This is to certify that the

dissertation entitled

The role of platelets in

lipopolysaccharide-induced liver injury

presented by

Julia Meryl Pearson

has been accepted towards fulfillment
of the requirements for

Doctorate degree in Pharmacology,
Toxicology and Environmental
Toxicology

Ronald A. Rippy
Major professor

Date 4/24/96

THE ROLE OF PLATELETS IN LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY

Volume I

by

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1996

ABSTRACT

THE ROLE OF PLATELETS IN LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY

By

Julia Meryl Pearson

Exposure to lipopolysaccharide (LPS) from gram-negative bacteria leads to an array of pathophysiologic alterations, including multiple organ failure and liver injury. The development of hepatocellular necrosis entails contributions from both cellular and soluble inflammatory mediators, including neutrophils, Kupffer cells, TNF-α and components of the coagulation system. Much remains unknown about the role of platelets and their interactions with these mediators in the pathogenesis of liver injury *in vivo*. Time course studies revealed that neutrophils and platelets accumulate in the liver within 1 hour after LPS administration. These events preceded the appearance of TNF-α in plasma, thrombocytopenia, activation of the coagulation system and the onset of hepatocelluar damage. Prior depletion of platelets with an antiserum attenuated LPS-induced hepatotoxicity and suggested that platelets contribute to the activation of the coagulation system. To address the mechanism by which platelets accumulate within the liver, studies were conducted in neutrophil-depleted or Kupffer cell-inactivated animals. Results from these studies suggested that Kupffer cells, but not neutrophils, contribute to the hepatic platelet accumulation and thrombocytopenia observed after LPS exposure. The

coagulation system, and thrombin in particular, can stimulate platelets. Pretreatment with heparin, or the thrombin inhibitor, hirudin, afforded protection from liver injury but did not alter hepatic platelet accumulation and thrombocytopenia in LPS-treated animals. Furthermore, thrombin is a distal mediator of liver injury, since the administration of heparin or hirudin 2.5 hours after the administration of LPS afforded protection from hepatocellular injury. Lipid mediators, including platelet activating factor (PAF), leukotrienes and cyclooxygenase products are released from platelets and could contribute to the activation of other inflammatory cells and the development of hepatocellular damage. To address these possibilities, studies were conducted with inhibitors of 5-lipoxygenase, cyclooxygenase and thromboxane synthase and a PAF receptor antagonist. Results suggested that PAF, lipoxygenase products and thromboxane are not critical mediators in this model, but suggested some cyclooxygenase products partially contribute to liver injury. In conclusion, results of these studies provide novel insight into the mechanisms of LPS-induced liver injury. The findings suggest platelet interact with other inflammatory mediators in the pathogenesis of hepatocellular damage.

In loving memory of my grandfather

Bud Boedeker

and for Mom, Dad and Hazel Marie

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my friend, mentor and advisor Dr. Robert Roth for all of his support and encouragement. I am especially grateful THAT he spent so much time helping me become less grammatically challenged. I would also like to thank the other members of my thesis committee Drs. Larry Fischer, Greg Fink, Ken Schwartz and Tom Bell for all of their assistance and encouragement.

I am extremely grateful to Dr. Jim Hewett for showing me how to handle laboratory animals and for sparking my interest in this project.

A number of unique individuals provided wonderful technical assistance and daily entertainment. Special thanks to my graduate student mentor, Dr. Marc Bailie. In addition, thanks to all the other past and present Rothonians Drs. Cindy Hoorn, Albert Eric Schultze, Pat Lappin, Patti Tithof, Fredo Moulin, Rosie Sneed, Alan Brown and Dwayne Hill and the other non-veterinarians Jim Wagner, John Buchweitz and Dr. Patti Ganey. In addition, I am grateful for the technical assistance provided by Kerri Ross. This work could have never been completed without the long hours of dedication and hard work put in by Therese Schmidt and Randy Westrick. If it wasn't for these two I would be knee deep in indium and would have never published a

paper. I am indebted to Diane Hummel, Nelda Carpenter and Mickie Vanderlip for limiting my interaction with the Administrative Offices at MSU.

Special thanks to the ladies of the histology lab, Leann Lumbert and Cathy Campbell, for their assistance with immunocytochemistry. I am also thankful to Ralph Commons for his help with the photography of histology sections. I am very grateful to Dr. Eric Schultze for his painstaking hours on the microscope and his meticulous histopathologic and morphometric analyses that are presented thoughout this thesis.

I would especially like to express my gratitude to John Davis and Drs. Ken Schwartz and Michael Scott for their assistance with the platelet and coagulation studies. In addition, special thanks to Dr. Greg Fink for his expertise with statistics, hemodynamics and cigars.

Special thanks to Mike for years of companionship and teaching me perseverance, perspective and priorities.

Finally, my unending love to Mom, Dad and Laura for years of support, love and motivation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

AA	arachidonic acid
ADP	adenosine diphosphate
ALT	alanine aminotransferase
ANOVA	analysis of variance
APTT	activated partial thromboplastin time
APS	antiplatelet serum
ATP	adenosine triphosphate
ß-TG	beta-thromboglobulin
C	Celsius
Ca	calcium
CS	control serum
EIA	enzyme immunoassay
DIC	disseminated intravascular coagulation
diHETE	5,12-hydroxyeicosatetraenoic acid
dl	deciliter
DTS	dense tubular system
E. coli	*Escherichia coli*
EGF	epidermal growth factor
fmlp	N-formyl-methionyl-leucyl-phenylalanine
g	gram
$GdCl_3$	gadolinium chloride
Gp	glycoprotein
GSH	glutathione
GSSG	oxidized glutathione
HEP	heparin
5-HETE	5-hydroxyeicosatetraenoic acid
12-HETE	12-hydroxyeicosatetraenoic acid
5-HPETE	5-hydroperoxyeicosatetraenoic acid
12-HPETE	12-hydroperoxyeicosatetraenoic acid
15-HPETE	15-hydroperoxyeicosatetraenoic acid
5-HT	serotonin
HDL	high density lipoprotein
HMW	high molecular weight
ICAM-1	intracellular adhesion molecule-1
Ig	immunoglobulin
IL-1	interleukin-1
IL-6	interleukin-6
In	indium
ip	intraperitoneal
iv	intravenous
hr	hour

kDa	kilodalton
kg	kilogram
L	liter
LAPF4	low-affinity platelet factor 4
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LTs	leukotrienes
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTD_4	leukotriene D_4
LTE_4	leukotriene E_4
MC	methylcellulose
min	minutes
mg	milligram
Mg	magnesium
ml	milliliter
MOF	multiple organ failure
μCi	microcurie
μg	microgram
μl	microliter
μm	micrometer
NAP_2	neutrophil activating peptide 2
ng	nanogram
O_2^-	superoxide anion
OCS	open canalicular system
PAF	platelet activating factor
PBP	platelet basic protein
PDGF	platelet derived growth factor
PF4	platelet factor 4
pg	picogram
PGE_2	prostaglandin E_2
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
PGJ_2	prostaglandin J_2
PLA_2	phospholipase A_2
po	*per os*
PPP	platelet poor plasma

PRP	platelet rich plasma
SAL	saline
sc	subcutaneous
SEM	standard error of the mean
TGF-ß	tissue growth factor-beta
TNF-*α*	tumor necrosis factor-alpha
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
S. enteritidis	*Salmonella enteritidis*
U	unit
VEH	vehicle
vWF	von Willebrand factor
WBCs	white blood cells

Chapter 1

General Introduction

In the late 1870's, Robert Koch established the link between infectious disease and microbes. Decades later, Koch's student, Richard Pfeiffer discovered that lysates from heat-inactivated *Vibrio chlorae* bacteria induced death in experimental animals. Pfeiffer hypothesized that this toxic component was found within the bacteria and therefore called it endotoxin (Morrison *et al.*, 1979). Years later, the structure of endotoxin was characterized and found to be a lipopolysaccharide (LPS) located within the cell wall of gram-negative bacteria. A century later, much remains unknown about the specific mechanisms by which endogenous inflammatory mediators contribute to the pathogenesis of tissue injury during LPS exposure *in vivo*.

The purpose of this introduction is to discuss LPS and the host-derived responses to LPS that contribute to tissue injury. Section I will introduce LPS and will provide descriptions of its structure, routes of exposure, clearance and some of its pathophysiologic effects. In Section II, the effects of LPS on the liver will be described in detail. In particular, this chapter will focus on some of the cellular and soluble mediators that are critical in the pathogenesis of hepatic injury. Section III will focus on platelets, with emphasis on their function, potential roles in the development of tissue injury and their interactions with other inflammatory mediators.

I. LPS: STRUCTURE, EXPOSURE, CLEARANCE AND EFFECTS

I. A. LPS Structure

LPS is an endotoxin isolated from the cell walls of gram-negative bacteria. It consists of a lipid region, called lipid A, covalently bound to a polysaccharide region. The polysaccharide region, which extends outward from the bacteria, is composed of two regions: the core polysaccharide and the O-antigen polysaccharide (Morrison *et al.*, 1979). The core polysaccharide region is similar among different strains of bacteria and contains several sugar moieties with a unique deoxysugar, 2-keto-3-deoxyoctonic acid. The O-antigen polysaccharide is unique for each type of bacteria and LPS. Consisting of several repeating oligosaccharide units, the number of these units varies.

The lipid A region is amphipathic and embedded within the cell wall lipid bilayer. It consists of a two glucosamine residues joined by a ß1-6 linkage to form a backbone. Amide and ester bonds link long-chain fatty acids to this backbone, along with charged pyrophosphate groups that contibute to its amphipathic nature. The lipid A region is remarkably similar among gram-negative bacteria. This conserved region of the LPS molecule appears to mediate many of the pathophysiologic effects of LPS, since the administration of lipid A can mimic the effects of LPS (Nowotny, 1987). In addition, monoclonal antibodies to this region inhibit the biological activities of LPS (Rietschel *et al.*, 1977).

I. B. Routes of LPS exposure

The most obvious mode of LPS exposure is from infection with gram-negative bacteria. Since LPS is embedded within the cell walls of the bacteria, it is not normally released. However, it can be released during cell division and death. In addition, treatment of bacterial infections with antibiotics can liberate LPS (Shenep *et al.*, 1988; Shenep *et al.*, 1984). Thus, antimicrobial therapy can enhance exposure to LPS and may contribute to the steadily worsening course of disease in some patients.

The gastrointestinal tract contains indigenous gram-negative bacterial flora and large quantities of LPS. The intestinal wall inhibits the release into blood of bacteria and most of the LPS from the gut. However, low levels of LPS can be detected in portal blood, but not in the systemic blood, under normal physiologic conditions in humans (Jacob *et al.*, 1977). Since this portal LPS exposure is not associated with disease and is not detected in the systemic circulation, it has been proposed that normal liver functions include the removal of LPS from the portal blood.

Certain pathophysiolgical conditions such as portal vein occlusion enhance the absorption of LPS from the gastrointestinal tract (Gans *et al.*, 1974). In addition, intestinal ischemia (Caty *et al.*, 1990), dietary cirrhosis (Broitman *et al.*, 1964) and chemically induced liver injury (Nolan, 1975; Nolan *et al.*, 1989) can promote LPS absorption from the gut. Taken together, these findings suggest that under normal conditions, some LPS is released from the

gut into the portal circulation and that the liver removes most of it. Hepatic diseases may contribute to elevated LPS in the systemic circulation by impairing hepatic clearance of LPS.

