagulation system contributes to the pathogenesis of LPS-induced liver injury. Activation of the coagulation system by LPS administration

occurred prior to the onset of liver injury. Anticoagulants inhibited activation of the coagulation system and afforded protection against liver injury after LPS exposure. Although much remains unknown about the mechanism of liver injury by the coagulation system after LPS exposure, the actions of specific anticoagulants pointed to the importance of a coagulation factor(s) proximal to fibrinogen in the coagulation cascade. It is tempting to speculate that this factor may by thrombin.

6.2 Relationship between host-derived mediators in LPS hepatotoxicity.

6.2.a Relationship between PMNs and TNF-*alpha*

An interaction between PMNs and TNF-*alpha* appears to be necessary for the full manifestation of LPS hepatotoxicity. For example, PMN depletion afforded protection against LPS-induced liver injury but did not prevent the LPS-induced increase in circulating TNF-*alpha* concentration. Indeed, exposure to LPS after PMN depletion was associated with a > 3-fold enhancement of TNF-*alpha* concentration in plasma. Conversely, attenuation of the increase in plasma TNF-*alpha* concentration after LPS exposure protected against liver injury but did not prevent the accumulation of PMNs in the liver. Taken together, these results suggest that neither TNF-*alpha* nor PMNs alone are sufficient to cause liver injury after exposure to LPS.

6.2.b Relationship between PMNs, TNF-*alpha* and the coagulation system

In addition to an interaction between PMNs and TNF-*alpha*, evidence from chapter 5 suggests that both PMNs and TNF-*alpha* interact with the coagulation system after LPS exposure. This is suggested by studies which showed that activation of the coagulation system was inhibited by PMN depletion or by manipulations that attenuated the increase in plasma TNF-*alpha* concentration after LPS administration.

The nature of the interactions between PMNs, TNF-*alpha* and the coagulation system in the pathogenesis of LPS hepatotoxicity remains unknown. TNF-*alpha* promotes the release of reactive oxygen metabolites from PMNs *in vitro*. Because reactive oxygen metabolites have been implicated in the activation of the coagulation system after LPS exposure, it seems possible that TNF-*alpha* may contribute to the activation of the coagulation system by inducing PMNs to release reactive oxygen metabolites. Further studies are necessary to test this possibility.

The mechanism of liver injury by the coagulation system after LPS exposure remains to be elucidated. However, this system does not appear to contribute to liver injury by promoting hepatic PMN accumulation since heparin afforded protection against LPS hepatotoxicity without attenuating PMN accumulation in the liver. Also, because defibrinogenation with ancrod did not afford protection against LPS hepatotoxicity, the coagulation system probably

does not mediate liver injury by a mechanism which is dependent on the thrombin-catalyzed conversion of fibrinogen to fibrin monomers. This result argues against the hypothesis proposed in Specific Aim 3 which stated that the coagulation system mediates liver injury by a mechanism which is dependent on circulating fibrinogen.

Thrombin has a number of biologic activities which appear to be independent of its proteolytic action on circulating fibrinogen. Among these is the stimulation of thromboxane A_2 release from platelets. Evidence from several studies suggests that this potent, vasoactive arachidonic acid metabolite may contribute to the pathogenesis of LPS-induced liver injury. Although the source of thomboxane A_2 is not known, it seems possible that it could be derived from activated platelets. Indeed, an early morphologic change in the hepatic sinusoids after LPS administration is the appearance of aggregates of activated platelets. Thus, the elaboration of thrombin during activation of the coagulation system after LPS exposure could result in liver injury by a mechanism which was dependent on platelet-derived thromboxane A_2. Further studies are necessary to test this possibility.