I. C. Clearance of LPS

Studies with radiolabeled LPS suggest that LPS is cleared from the circulation primarily by the liver, although radiolabeled LPS is also detected in lungs, spleen, kidneys and adrenal glands. The kinetics of LPS removal is biphasic and dose-dependent. The intravenous administration of low doses of LPS (μg/kg) results in a rapid clearance of LPS within 30 minutes followed by a slower clearance phase of the remaining LPS over a period of hours (Mathison *et al.*, 1979). The administration of larger doses of LPS (mg/kg) prolongs the slower phase of LPS clearance over 48 hours (Freudenberg *et al.*, 1985). The radiolabeled LPS is localized within tissue macrophages and is found predominantly within the hepatic Kupffer cells (Mathison *et al.*, 1979; Freudenberg *et al.*, 1985).

Ruminants have phagocytic pulmonary intravascular macrophages and are very susceptible to lung injury after LPS exposure. Following intraportal administration, most of the LPS is cleared by the liver. However, the intrajugular administration results in the clearance of LPS by the pulmonary intravascular macrophages. Thus, in ruminants, the route of LPS administration

defines the organ responsible for LPS removal from the circulation. This finding may explain why ruminants are particularly susceptible to pulmonary injury following LPS exposure (DeCamp *et al.*, 1992).

The clearance of LPS is influenced by plasma binding proteins. LPS binds to high density lipoproteins (HDLs) and the lipopolysaccharide binding protein (LBP). The binding of LPS to HDLs prolongs the half-life of LPS and also inhibits its biologic activity (Ulevitch *et al.*, 1981). The binding of the lipid A region of LPS to LBP can also influence its clearance from plasma (Tobias *et al.*, 1988). This LPS-LBP complex will be discussed in section I. E. 1.

I. D. Pathophysiologic effects of LPS

Systemic gram-negative bacterial infections (sepsis or septicemia) often result in circulatory shock, disseminated intravascular coagulation (DIC), multiple organ failure (MOF) and death. Indeed, sepsis and MOF are major causes of death in hospitalized patients. Several studies report mortality rates of 50-74% in septic patients with MOF (Fry *et al.*, 1980; Carrico *et al.*, 1986). LPS is proposed to be a major contributing factor to the high mortality rates associated with sepsis, since antibodies which neutralize LPS afford protection from lethality in both animals and patients (Dunn *et al.*, 1984; Baumgartner, 1992; Ziegler *et al.*, 1991).

The mechanisms by which LPS exposure results in tissue injury are

complex and vary among tissues. However, the involvement of host-derived, cellular and soluble mediators in the pathogenesis of LPS-mediated tissue injury is a universal finding. For the sake of brevity, the roles of inflammatory mediators in the development of pathophysiolgic alterations in extrahepatic tissues will not be described in detail. Section II will focus on the role of these inflammatory mediators in the pathogenesis of hepatocellular injury.

I. D. 1. Circulatory shock

LPS exposure results in hypotension and decreased cardiac output and tissue perfusion. Within minutes after the intravenous administration of LPS, mean arterial pressure decreases. This is associated with a similar decrease in cardiac output. Later, a slight rebound in both mean arterial pressure and cardiac output is often observed (Moore *et al.*,1991; Casals-Stenzel,1987). Tachycardia, a reflex response to decreased mean arterial pressure, is observed as well (Qi *et al.*,1990). The hypotension and decreased cardiac output contribute to alterations in tissue perfusion (Mozes *et al.*,1991). The exposure to LPS also results in increased vascular permeability, particularly in the lungs (Olson *et al.*,1990; Chang,1992). The nature and magnitude of these cardiovascular responses are dependent upon species, doses and sources of LPS and routes of exposure.

I. D. 2. Disseminated intravascular coagulation

LPS can directly activate both the intrinsic and extrinsic pathways of coagulation *in vitro*, through activation of factor XII and release and/or expression of tissue factor, respectively (Morrison *et al.*,1974; Stern *et al.*,1985; Simchowitz,1985). LPS administration *in vivo* results in disseminated intravascular coagulation (DIC). This is characterized by decreases in plasma fibrinogen and blood platelet concentrations, prolongation of both prothrombin and activated partial thromboplastin times and elevations in fibrin degradation products. These changes are accompanied by the deposition of fibrin within the microcirculation of several tissues (Ito *et al.*,1990; Kramer *et al.*,1977). LPS-induced DIC can be inhibited by the administration of anticoagulants or inhibition or removal of platelets (Ito *et al.*,1990; Kramer *et al.*,1977; Margaretten *et al.*,1967).

DIC is a critical event in the lethality after LPS exposure, and the coagulation system contributes to the pathogenesis of tissue injury (Emerson *et al.*,1987; Hauptman *et al.*,1988). In addition, DIC is an early manifestation of sepsis and is often used as an indicator of infection with gram-negative bacteria (Bone,1992). An association between DIC and lethality is supported by studies in which anticoagulants and fibrinolytic agents afford protection from death after LPS exposure (Smith *et al.*,1988; Emerson *et al.*,1987). The administration of LPS induces fibrin thrombi formation in numerous tissues, including the lungs, liver, spleen and kidney (Koth *et al.*,1980; Kramer *et al.*,1977). The deposition of thrombi within tissues may contribute to organ

injury by disrupting local blood flow.

I. D. 3. Multiple organ injury

LPS exposure results in injury to several organs, including the lungs, gastrointestinal tract, kidneys and liver. Pulmonary alterations after LPS administration are biphasic. Early in endotoxemia, mean pulmonary arterial pressure and pulmonary vascular resistance increase dramatically. These parameters return toward control levels, followed by a second gradual increase in pulmonary arterial pressure. This second phase is accompanied by increased pulmonary microvascular hydrostatic pressure and increased alveolar-capillary permeability (Olson *et al.*, 1985; Olson *et al.*, 1990).

LPS exposure and subsequent DIC culminates in renal tubular necrosis (Ou *et al.*, 1994). This is associated with renal dysfunction, as marked by elevated blood urea nitrogen and serum creatinine. The hypotensive effects of LPS are believed to contribute to marked decreases in renal blood flow and glomerular filtration rate (Cochrane, 1978).

Gastrointestinal alterations include increased microvascular permeability, edema and gastric bleeding (Masuda *et al.*, 1989). LPS-mediated hypotension and subsequent alterations in mucosal blood flow are believed to contribute to sloughing of intestinal mucosal cells and decreased mucus production. There is increasing evidence that endotoxemia, due to its effects on gastrointestinal permeability, can promote the additional absorption of bacteria and LPS from the gut, thereby enhancing endotoxemia and bacteremia (Carrico *et al.*, 1986).

I. E. Cellular effects of LPS

I. E. 1. Cellular and soluble LPS binding proteins

LPS can bind to endogenous serum proteins such as HDL. This binding decreases the biologic activity of LPS (Ulevitch *et al.*, 1981). LPS also binds to the lipopolysaccharide binding protein (LBP), which dramatically enhances the proinflammatory response to LPS. Small quantities of LBP are present in serum under normal conditions. Exposure to LPS induces the expression of acute phase proteins, which includes LBP. The presence of LBP enhances macrophage and neutrophil responses to LPS 100-1000 fold (Mathison *et al.*, 1992). The enhanced responsiveness of leukocytes to the LPS-LBP complex is believed to be a consequence of binding of this complex to the CD14 receptor, since this effect is inhibited with anti-CD14 receptor monoclonal antibodies (Yang *et al.*, 1994). The CD14 receptor is found on monocytes, macrophages and neutrophils and its activation contributes to the expression of CD11b/CD18 integrin (Mac-1), tissue factor, TNF-α, interleukin-1 and interleukin-6 (Heumann *et al.*, 1992; Yang *et al.*, 1994).

LPS can also bind to other receptors in a LBP-independent manner. A 73 kDa protein, originally described on mouse macrophages and T and B lymphocytes, binds specifically to the lipid A moiety (Halling *et al.*, 1992). This protein functions as a receptor since activation of this receptor with a monoclonal antibody stimulates the tumoricidal activity of macrophages (Chen *et al.*, 1990). This receptor has also been identified on human peripheral blood

monocytes, lymphocytes, neutrophils and platelets (Halling *et al.*,1992). Additional binding sites of 20, 31, 38 and 50 kDa have been identified on human blood cells, but their role in LPS-induced responses is not known (Halling *et al.*,1992). In addition to the 73 kDa binding protein, proteins of 96 kDa on the macrophage RAW263 cell line (Golenbock *et al.*,1990), 65 and 55 kDa on J774 cells (Hara-Kuge *et al.*,1990), 47 kDa on hepatocytes (Parent,1989) and 18 kDa on mouse 70Z/3 pre-B cell line (Kirkland *et al.*,1990) have been identified. Although numerous LPS-binding proteins have been identified, several appear to be unique for specific cell populations. Their precise roles in the various cellular responses to LPS remain to be established.

LPS exposure upregulates the expression of CD11b/CD18 by the interaction of LPS with CD14. CD11b/CD18 is present on leukocytes and is involved in adherence to endothelium and diapedesis into tissues. The interaction of LPS with this receptor is believed to promote the phagocytosis of gram-negative bacteria by macrophages and neutrophils (Lynam *et al.*,1994).

I. E. 2. Cytotoxicity of LPS

LPS is cytotoxic to cultured macrophages (Glode *et al.*,1977) and endothelial cells (Harlan *et al.*,1983b). Endothelial toxicity *in vitro* is characterized by detachment, leakage of cytoplasmic enzymes and by alterations in protein, DNA and RNA synthesis (Meyrick,1986). The cytotoxicity is associated with oxygen radicals both *in vitro* and *in vivo.* LPS-induced injury to cultured endothelial cells is inhibited with oxygen radical

scavengers (Brigham *et al.*, 1987). Antioxidants also protect the sinusoidal endothelium from injury after LPS administration to rats (Deaciuc *et al.*, 1993a; Deaciuc *et al.*, 1993b; Liu *et al.*, 1994).

I. E. 3. Stimulation of inflammatory mediator release

Cell culture studies have helped elucidate some of the inflammatory mediators released from cells exposed to LPS. These potent bioactive compounds have also been detected *in vivo* and are associated with the pathogenesis of LPS-induced alterations.

Macrophages, neutrophils and endothelial cells are major sources of inflammatory mediators. For example, exposure to LPS stimulates macrophage expression of tissue factor (Yang *et al.*, 1994), TNF-α (Yang *et al.*, 1994), interleukin-1 (Doide *et al.*, 1987), arachidonic acid metabolites (Decker *et al.*, 1989), nitric oxide (Marletta *et al.*, 1988), lysosomal enzymes (Decker, 1990), oxygen radicals (Jaeschke *et al.*, 1992b) and platelet activating factor (Decker, 1990). These inflammatory mediators can promote the development of pathophysiolgic alterations independently and also in a synergistic fashion with other inflammatory mediators and with LPS. Many of these will be discussed as they relate to the development of hepatocellular injury during LPS exposure in Section II.

LPS can stimulate inflammatory mediator release by several mechanisms. For instance, LPS-induced activation of phospholipase A_2 results in the cleavage

of membrane phospholipids into lysophospholipids and arachidonic acid (Mohri *et al.*, 1990). This leads to the formation of platelet activating factor, and to leukotrienes and prostaglandins by the lipoxygenase and cyclooxygenase pathways, respectively. LPS can also stimulate inflammatory mediator release by the induction of gene expression. For example, LPS exposure results in increased TNF-α mRNA expression (Mohri *et al.*, 1990). Gene expression is involved in the expression of adhesion molecules as well (Colucci *et al.*, 1983). In addition, inflammatory mediator release can be influenced by other mediators, which can either inhibit or promote the release of bioactive compounds. For example, prostaglandin E_2 inhibits TNF-α release from macrophages (Streiter *et al.*, 1988). In contrast, PAF enhances LPS-induced superoxide production (Gardner *et al.*, 1995) and expression of tissue factor (Osterud, 1992).

In summary, LPS can stimulate the release of inflammatory mediators, which can directly contribute to LPS-mediated tissue injury and also promote or inhibit the production of other inflammatory substances. Thus, the possible mechanisms by which these mediators contribute to the pathophysiologic alterations observed during LPS exposure *in vivo* are multifarious.

The focus of this dissertation is on the mechanisms by which LPS exposure culminates in hepatocellular injury. Section II will include comprehensive discussion of the direct effects of LPS on the liver. In addition, as discussed in this chapter, host-derived cellular and soluble mediators appear to be critical in the biological responses to LPS. A main focus of the following

section will be the possible roles of some of these mediators in the development of hepatotoxicity.

II. LPS-MEDIATED HEPATOTOXICITY

II. A. Liver structure and function

The liver comprises several cell types. The hepatic parenchymal cells are responsible for most hepatic functions and consistitute approximately 80% of the cells within the liver (Lautt *et al.*, 1987). The remaining hepatic cells consist of sinusoidal endothelial cells, Kupffer cells (resident liver macrophages), Ito cells (fat storing cells) and bile duct epithelial cells.

The liver receives a dual blood supply that represents about 25% of cardiac output. The hepatic artery only supplies one-third of the total hepatic blood flow in humans but furnishes nearly 75% of the oxygen supply (Lautt *et al.*, 1987). The portal vein provides a majority of blood supply to the liver. The portal vein contains blood that has passed through the mesenteric system and is therefore rich in nutrients but relatively poor in oxygen.

The basic structural unit of the liver is the lobule. The central vein lies in the center of the lobule from which cords of parenchymal cells radiate outward to the portal triads, which contain branches from the portal vein,

hepatic artery, lymph nodes and bile ducts. The parenchymal cells surrounding the central vein and portal triad are termed centrilobular and periportal cells, respectively. The cells in between are referred to as midzonal cells.

Blood enters the liver lobule at the portal triads and flows through sinusoids to the centrilobular regions, where blood enters the central vein and eventually leaves the liver via the hepatic vein. Fenestrated endothelial cells line these sinusoids allowing for contact between the plasma and hepatocytes. Kupffer cells are found scattered throughout the liver sinusoids. Their main function is to remove particulate debris, senescent blood cells and foreign substances such as bacteria and LPS. Bile produced by the parenchymal cells is transported to the portal triad in bile canaliculi.

Main functions of the liver include synthesis of plasma proteins, regulation of carbohydrate metabolism, storage of glycogen and vitamins, metabolism of lipids, degradation of hormones, formation of bile constituents and metabolism and excretion of xenobiotic agents.

II. B. Hepatic alterations after LPS exposure

II. B. 1. Morphologic changes

Kupffer cells are the first hepatic cells to be altered after LPS exposure. These alterations include swelling and an increase in both number and size of phagocytic vacuoles followed by the loss of primary lysosomes. Platelets, with

secretory granules intact, are frequently observed within phagocytic vacuoles of Kupffer cells (Levy *et al.*, 1968a; Levy *et al.*, 1968b). These changes occur within 15 minutes after systemic LPS exposure. Thirty minutes after exposure, significant numbers of neutrophils accumulate within the sinusoids, and a number of neutrophils are located within Kupffer cell phagosomes. Subsequent to alterations in Kupffer cells, swelling of endothelial cell processes occurs along with the appearance of fibrin clumps and platelet thrombi within the sinusoids (Levy *et al.*, 1968a; Levy *et al.*, 1968b; Durham *et al.*, 1990).

Hepatic parenchymal cells do not appear to be altered until 2-4 hours after LPS exposure. Parenchymal cell changes include cytoplasmic invagination of nuclei, mitochondrial and microvilli swelling, vacuolation and the presence of spherical eosinophilic cytoplasmic droplets (Levy *et al.*, 1968a; Levy *et al.*, 1968b; Durham *et al.*, 1990). Four hours after LPS exposure, there is evidence of parenchymal degeneration and necrosis. Hepatocellular lesions are characterized by multifocal, irregularly shaped areas of midzonal hepatocellular necrosis (Hewett *et al.*, 1992). Necrotic cells are hypereosinophilic with small, pyknotic nuclei or pale with indistinct or absent nuclei and indistinct cytoplasmic borders. These lesions contain degenerate neutrophils and small amounts of hemorrhage (Hewett *et al.*, 1992). Between four and six hours after LPS administration, liver-specific enzymes such as aspartate aminotransferase and alanine aminotransferase are elevated in plasma, indicating a loss in hepatic parenchymal cell integrity.

II. B. 2. Functional changes

LPS exposure decreases gluconeogenesis and stimulates glycogenolysis. These changes contribute to a marked reduction in parenchymal cell glycogen content within 30 minutes after systemic LPS exposure (Levy *et al.*, 1968a; Levy *et al.*, 1968b; Filkins *et al.*, 1977; Filkins *et al.*, 1974).

Results from studies *in vitro* suggest that LPS exposure alters hepatic lipid metabolism. The addition of LPS to isolated parenchymal cells increases cellular lipid content (Victorov *et al.*, 1989). This is associated with enhanced secretion of lipids into the culture medium. These changes are believed to result from increased lipid synthesis, since hepatocytes incorporate radiolabeled acetate into neutral lipids. These alterations may contribute to the accumulation of lipids within the liver and may explain fatty changes observed in livers 24 hours after the administration of LPS (Levy *et al.*, 1968a; Levy *et al.*, 1968b).

Cholestasis is defined as a decrease in bile flow. This is characterized by elevated plasma bilirubin and decreased clearance of sulfobromophthalein and indocyanine green. In isolated livers perfused with buffer, the addition of LPS to the buffer results in cholestasis (Utili *et al.*, 1976; Utili *et al.*, 1977). This appears to be mediated directly by LPS since blood elements are not present in the buffer. In addition, cholestasis develops without significant release of parenchymal cell enzymes into the perfusate, suggesting that changes in bile flow occur in the absence of hepatocellular damage (Utili *et al.*, 1977).

Hepatic parenchymal cells produce bile and export it into the bile

canaliculi, where it is collected into bile ducts in the portal triad and transported to the gall bladder for storage. Bile is produced by two mechanisms, one bile salt-dependent and the other bile salt-independent (Erlinger *et al.*, 1974). The bile salt-dependent fraction is formed by the secretion of bile into the canaliculi by the formation of an osmotic gradient. In the bile salt-independent fraction, bile is actively transported into the canaliculi by a Na^+/K^+ ATPase located on the parenchymal cell membrane (Erlinger *et al.*, 1974). Some evidence suggests that LPS can inhibit this Na^+/K^+ ATPase in isolated membranes (Utili *et al.*, 1976). In addition, although bile flow is reduced after LPS exposure, this is not associated with decreased secretion of bile salts (Utili *et al.*, 1976). Taken together, these studies suggest that LPS can directly alter the formation of the salt-independent fraction of bile.

As mentioned in Section I, Kupffer cells are the primary cells responsible for the removal of LPS from the circulation after the intravenous administration of LPS to rodents. In addition, endothelial cells can remove LPS by a process of micropinocytosis. Some LPS is also associated with hepatic parenchymal cells. This association may be responsible for some of the alterations in hepatocellular function. However, exposure to LPS does not appear to result in hepatocellular toxicity, although it is possible that LPS exposure may predispose the hepatocytes to injury from inflammatory mediators. The remaining sections in this chapter will focus on some of the cellular and soluble mediators that may contribute to the pathogenesis of hepatocellular damage after LPS exposure.

II. C. Roles of cellular and soluble mediators in LPS-mediated heptotoxicity

The following subsections will focus on the mechanisms by which inflammatory mediators contibute to the pathogenesis of hepatocellular injury, as determined by both *in vitro* and *in vivo* studies. Particular attention will be centered on the roles of neutrophils, Kupffer cells, cytokines, coagulation factors, lipid mediators and platelets.

As will be discussed in this section, the role of some of these endogenous mediators in the development of LPS-mediated hepatotoxicity is often model-dependent. Although the cellular mediators involved in LPS-induced hepatotoxicity are consistent among models, there are considerable differences regarding the critical nature of soluble mediators among animal models in the response to LPS *in vivo.* Some of the variable factors include species differences, route and duration of LPS exposure, and differences in doses and bacterial sources of LPS. The following discussion on the roles of inflammatory mediators in LPS-induced hepatocellular damage will highlight some of these differences.

II. C. 1. Neutrophils

The accumulation of neutrophils within the sinusoids is one of the earliest alterations observed in livers of animals exposed to LPS (Levy *et al.*, 1968a; Levy *et al.*, 1968b). The most compelling evidence for a role for neutrophils in

LPS-mediated hepatotoxicity comes from studies in which prior depletion of neutrophils with antibodies prevents the manifestation of hepatotoxicity after LPS exposure (Hewett *et al.*, 1992; Jaeschke *et al.*, 1991b). These findings are supported by studies using chemotherapeutic agents. Large doses of vinblastine inhibit white blood cell formation in bone marrow and result in a prounounced leukopenia. This leukopenia is associated with protection from the hepatotoxic effects of LPS exposure in rats (Yoshikawa *et al.*, 1992).

The mechanism by which neutrophils accumulate within the liver remains unclear. However, LPS is directly toxic to cultured endothelial cells (Harlan *et al.*, 1983b; Harlan *et al.*, 1983a) and evidence from LPS-treated rats indicates an early and pronounced decrease in sinusoidal cells that resemble endothelium and a loss in endothelial cell functions (Hewett *et al.*, 1992; Deaciuc *et al.*, 1993a; Deaciuc *et al.*, 1993c). Injury to the endothelium and subsequent exposure of the subendothelium might promote the adherence and accumulation of neutrophils within the sinusoids.

LPS exposure can upregulate the expression of adhesion molecules on a variety of cells, including endothelial cells (Osborn, 1990). These adhesion molecules promote leukocyte adherence to such cells. Although one such adhesion molecule, ICAM-1, is upregulated after LPS exposure (Essani *et al.*, 1995), antibodies to this molecule do not inhibit hepatic neutrophil infiltration after LPS administration (Essani *et al.*, 1995). Another important adhesion molecule is P-selectin (GMP-140). This lectin containing molecule is packaged within the α-granules of platelets and Weibel-Palade bodies in

endothelial cells. Stimulation of these cells results in the rapid expression of this molecule on the cell surface. LPS exposure results in the expression of P-selectin on endothelial cells (Coughlan *et al.*, 1994). P-selectin is suggested to mediate hepatic neutrophil accumulation in rats exposed to LPS, since an antibody to P-selectin completely blocks sinusoidal neutrophilia (Coughlan *et al.*, 1994). Exposure to LPS stimulates the release of cytokines, such as IL-1, TNF-α and PAF. Each of these mediators could contribute to the adherence of neutrophils in the liver since they all upregulate adhesion molecule expression on endothelium (see below). It is possible that several inflammatory mediators and adhesion molecules contribute to hepatic sinusoidal neutrophilia.