6.3 Proposed mechanism of LPS hepatotoxicity: an hypothesis

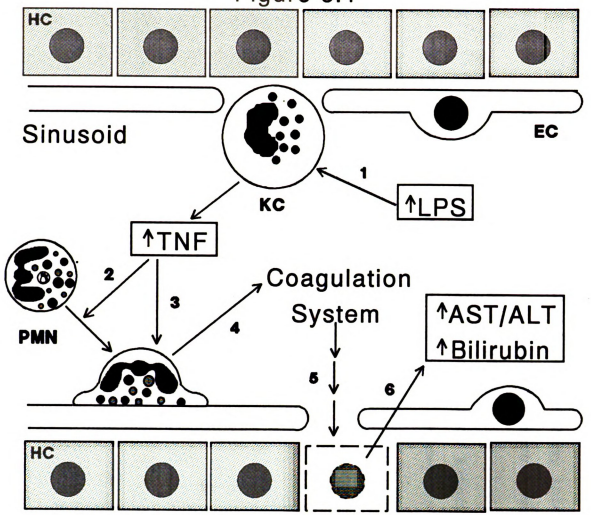
The results from this dissertation indicate that PMNs, TNF-*alpha* and the coagulation system contribute to the

pathogenesis of LPS hepatotoxicity. Furthermore, full manifestation of liver injury after LPS exposure appears to be dependent on complex interactions between these host-derived factors. Figure 6.1 summarizes the results from this dissertation and shows proposed relationships between PMN activation, TNF-*alpha* release and activation of the coagulation system in LPS-induced liver injury.

Exposure to LPS is associated with the accumulation of PMNs in the liver and increased circulating TNF-*alpha*, which is probably derived from hepatic Kupffer cells. The mechanism of recruitment of PMNs in the liver remains unknown. However, it does not appear to be mediated by the coagulation system since hepatic PMN accumulation was not prevented by heparin. Both blood PMNs and TNF-*alpha* appear to be required for activation of the coagulation system after LPS exposure. Because TNF-*alpha* stimulates PMNs to release oxygen metabolites *in vitro* and because reactive oxygen metabolites have been implicated in the activation of the coagulation system after LPS exposure, TNF-*alpha* may contribute to the activation of the coagulation system by inducing PMNs to release reactive oxygen metabolites. The coagulation system mediates liver injury by an unknown mechanism which might involve thrombin but which is independent of the thrombin-catalyzed conversion of fibrinogen to fibrin monomers.

Figure 6.1. Proposed mechanism of LPS-induced liver injury: an hypothesis. Circulating LPS activates Kupffer cells (KC) [1], resulting in the release of TNF-*alpha* which promotes recruitment [2] and activation [3] of PMNs in the liver. PMN activation leads to activation of the coagulation system [4], possibly by an oxygen radical- or lysosomal enzyme-dependent mechanism. Factors derived from the coagulation system subsequently lead, by poorly defined mechanisms, to parenchymal cell injury [5] detectable as increased aminotransferase activity and bilirubin concentration [6]. Abbreviations: LPS, lipopolysaccharide; HC, hepatocytes; KC, Kupffer cells; EC, endothelial cells; PMN, neutrophils; TNF, tumor necrosis factor-*alpha*; AST/ALT, aspartate/alanine aminotransferases.

Figure 6.1


HC
Sinusoid
EC
KC
1
↑LPS
↑TNF
2
3
4
PMN
Coagulation
System
↑AST/ALT
↑Bilirubin
5
6
HC

The results described in this dissertation provide new insight into the mechanisms of liver injury from bacterial LPS by showing that blood PMNs, circulating TNF-*alpha* and the coagulation system each play important roles. While much remains unknown about the specific mechanisms by which each of these factors is involved, it is clear that they interact to result in liver injury. In addition, evidence suggests that PMNs, TNF-*alpha*, and the coagulation system may contribute to other pathophysiologic alterations associated with systemic endotoxemia. Among these is injury to the lungs. Thus, the results from studies in this dissertation may provide insight into the mechanisms contributing to LPS-induced alterations in extrahepatic tissues.

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