Although neutrophils accumulate within the liver rapidly after LPS exposure, an unresolved question is how and when these cells become activated. Numerous inflammatory mediators and adhesion molecles can prime or directly stimulate neutrophil activation. For example, an antibody to ICAM-1 does not inhibit the hepatic accumulation of neutrophils yet affords protection from liver injury (Essani *et al.*, 1995), suggesting that ICAM-1 is involved in the activation of neutrophils. PAF is chemotactic for neutrophils and can stimulate the production of superoxide anion (Worthen *et al.*, 1983; Montrucchio *et al.*, 1993; Takahashi *et al.*, 1991). Other mediators, such as TNF-α, prime neutrophils for activation and enhance superoxide generation and degranulation in response to other stimuli (Klebanoff *et al.*, 1986). Several mediators, and their effects on neutrophils, will be discussed in the following subsections.

The mechanisms by which neutrophils mediate hepatocellular damage *in*

vivo remain unclear. Stimulated neutrophils can release numerous inflammatory substances that could alter parenchymal cell integrity. These include reactive oxygen metabolites, such as superoxide, hydrogen peroxide and hydroxyl radical. These reactive oxygen species can initiate lipid peroxidation and result in membrane damage. Lipid peroxidation products have been detected in livers 8-16 hours after LPS exposure (Ogawa *et al.*, 1982; Sugino *et al.*, 1987), however the magnitude of lipid peroxidation in the liver is significantly lower than that observed during ischemia/reperfusion injury (Jaeschke *et al.*, 1992d; Jaeschke *et al.*, 1991a; Liu *et al.*, 1995). Concurrent with the increase in lipid peroxidation products is a decrease in hepatocellular ATP content (Sugino *et al.*, 1989), suggesting lipid peroxidation results in mitochondrial dysfunction. The administration of antioxidants such as catalase, superoxide dismutase and α-tocopherol attenuate lethality and hepatotoxicity in LPS-treated mice (Neihorster *et al.*, 1992; Sugino *et al.*, 1987). However, oxygen radicals are not critical in all models of endotoxemia since oxygen radical scavengers do not inhibit the cardiovascular effects of LPS in dogs (Novotny *et al.*, 1988). Thus, the role of oxygen radicals in LPS-induced liver injury remains unclear.

Activation of neutrophils also results in degranulation and release of lysosomal constituents into the extracellular mileu. These lysosomes contain proteases, lactoferrin and myeloperoxidase, an enzyme that can generate the potent oxident, hypochlorous acid. Studies with protease inhibitors suggest that the neutrophil protease, elastase, promotes renal dysfunction in isolated perfused kidneys (Linas *et al.*, 1991). In addition, f-met-leu-phe (fmlp)

stimulated neutrophils mediate hepatocellular injury *in vitro* by releasing cathepsin G and elastase (Ho *et al.*, 1995). The role of proteases will be discussed in greater detail in Section III. Further study is needed to evaluate the roles of these neutrophil-derived proteases in LPS-induced liver injury. Upon activation, neutrophils also release potent inflammatory mediators such as the cysteinyl leukotrienes and PAF (see below), which could contribute to cytotoxicity.

II. C. 2. Kupffer cells

Kupffer cells, the resident macrophages of the liver, are part of the innate immune response and their phagocytic activity is critical in the removal of invading microbial pathogens. As discussed in Section I, they rapidly remove LPS from the circulation and thereby prevent gut-derived LPS from reaching the systemic circulation. Although this phagocytic activity of Kupffer cells prevents systemic endotoxemia, several studies suggest these cells promote the pathogenesis of LPS-induced hepatotoxicity. For example, gadolinium chloride ($GdCl_3$) forms insoluble carbonate and phosphate precipitates in blood which are phagocytosed by Kupffer cells and results in their inactivation (Dean *et al.*, 1988; Hardonk *et al.*, 1992). Pretreatment of rats with $GdCl_3$ attenuates the hepatotoxicity observed after LPS administration. Why Kupffer cell inhibition with $GdCl_3$ leads to protection from liver injury remains unclear. However, $GdCl_3$ administration does not reduce the elevation in plasma TNF-α activity (see below) or hepatic neutrophil accumulation, suggesting that the presence

of neutrophils and TNF-α is insufficient to produce liver injury in the absence of functional Kupffer cells.

Further evidence for a role for Kupffer cells in LPS-mediated hepatotoxicity comes from studies with C3H/HeJ mice. These mice are exceptionally resistent to the lethal effects of LPS. This resistance may be mediated by alterations in macrophage functions. In contrast to other strains of mice and animal species, the macrophages isolated from C3H/HeJ are less responsive to LPS exposure and do not release many inflammatory mediators (Wahl *et al.*, 1979; Tracey *et al.*, 1986). Macrophage-derived mediators have been implicated in the pathogenesis of hepatotoxicity in other models (see below). Thus, alterations in macrophage function may explain the reduced responsiveness to LPS in these resistent mice. This finding provides evidence that macrophages contribute to LPS-mediated liver injury under normal circumstances. Kupffer cells from C3H/HeJ mice also have reduced phagocytic activity compared to LPS-sensitive mice (McCuskey *et al.*, 1984a; McCuskey *et al.*, 1984b). The decreased phagocytic activity may be partly related to deficiencies in lysosomal enzymes.

Activated Kupffer cells can release numerous inflammatory mediators that could contribute to hepatocellular injury. Kupffer cell activation results in the release of lipoxygense products and oxygen radicals, which can directly injure endothelial cells (Deaciuc *et al.*, 1993a; Deaciuc *et al.*, 1993c; Liu *et al.*, 1994). The release of reactive oxygen species by Kupffer cells may also contribute to oxidative stress in hepatocytes (Jaeschke, 1992a; Liu *et al.*, 1994;

limuro *et al.*,1994), as measured by a decrease in total hepatocellular glutathione content (GSH + GSSG) and an increase in oxidized glutathione (GSSG). Glutathione is an endogenous free radical scavenger that is important in protecting the cell from injurious oxygen radicals. An oxidative stress could predispose liver cells to the actions of LPS or other endogenous chemical mediators (Adamson *et al.*,1992; Liu *et al.*,1994).

II. C. 3. Cytokines

LPS exposure stimulates the expression of various cytokines such as TNF-α and interleukins -1 and -6. A transient increase in circulating TNF-α concentration occurs between 1 and 2 hours after LPS exposure (Hewett *et al.*,1993). TNF-α appears to contribute to development of several LPS-mediated pathophysiologic alteration since neutralization of TNF-α with antibodies affords protection against hypotension (Tobias *et al.*,1988), lung injury (Caty *et al.*,1990; Remick *et al.*,1990) and lethality (Beutler *et al.*,1985). In addition, the administration of recombinant TNF-α can mimic some of the pathophysiologic effects of LPS exposure (Remick *et al.*,1987).

TNF-α is critical for the pathogenesis of hepatocellular injury since inhibition of its release with pentoxifylline or neutralization of it with antibodies attenuates LPS-mediated liver necrosis (Hewett *et al.*,1993). The mechanism by which TNF-α contributes to liver injury are not known. TNF-α promotes synthesis of acute phase proteins in the liver (Andus *et al.*,1991) which are important in the regulation of inflammatory processes. TNF-α also stimulates

the expression of adhesion molecules on both the endothelium and neutrophils *in vitro* (Bevilacqua *et al.*, 1985; Vadas *et al.*, 1990; Salyer *et al.*, 1990). Such events could promote the accumulation of neutrophils within the liver sinusoids. However, neutralization of TNF-α with antibodies does not prevent the hepatic neutrophil sequestration observed after LPS exposure (Hewett *et al.*, 1993).

TNF-α can prime neutrophils for the release of lysosomal proteases and reactive oxygen species *in vitro* (Klebanoff *et al.*, 1986). This suggests that TNF-α might contribute to liver injury by a mechanism involving activation of neutrophils. However, results from studies with $GdCl_3$ suggest that the effects of TNF-α do not appear to be mediated through neutrophils since Kupffer cell inhibition prevents the development of liver injury despite the presence of elevated TNF-α concentrations in the systemic circulation and pronounced sinusoidal neutrophilia. It is not known if the hepatic concentrations of TNF-α are similar to those detected in the blood.

Interleukins -1 and -6 are also markedly elevated within 2-6 hours after LPS exposure. The elevations in IL-1 occur prior to elevations in IL-6 (Chensue *et al.*, 1991). These interleukins are critical for the lethality observed after LPS exposure since IL-6 neutralizing antiserum and IL-1 receptor antagonists afford protection (Ohlsson *et al.*, 1990; Starnes *et al.*, 1990). The activities of IL-1 are similar to those produced by TNF-α. Although these cytokines have been implicated in the development of renal microcirculatory injury in response to LPS (Movat, 1987), their roles in LPS-induced hepatotoxicity are unknown.

II. C. 3. Coagulation system

Numerous studies have focused on LPS-induced activation of the coagulation system and DIC in the pathogenesis of tissue injury. Within 2-3 hours after the intravenous administration of LPS to rats, plasma fibrinogen concentrations decrease, suggesting that the coagulation system is activated. This event precedes the onset of liver injury (Hewett *et al.*, 1995).

Results of several studies suggest that activation of the coagulation system is necessary for the pathogenesis of LPS-induced liver injury. For instance, the infusion of thrombin into the portal vein of rats produces liver injury that is morphologically similar to that induced by LPS (Shibayama, 1987). In addition, pretreatment of animals with anticoagulants such as warfarin or heparin prevents activation of the coagulation system as well as liver injury in LPS-treated rats (Hewett *et al.*, 1995; Margaretten *et al.*, 1967). Both of these agents interfere by different mechanisms with thrombin, thereby inhibiting the formation of insoluble fibrin clots. Consequently, it has been suggested that the formation of occlusive fibrin thrombi and consequent ischmia underlie tissue injury (Shibayama, 1987; Margaretten *et al.*, 1967), yet there is little direct support for this hypothesis, and the mechanism by which the coagulation system contributes to liver injury remains unclear. Indeed, pretreatment of rats with ancrod, which anticoagulates by depleting plasma fibrinogen but does not interfere with thrombin, fails to protect rats against LPS-induced hepatocellular necrosis (Hewett *et al.*, 1995), suggesting that insoluble fibrin clot formation does not explain the critical role of the coagulation system in liver injury.

Taken together, these findings raise the possibility that thrombin is involved in the pathogenesis of liver injury by a mechanism that is independent of its ability to form occlusive fibrin thrombi. In addition to its role in the coagulation system, thrombin affects many cells through receptor-mediated actions and has proinflammatory effects that could contribute to tissue injury. For example, thrombin can influence endothelial cells, resulting in expression of adhesion molecules and the release of cytokines (Bizios *et al.*, 1986; DeMichele *et al.*, 1992). Such events could contribute to the accumulation of neutrophils and other inflammatory cells within tissues. Thrombin is also an activator of platelets and a chemoattractant and activator of neutrophils (Drake *et al.*, 1992; Bizios *et al.*, 1986). It stimulates thromboxane A_2 release from platelets (Feinstein *et al.*, 1977), and several studies suggest that this arachidonic acid metabolite is important in the pathogenesis of liver injury in some animal models (see below).

Thrombin stimulates the production of cytokines and arachidonic acid metabolites from macrophages *in vitro* (Bar-Shavit *et al.*, 1983a; Bar-Shavit *et al.*, 1983b; Jones *et al.*, 1990; Podjarny *et al.*, 1989). Accordingly, thrombin might contribute to the pathogenesis of liver injury by stimulating the release of inflammatory mediators from Kupffer cells.

It is possible that thrombin contributes to parenchymal cell necrosis by a direct effect on these cells. High and low affinity binding sites for thrombin on hepatocytes have been reported, although the functions of these "receptors" is not known (Weyer *et al.*, 1988). Thrombin stimulates glycogenolysis in

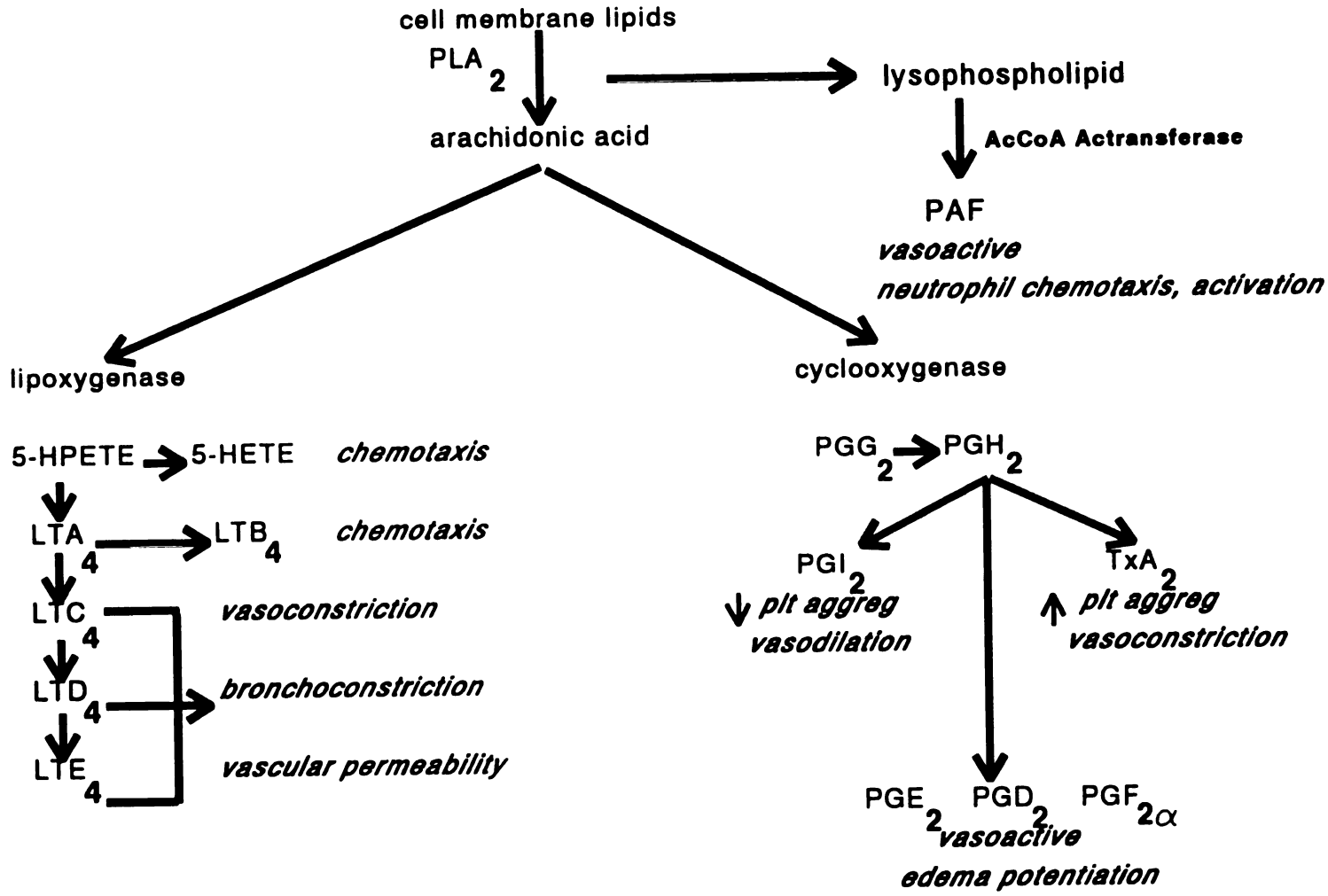
isolated, perfused livers by a cyclooxygenase-dependent mechanism (Yamanaka *et al.*, 1992). However, the mechanism by which thrombin contributes to hepatocellular injury remains to be determined.

II. C. 4. Lipid mediators

Arachidonic acid (AA) is a 20-carbon, polyunsaturated fatty acid (5,8,11,14-eicosatetraenoic acid) that is derived directly from dietary sources or by biosynthesis from the essential fatty acid, linoleic acid. AA is incorporated into cell membranes where it is esterified to phospholipids, particularly in the carbon 2 position of phophatidylcholine and phosphatidylinositol. LPS exposure activates phospholipase A_2, which cleaves these phospholipids into free AA and lysophospholipids. The free AA is then further metabolized by lipoxygenases and cyclooxygenase, resulting in the production of leukotrienes, prostaglandins and other lipid mediators (see Figure 1.1). The lysophospholipid released from phosphatidylcholine can be acetylated to form PAF (1-O-alkyl-2(R)-acetyl-glycero-3-choline).

The critical nature of lipid mediators in the pathogenesis of liver injury after LPS exposure is supported by studies in rats maintained on a diet deficient in essential fatty acids. These animals are devoid of AA and resistant to the hepatotoxic effects of *Salmonella enteritidis* LPS (Cook *et al.*, 1982; Cook *et al.*, 1980). In addition, dexamethasone, which inhibits the actions of phospholipase A_2, affords protection from LPS hepatotoxicity in galactosamine-pretreated mice (Tiegs *et al.*, 1988). Taken together, these findings support the

Figure 1.1. Arachidonic acid metabolic pathways. Arachidonic acid is liberated from membrane lipids after cleavage by phopholipase A_2. The free arachidonate is then further metabolized by the cyclooxygenase and lipoxygenase pathways. Some of the key lipid mediators are shown, along with their main biologic effects.


cell membrane lipids
PLA2
arachidonic acid
lysophospholipid
AcCoA Actransferase
PAF
vasoactive
neutrophil chemotaxis, activation
lipoxygenase
cyclooxygenase
5-HPETE
5-HETE
chemotaxis
LTA4
LTB4
chemotaxis
LTC4
vasoconstriction
LTD4
bronchoconstriction
LTE4
vascular permeability
PGG2
PGH2
PGI2
plt aggreg
vasodilation
TxA2
plt aggreg
vasoconstriction
PGE2
PGD2
PGF2α
vasoactive
edema potentiation

contention that AA metabolites contribute to the pathogenesis of hepatocellular injury. The next sections will focus on the critical roles of AA metabolites and PAF in liver injury resulting from LPS exposure.

Lipoxygenase pathways. Free AA can be converted into hydroperoxy derivatives by lipoxygenases. 5-lipoxygenase, abundant in neutrophils and macrophages, converts AA into 5-HPETE. In neutrophils, 5-HPETE can then undergo further metabolism by different routes: glutathione peroxidase converts 5-HPETE to 5-HETE, a potent neutrophil chemoattractant; 5-lipoxygenase, using 15-HPETE as a substrate, can introduce another hydroxyl group to produce lipoxins; and 5-lipoxygenase can convert 5-HPETE to leukotriene A_4 (Decker,1990). LTA_4 is unstable and is either enzymically converted to LTB_4, a potent chemotactic agent, or conjugated to glutathione to form LTC_4, LTD_4 and LTE_4, which cause vasoconstriction, bronchospasm and increased vascular permeability (Cotran *et al.*, 1989; Ball *et al.*,1986).

Platelets, which contain 12-lipoxygenase, can convert AA into 12-HPETE, which is further metabolized to 12-HETE (Cotran *et al.*, 1989; Ball *et al.*,1986). 12-HPETE can stimulate 5-lipoxygenase in leukocytes, resulting in the formation of 5-HETE, LTB_4 and diHETE (5, 12 HETE) (Maclouf *et al.*,1982).

LTs are critical for several LPS-mediated alterations. LTC_4 and LTD_4 are present in the bile of LPS-treated rats (Hagmann *et al.*,1985), and the intravenous administration of LPS results in LT formation *in vivo* through a mechanism that depends upon complement (Jaeschke *et al.*,1992c). LTs are

critical for LPS-induced leukopenia (Cook *et al.*, 1985). In addition, they contribute to liver injury in an experimental model of endotoxemia in which mice are sensitized with galactosamine (Tiegs *et al.*, 1988). Some evidence suggests that liver injury resulting from the intraperitoneal administration of LPS is not mediated by 5-lipoxygenase products alone, but rather by the combination of LTs and PAF (Yoshikawa *et al.*, 1992). These findings suggest that the role of LTs in LPS-induced hepatotoxicity is dependent upon the experimental model used.

Cyclooxygenase pathway. Cyclooxygenase can rapidly transform AA into the prostaglandin endoperoxide, PGG_2, which is enzymically converted to PGH_2. PGH_2 is then metabolized to several other products which have various and sometimes opposing biologic effects. Thromboxane synthase converts PGH_2 into TxA_2. Thromboxane, a potent vasoconstrictor and platelet aggregator, is released from Kupffer cells and platelets, as well as other cells (Kawada *et al.*, 1992; Moscat *et al.*, 1987). PGI_2 and PGE_2 are potent inhibitors of platelet aggregation and are vasodilators. $PGF_{2\alpha}$ is vasoconstrictive and PGD_2, the major prostanoid released from Kupffer cells, is a vasoconstrictor or vasodilator, depending upon the tissue (Quiroga *et al.*, 1993; Ball *et al.*, 1986; Cotran *et al.*, 1989).

Numerous studies have implicated cyclooxygenase products in the pathogenesis of LPS-induced lethality in several animal models. In rats, LPS administration stimulates the production of PGI_2 and TxA_2, as measured by the

stable metabolites 6-keto-$PGF_{1\alpha}$ and TxB_2 in plasma, respectively (Cook *et al.*, 1980; Olanoff *et al.*, 1985; Wise *et al.*, 1980; Halushka *et al.*, 1981; Ishiguro *et al.*, 1994). Inhibition of the formation of cyclooxygenase metabolites with aspirin or ibuprofen attenuates the lethal effects of LPS (Cook *et al.*, 1982; Wise *et al.*, 1980; Halushka *et al.*, 1981; Ishiguro *et al.*, 1994). In addition, pretreatment with aspirin attenuates liver injury after the intravenous administration of LPS in rats (Ishiguro *et al.*, 1994). However, the importance of cyclooxygenase metabolites in the pathogenesis of LPS-induced liver injury is model-dependent, since aspirin and ibuprofen do not afford protection from the hepatotoxic effects of LPS in mice that have been sensitized with galactosamine (Tiegs *et al.*, 1988; Wendel *et al.*, 1986).

With the development of newer pharmacologic interventions, considerable attention has focused on the role of TxA_2 in endotoxemia and, in particular, its role in LPS-induced hepatotoxicity. Studies with thromboxane synthase inhibitors and thromboxane receptor antagonists have supported a role for TxA_2 in the development of liver injury in rats treated with intravenous *Salmonella enteritidis* LPS (Cook *et al.*, 1982; Wise *et al.*, 1980) and in mice treated with LPS one week after priming with *C. parvum* (Nagai *et al.*, 1989). However, TxA_2 may not be critical for the development of LPS-induced hepatotoxicity in all models, since thromboxane synthase inhibition does not afford protection in a rat model of sepsis in which a fecal suspension is instilled into the peritoneal cavity (Cook *et al.*, 1982; Butler *et al.*, 1983) or in a model of *E. coli* LPS infusion in the rat (Furman *et al.*, 1984). Thus, the role and

importance of TxA_2 as a determinant of liver injury during endotoxemia may vary with biological and/or experimental differences among animal models.

The intravenous administration of *E. coli* LPS to rats results in a pronounced thrombocytopenia and activation of the coagulation system, as marked by a decrease in plasma fibrinogen concentration (Hewett *et al.*, 1995; Margaretten *et al.*, 1967). TxA_2 is a potent aggregator of platelets. In models in which thromboxane synthase inhibitors or receptor antagonists afford protection from lethality and liver injury, results suggest that TxA_2 contributes to activation of the coagulation system and thrombocytopenia (Olanoff *et al.*, 1985; Taneyama *et al.*, 1989; Cook *et al.*, 1982; Wise *et al.*, 1980). Thus, in these models, TxA_2 may contribute to hepatocellular injury by a mechanism involving its interactions with platelets and/or the coagulation system.

PAF. Subsequent to the activation of phospholipase A_2, lysophospholipids can be converted to PAF by acetyl CoA acetyltransferase (Bratton *et al.*, 1989). PAF is critical for the development of numerous LPS-mediated alterations, including hypotension (Casals-Stenzel *et al.*, 1988), lung injury (Olson *et al.*, 1990; Yue *et al.*, 1991) and death (Casals-Stenzel *et al.*, 1988; Salari *et al.*, 1990; Rabinovici *et al.*, 1990). PAF is produced by leukocytes, platelets and endothelial cells and has proinflammatory properties. For example, it can mediate neutrophil chemotaxis and activation (Worthen *et al.*, 1983; Montrucchio *et al.*, 1993; Takahashi *et al.*, 1991), increase vascular permeability and alter vascular tone (Hosford *et al.*, 1993; Buxton *et al.*, 1986).

PAF also stimulates the production of other soluble mediators such as eicosanoids, cytokines and superoxide anion (Hosford *et al.*, 1993; Snyder,1990; Takahashi *et al.*,1991).

PAF can influence the liver, since the intravenous administration of PAF increases hepatic vascular resistance, glucose production, oxygen uptake and free radical generation (Lapointe *et al.*,1989; Zhou *et al.*,1992). It can affect non-parenchymal cells of the liver by stimulating superoxide production from both Kupffer cells and the endothelium (Gardner *et al.*,1995). Since PAF alters hepatic functions and can be produced by and stimulate inflammatory cells that are required for the genesis of liver injury, it is possible that PAF may be a critical mediator of hepatocellular injury during LPS exposure.

A recent study indicates that antagonism of PAF receptors alone is insufficient to prevent liver injury in the rat after the intraperitoneal administration of LPS (Yoshikawa *et al.*,1992). Similarly, administration of a 5-lipoxygenase inhibitor affords no protection. However, one study suggests that the combination of a PAF receptor antagonist and a 5-lipoxygenase inhibitor prevents hepatic injury and lethality (Yoshikawa *et al.*,1992). This study supports the hypothesis that PAF in combination with LTs is critical for the development of hepatic injury and suggests that PAF and LTs have redundant actions in this model. This finding contrasts results observed in mice sensitized with galactosamine. In the galactosamine/LPS model, inhibition of LT biosynthesis prevents liver injury, whereas PAF antagonism does not. The contrasts between these studies may result from species differences or from

other biologically significant differences among animal models.

As mentioned, the coagulation system contributes to the pathogenesis of LPS-induced liver injury. Some studies suggest that PAF may be involved in the LPS-induced activation of the coagulation system and thrombocytopenia. PAF receptor antagonism attenuates DIC induced by the infusion of LPS to rats (Imanishi *et al.*, 1991; Imura *et al.*, 1986). However, blockade of PAF's actions does not inhibit DIC in septic rabbits (Ou *et al.*, 1994) or thrombocytopenia in rats after the administration of large doses of LPS (Rabinovici *et al.*, 1990). Thus, similar to the findings with liver injury, the role of PAF in LPS-induced DIC is model-dependent.

II. C. 5. Platelets

Several studies suggest a role for platelets in the pathogenesis of some LPS-induced responses. Within 2 hours after the intravenous administration of LPS in the rat, blood platelet numbers decrease by 75%. This thrombocytopenia occurs prior to the onset of liver injury (Olanoff *et al.*, 1985). In addition, electron micrographs from livers of LPS-treated mice show platelets within liver sinusoids and within Kupffer cells (Levy *et al.*, 1968a; Levy *et al.*, 1968b).

LPS exposure results in a redistribution of platelets from the blood into hepatic and pulmonary tissues. Based on measurements of tissue platelet

distribution in dogs with ^{111}indium-labeled platelets, LPS exposure causes a rapid, transient accumulation of platelets within the lungs that is maximal 10 minutes after LPS administration. One hour after the administration of LPS, most of the accumulated platelets leave the lungs and are deposited within the liver (Gutmann *et al.*, 1987; Sostman *et al.*, 1983). In mice, the injection of LPS stimulates a dose-dependent increase in hepatic serotonin (5-HT), a constituent of platelet dense granules. This increased hepatic 5-HT is temporally associated with a fall in blood platelet numbers and in blood 5-HT. Therefore, the elevated hepatic 5-HT is suggested to reflect the accumulation of platelets within the liver (Endo *et al.*, 1993; Endo, 1984). Thus, different techniques used in several animal models suggest that LPS administration results in hepatic platelet accumulation.

Platelets appear to contribute to several LPS-mediated alterations. For instance, platelet depletion with antibodies affords protection from DIC in rabbits challenged with two intravenous injections of LPS (generalized Schwartzman reaction)(Kramer *et al.*, 1977). Drugs that inhibit platelet function also prevent DIC in rats infused with *E. coli* LPS over a 4 hour period (Ito *et al.*, 1990). In galactosamine sensitized mice, prior removal of platelets with an antibody protects animals from the lethal effects of LPS (Piguet *et al.*, 1993).

Although these studies suggest that platelets are critical for the pathogenesis of LPS-induced DIC and lethality and show that platelets rapidly accumulate within the liver after LPS exposure, the role of platelets in liver injury has not been evaluated. A recent study suggests a relationship between

the degree of LPS-mediated thrombocytopenia and hepatocellular injury (Shibayama *et al.*, 1995). Taken together, these observations support the possibility that platelets may play a role in the pathogenesis of LPS-mediated hepatotoxicity. Section III will therefore focus on platelets and how platelets might contribute to the pathogenesis of hepatocellular injury. In particular, this section will focus on platelet functions and products and their potential interactions with the inflammatory mediators of liver injury discussed above.

III. PLATELETS

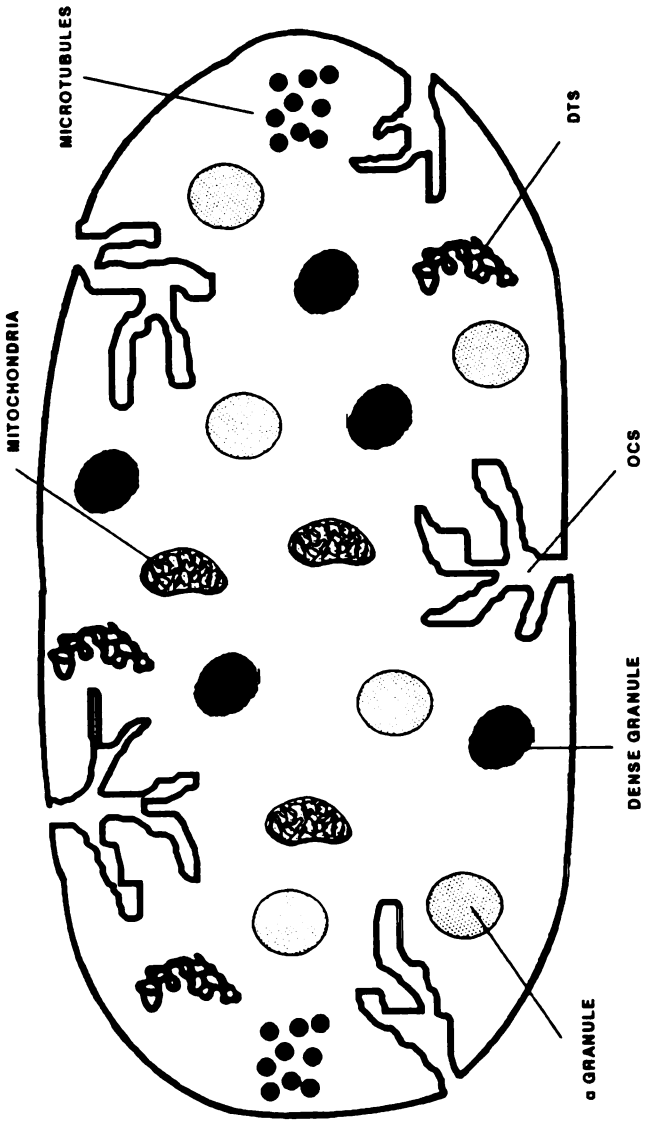
III. A. Platelet Structure

Platelets are the smallest (2 μm) yet one of the most numerous blood elements. These small, anucleate discoid cells are derived from megakaryocytes in the bone marrow and have a circulating half-life of approximately 2.5 days in rats (White *et al.*, 1988). In humans, nearly one-third of platelets are stored in the spleen as an interchangeable pool that can be rapidly released into the circulation (Mackie *et al.*, 1988).

To simplify the complicated structures of the platelet and relate them to functional activities, the platelet can be divided into four main regions: the

peripheral zone, the sol-gel zone, the organelle zone and the membrane systems (Mackie *et al.*, 1988; White *et al.*, 1994). The peripheral zone is composed of the membranes and associated structures which constitute the surface of the platelet and the walls of platelet-specific channels termed the open canalicular system (OCS). These membranes are rich in glycoproteins and contain receptors for prostanoids, coagulation factors, immunoglobulins and complement. The peripheral zone includes the submembrane region that contains filaments which are closely linked to membrane glycoproteins and which are important for the translation of chemical signals received on the platelet exterior. The sol-gel zone is the matrix of the platelet cytoplasm. It contains fibrous systems of microfilaments and microtubules. This region supports the platelet discoid shape and provides the contractile system involved in shape change, pseudopod formation and secretion. The organelle zone consists of mitochondria, lysosomes, peroxisomes, dense bodies and granules. These organelles are critical for metabolic processes and the storage of enzymes, adenine nucleotides, serotonin and calcium which are destined for release upon platelet activation. The membrane system contains two distinct structures: the OCS and the dense tubular system (DTS). The OCS, although intimately associated with the peripheral zone, is described here in this section since it is a unique platelet structure. It forms a network of canaliculi through the platelet that is connected to the exterior surface. This network greatly increases the surface area of the platelet and provides an egress route for products of the release reaction. The OCS remains intact during platelet

Figure 1.2 Platelet structure. This diagram summarizes some of the ultrastructural features of a non-activated platelet. Components of the peripheral zone include the cell membrane and the surface connected OCS. The sol-gel zone contains microfilaments and microtubules. Embedded within the sol-gel zone are mitochondria, granules, dense bodies and lysosomes that constitute the organelle zone. Specialized membrane systems include the OCS and DTS.


MICROTUBULES
DTS
MITOCHONDRIA
OCS
DENSE GRANULE
α GRANULE

aggregation and shape change, which suggests that shape change is not a consequence of the OCS opening and fusing with the platelet membrane. DTS channels are derived from smooth endoplasmic reticulum, are located in close association with the OCS and provide a storage site for calcium. In addition, the DTS is the site for the synthesis of eicosanoids, as it contains enzymes from the cyclooxygenase and lipoxygenase pathways (Mackie *et al.*, 1988; White *et al.*, 1994; Niewiarowski *et al.*, 1994; Gordon, 1981).

III. B. Platelet Activation

Numerous particulate and soluble substances can stimulate platelet activation. These include ADP, norepinephrine, thrombin, arachidonic acid, collagen, TxA_2, PGG_2, serotonin, vasopressin, calcium ionophores, immune complexes and PAF (Mackie *et al.*, 1988; White, 1994; Niewiarowski *et al.*, 1994). However, with regard to this thesis, it is important to note that although rat platelets can produce PAF, they do not respond to PAF (Klee *et al.*, 1991). This biological difference may explain some of the contrasting findings using PAF receptor antagonists among animal models of endotoxemia.

Upon activation with these stimuli, platelets undergo a complex series of events described as adherence, shape change, secretion and aggregation. When the vascular endothelium is injured, the subendothelial matrix containing collagen is exposed and platelets rapidly adhere to this matrix, change shape

and spread across the site of damage. The platelets then secrete their granular constituents that modify vascular tone and promote further platelet accumulation. The result is a aggregate of platelets. Stimulated platelets also express a procoagulant surface which promotes the activation of the coagulation system and subsequent fibrin formation. The fibrin adds mechanical support to the platelet thrombus by forming links between platelet surface glycoproteins (Stormorken,1969; Bennett,1992; Mackie *et al.*,1988).

Platelet adherence to the subendothelium involves complex interactions between plasma proteins and glycoproteins on the platelet surface. Some of the critical plasma proteins include von Willebrand factor (vWF), fibronectin, fibrinogen and thrombospondin. Resting platelets do not interact with fibrinogen since the glycoprotein IIb/IIIa (or $\alpha_{IIb}ß_3$) is in an inactive conformation and has low affinity for fibrinogen (Calvete,1994). However, upon platelet activation, the IIb/IIIa complex undergoes measurable conformational changes and becomes a high-affinity receptor for soluble fibrinogen and other plasma adhesive proteins, including fibronectin, vitronectin and vWF (Calvete,1994). This complex is important for crosslinking platelets to each other and also for the adherence of platelets to subendothelium.

Another important mediator of platelet adherence to exposed subendothelium is vWF. This protein is synthesized in endothelial cells and secreted in a controlled fashion. The vascular endothelium releases some vWF directly into the plasma, but a majority of vWF is released into the subendothelium where it binds to the matrix to provide a surface for platelet

attachment should the endothelium be damaged (Pearson,1991; Ware *et al.*,1993). vWF can bind to glycoprotein IIb/IIIa, but it primarily binds to platelet glycoprotein Ib (Kainoh *et al.*,1992). The importance of vWF binding to glycoprotein Ib in hemostasis and thrombosis is emphasized by the Bernard Soulier syndrome, in which the absence of this glycoprotein from platelets results in bleeding disorders (Charo *et al.*, 1994; Bennett,1992).

Other important mediators of adhesion include the selectins. These lectins mediate cell-cell interactions. E- and L-selectins mediate the adhesion of neutrophils and lymphocytes, respectively, to the endothelium. P selectin, or GMP-140 (PADGEM), is found within the Weibel-Palade bodies of endothelial cells and within α-granules of platelets. Upon activation of these cells, the preexisting P-selectin is rapidly mobilized to the cell surface and mediates the adhesion of neutrophils to endothelium and of leukocytes to platelets (Sanders *et al.*,1992; 37). P-selectin appears to mediate platelet aggregation as well, since it has been localized between adjacent platelets undergoing aggregation and since a monoclonal antibody to it inhibits aggregation (Parmentier *et al.*,1991).

After adhesion to the subendothelium, platelets change shape from normal discoid to an irregularly shaped elongated cell with cytoplasmic projections. The presence of pseudopods on platelets can be used as a marker of platelet activation. The shape change allows platelets to spread across the exposed subendothelium.

III. C. Platelet degranulation

Activation of platelets results in the release of platelet granular constituents. These granule components are important as they modify vascular tone, recruit more platelets to the site of injury and enhance aggregate formation. The platelet release reaction involves the fusion of platelet granules and dense bodies to the OCS in a calcium-dependent process and the release of constituents into the OCS followed by secretion into the extracellular environment (Mackie *et al.*, 1988; Bennett,1992).

Table 1 summarizes some of the mediators released from platelets. The *α*-granules contain platelet-specific proteins such as platelet factor 4 (PF4) and ß-thromboglobulin (ßTG), as well as coagulation factors, growth factors and glycoproteins. Platelet basic protein (PBP) is a precursor of low affinity platelet factor 4 (LAPF4), PF4 and ßTG. PBP and LAPF4 are found within megakaryocytes and platelets, but only PF4 and ßTG are found in platelet releasates. PF4 binds heparin and can neutralize its activity. PF4 can also bind to hepatocytes, and the hepatocyte is involved in its degradation (Rucinski *et al.*,1986). Other activities of PF4 include stimulation of histamine release, potentiation of platelet aggregation and of digestion of elastin by elastase (Brindley *et al.*,1983; Capitanio *et al.*,1985). ßTG has some heparin-binding activity (Holt *et al.*,1988). Interestingly, the most characterized biologic activity of ßTG occurs when this protein is cleaved. The C-terminal fragment is known as ßTG-F or neutrophil activating peptide 2 (NAP_2). This fragment is

chemotactic for neutrophils and stimulates neutrophil degranulation (Walz *et al.*, 1989).

The dense granules of platelets contain large amounts of adenosine and guanosine di- and triphosphates, Ca, Mg and serotonin. Serotonin is vasoactive and may be important in limiting blood loss at the site of injury. Serotonin and ADP also promote further local platelet recruitment, as they stimulate platelet aggregation.

Platelets can phagocytose several kinds of particles such as collagen, fibrin and immune complexes (Gordon *et al.*, 1976) and have lysosomes which contain acid hydrolases that are used to degrade the phagocytosed material. Upon exposure to strong platelet stimulators, these lysosomal constituents are released. The secretion of these enzymes could have pathophysiologic consequences. For example, collagenase is proposed to function as a feedback mechanism that limits further thrombus formation (Chesney *et al.*, 1974). Elastase is believed to be critical in the development of atherosclerosis, since this disease features degradation of arterial elastic tissue (Niewiarowski *et al.*, 1994).

Table 1.1

Products Released from Activated Platelets

Constituents	Function	Reference
α-Granules		
albumin	plasma protein	(Holmsen, 1994)
α_2 macroglobulin	plasma protein	(Nachman *et al.* 1976)
fibrinogen	coagulation	(Holmsen, 1994)
factor V	coagulation	(Holmsen, 1994)
vWF	coagulation, adhesion	(Niewiarowski, 1985)
HMW kininogen	coagulation	(Schmaier *et al.* 1983)
PF4	antiheparin	(Walz *et al.* 1989)
plasminogen	fibrinolysis	(Niewiarowski, 1985)
α_2 antiplasmin	inhibits fibrinolysis	(Nachman *et al.* 1976)
ß-TG like proteins	neutrophil activation	(Walz *et al.* 1989)
thrombospondin	adhesion	(Niewiarowski, 1985)
fibronectin	adhesion	(Niewiarowski, 1985)
α_1 antitrypsin	protease inhibitor	(Nachman *et al.* 1976)
C1 inhibitor	complement inhibitor	(Schmaier *et al.* 1983)
PDGF	growth factor	(Holmsen, 1994)
EGF	growth factor	(Holmsen, 1994)
TGF-ß	growth factor	(Holmsen, 1994)
Dense Granules		
adenosine,ADP,ATP	aggregation	(Holmsen, 1994)
5-HT	vasoconstriction	(Holmsen, 1994)
calcium	signalling	(Holmsen, 1994)
magnesium	signalling	(Holmsen, 1994)
Lysosomes		
ß-glucuronidase	hydrolase	(Holmsen *et al.* 1970)
ß-galactosidase	hydrolase	(Holmsen *et al.* 1970)
N-acetylglucosaminidase	hydrolase	(Holmsen *et al.* 1970)
aryl sulfatase	hydrolase	(Holmsen *et al.* 1970)
elastase	protease	(James *et al.* 1985)
collagenase	protease	(Chesney, 1974)
cathepsins A, B and D	protease	(Holmsen *et al.* 1970)
Lipid Mediators		
TxA_2	aggregation	(Niewiarowski, 1994)
PAF	inflammation	(Bratton *et al.* 1989)
12-HPETE, 12-HETE	neutrophil stimulation	(Maclouf *et al.* 1982)
diHETE	leukocyte chemotaxis	(Thompson *et al.* 1993)

III. D. Platelet release products in tissue injury

The main hypothesis underlying the experiments described in this thesis is that platelets contribute to the pathogenesis of LPS-induced hepatotoxicity. Platelets might directly promote liver injury by several mechanisms. For example, platelets could contribute to hepatocellular damage through the formation of occlusive platelet thrombi. Indeed, as discussed above, platelet thrombi have been observed within the sinusoids of LPS-treated rats (Levy *et al.*, 1968a; Levy *et al.*, 1968b). It is therefore possible that platelets promote tissue injury by a mechanism involving the formation of occlusive thrombi and a resultant decrease in perfusion. This mechanism is believed to mediate the renal injury observed after the intravenous administration of LPS in rabbits (Schwartzman reaction), since platelet depletion affords protection from the development of glomerular microclots and renal injury (Kramer *et al.*, 1977). However, a similar mechanism is not likely to occur in the liver, since hepatic ischemia is characterized by centrilobular injury (Jaeschke *et al.*, 1990; Kehrer *et al.*, 1990; Jaeschke, 1992a), whereas LPS exposure results primarily in midzonal hepatocellular necrosis (Hewett *et al.*, 1992). Thus, the location of parenchymal cell damage suggests that LPS exposure and ischemia lead to liver injury by different mechanisms.

Platelets could contribute to liver injury by releasing platelet constituents. Of the mediators discussed in Table 1, those most likely to contribute directly to cell damage seem to be the lipid mediators and proteases. The roles of

TxA_2, PAF and leukotrienes in liver injury were discussed above. The proteases, such as elastase and the cathepsins, could injure cells by proteolytic action on cell membranes. The roles of platelet proteases in tissue injury is not well characterized, however neutrophil-derived proteases such as elastase and cathepsin G are critical mediators of cytotoxicity *in vitro*. For example, neutrophils stimulated with f-met-leu-phe (fmlp) are cytotoxic to cultured endothelial cells. This cytotoxicity is mimicked with the addition of purified elastase and inhibited with specific elastase inhibitors (Smedley *et al.*, 1986). In addition, similar studies have suggested that activated neutrophils mediate hepatic parenchymal cell killing by releasing cathepsin G and elastase (Ho *et al.*, 1995). It remains unknown whether platelet-derived elastase, although biochemically different from that released by activated neutrophils (James *et al.*, 1985), could have similar cytotoxic effects.

As studies *in vitro* suggest, neutrophil-derived elastase may contribute to the pathogenesis of tissue injury *in vivo*. For instance, the infusion of neutrophil-derived elastase and cathepsin G into the renal artery causes glomerular basement membrane injury and proteinuria (Johnson *et al.*, 1988). In plasma, depending upon the concentrations, neutrophil-derived elastase is unbound or complexed with the plasma protein, α_1-antitrypsin. These elastase-antitrypsin complexes are elevated in septic patients, and the plasma concentration of elastase correlates with the severity of infection (Fritz *et al.*, 1986). Thus, it has been hypothesized that extensive release of elastase from stimulated neutrophils can overcome the endogenous antiprotease activity

of α_1-antitrypsin and contribute to injury by non-specific proteolysis of structural elements. However, similar to the findings *in vitro*, the role of platelet-derived proteases in tissue injury *in vivo* is not fully understood.

III. E. Interactions between platelets and inflammatory mediators

The remaining platelet constituents released upon activation are unlikely to mediate hepatocellular injury directly, but they could contribute to parenchymal cell damage by interacting with other, critical inflammatory mediators. The following subsections will address some potential interactions of platelets with the coagulation system, neutrophils and Kupffer cells.

Platelet-coagulation system interactions. As mentioned previously, the coagulation system is critical for the pathogenesis of liver injury during LPS exposure (Hewett *et al.*,1995; Margaretten *et al.*,1967). Platelets may influence the coagulation system in several ways. As shown in Table 1, the α-granules of platelets contain coagulation proteins including vWF and vWF antigen, fibrinogen and high molecular weight kininogen. The surface of platelets can bind coagulation factors and can activate components of the intrinsic pathway, such as prothrombin and factors X, XI and XII. Platelets can also protect factors XIa and Xa from proteolytic inactivation (Walsh,1981a; Walsh *et al.*,1981b; Walsh *et al.*,1993), thereby promoting clot formation.

Thus, platelets could contribute to hepatocellular injury by promoting activation of the coagulation system.

Platelet-neutrophil interactions. Neutrophils have been localized within platelet thrombi *in vivo* (Wester *et al.*,1979) and neutrophils interact with platelet aggregates following desquamation of arteries (Ratliff *et al.*1979). In addition, neutrophils influence platelet deposition in tissues in response to intradermal injection of LPS, since prior neutrophil depletion inhibits this inflammatory response (Issekutz *et al.*,1983). Similarly, activated platelets can influence neutrophil accumulation in tissues by expressing P-selectin. The adhesion of neutrophils to a confluent monolayer of platelets in a flow chamber is completely inhibited with a P-selectin antibody (Yeo *et al.*,1994). In addition, the neutrophils bound to platelets expressing P-selectin appear to be activated, since Cd11b and CD18 surface expression is upregulated on adherent, but not on nonadherent neutrophils (Yeo *et al.*,1994). Similar findings are reported *in vivo*. A P-selectin antibody inhibits leukocyte adherence to platelets that have accumulated on grafts implanted with an arteriovenous shunt in mice (Palabrica *et al.*,1992).

Neutrophils are critical for LPS-induced liver injury (Hewett *et al.*, 1992; Jaeschke *et al.*,1991b), and several platelet constituents can influence neutrophil functions. As mentioned previously, PAF, which is produced from numerous inflammatory cells including platelets, is chemotactic for and

stimulates degranulation of neutrophils (Hosford *et al.*,1993). The ß-thromboglobulin family of proteins released from α-granules, including PBP, low affinity PF4 and NAP-2, are chemotactic for neutrophils and stimulate neutrophil intracellular calcium fluxes and degranulation (Walz *et al.*,1989; Holt *et al.*,1986; Holt *et al.*,1988). In addition, PF4 enhances the activity of elastase purified from human neutrophils (Lonky *et al.*,1981; Lonky *et al.*,1978). Such a mechanism might contribute to the pathogenesis of the adult respiratory distress syndrome (ARDS), since platelets are sequestered in the pulmonary tissue of patients with ARDS in association with elevated concentrations of PF4 and ß-TG in bronchoalveolar lavage fluid (Idell *et al.*,1989). It has been hypothesized that ßTG-like proteins contribute to ARDS by promoting pulmonary neutrophil infiltration and activation and increasing neutrophil-derived elastase activity (Idell *et al.*,1989).

Studies conducted *in vitro* suggest that platelets influence neutrophil activation. For instance, human platelets release a soluble factor that increases fmlp-induced O_2^- production in neutrophils (Wright *et al.*,1988; Naum *et al.*,1991). Similar co-incubation studies in sheep suggest interactions between neutrophils and platelets in response to thrombin. The addition of thrombin to neutrophils or platelets alone does not increase O_2^- production; however, when platelets and neutrophils are co-incubated, the addition of thrombin results in a 5-fold increase in stimulated O_2^- production (Moon *et al.*,1990). This enhanced response is dependent upon thrombin concentration and the platelet:neutrophil ratio and is suggested to be mediated by direct contact

between platelets and neutrophils.

Not only can platelets influence neutrophils but neutrophils can influence platelets too. Purified, neutrophil-derived cathepsin G stimulates platelet aggregation and the release of serotonin and TxA_2 (Evangelista *et al.*, 1992; Selak *et al.*, 1988). Similar findings are reported in a modified rosetting assay in which fmlp-stimulated neutrophils induce serotonin release from platelets. This effect is likely mediated by cathepsin G, since α-1antiprotease inhibits this effect (Evangelista *et al.*, 1993). The mechanism by which cathepsin G stimulates platelets is not clearly understood, however, cathepsin G is reported to alter the expression of platelet glycoproteins. For instance, this protease decreases the expression of glycoprotein Ib and vWF binding by a mechanism involving proteolysis and also a cytoskeleton-mediated redistribution of the glycoprotein (LaRosa *et al.*, 1994). In contrast, cathepsin G stimulates the expression of glycoprotein IIb/IIIa on the surface of platelets (Molino *et al.*, 1993). Taken together, these studies suggest that activated neutrophils and subsequent release of cathepsin G might reduce platelet binding to the endothelium while increasing their ability to aggregate.

Since TNF-α is a critical mediator of LPS-induced hepatotoxicity, it is interesting to note that priming of neutrophils with TNF-α enhances cathepsin G release in response to fmlp and also enhances platelet activation and serotonin release (Renesto *et al.*, 1991). Such an interaction could explain the critical nature of both neutrophils and TNF-α in the pathogenesis of hepatotoxicity.

Another neutrophil derived protease, elastase, has the opposite effect on platelets; it inhibits thrombin-induced platelet aggregation (Brower *et al.*, 1985). Immunocytochemistry suggests that elastase exposure reduces the number of high affinity thrombin binding sites on the platelet surface and, similar to cathepsin G, results in the proteolytic cleavage of Ib (Brower *et al.*, 1985). Thus, different neutrophil-derived proteases have some opposing effects on platelets.

Another interaction between neutrophils and platelets is transcellular metabolism of arachidonic acid and its metabolites *in vitro*. Platelet-derived arachidonate can serve as a precursor for LTB_4 and 5-HETE production in neutrophils (Marcus *et al.*, 1982). In addition, platelet-derived 12-HETE is metabolized to diHETE, a precursor of LTB_4, by neutrophils (Marcus *et al.*, 1982). On the other hand, neutrophil-derived 5-HETE and LTA_4 are converted to diHETE and LTC_4, respectively, by platelets (Maclouf *et al.*, 1990; Maclouf *et al.*, 1982). The role and significance of this transcellular metabolism *in vivo* is not understood.

Platelet-Kupffer cell interactions. As mentioned, electron micrographs of murine livers after LPS exposure reveal that platelets are present within the liver sinusoids and also within Kupffer cells (Levy *et al.*, 1968b; Endo *et al.*, 1993). Kupffer cells could contribute to platelet accumulation by several mechanisms. The hepatic reticuloendothelial system, consisting of Kupffer cells and endothelial cells, can clear senescent platelets as well as platelet-immune

complexes, as observed during immune thrombocytopenia purpura (du P Heyns *et al.*, 1986). The clearance of platelets by Kupffer cells may be mediated in part by the C1q receptor located on Kupffer cells. This receptor complex contributes to the clearance of fibronectin coated cells, proteins and particles. Both stimulated Kupffer cells and platelets release fibronectin (Toth *et al.*, 1992; Niewiarowski *et al.*, 1994). In addition, LPS exposure increases serum fibronectin concentration (Richards *et al.*, 1985). Moreover, the enhanced Kupffer cell phagocytic activity observed after challenge with a small dose of LPS correlates with elevated plasma fibronectin concentration (Richards *et al.*, 1985). Accordingly, both platelets and Kupffer cells, through the release of fibronectin, could stimulate Kupffer cell phagocytosis.

Kupffer cells from normal rat livers produce TxA_2, PGE_2 and primarily PGD_2. When exposed to LPS, these cells produce primarily PGE_2 (Kawada *et al.*, 1992). In addition, the stimulation of Kupffer cells with calcium ionophore, phorbol ester or opsonized zymosan stimulates both PGD_2 and TxA_2 production (Dieter *et al.*, 1989). Since these prostanoids have various effects on platelet aggregation, it is possible that their balance modifies platelet reactivity.

Activated Kupffer cells can directly injure the sinusoidal endothelium through the release of lipoxygenase metabolites and oxygen radicals (Deaciuc *et al.*, 1993a; Deaciuc *et al.*, 1994; Deaciuc *et al.*, 1993c; Liu *et al.*, 1995; Liu *et al.*, 1994), and endothelial cell injury promotes platelet adherence and activation. Moreover, oxygen radical-mediated injury to the endothelium, through changes in the production of platelet inhibitors such as prostacyclin and nitric oxide, may

alter the activity of platelets (Salvemini *et al.*, 1993). In addition, oxygen radicals can directly influence platelets, inasmuch as large concentrations of hydrogen peroxide stimulate platelet aggregation (Salvemini *et al.*, 1993). These potential interactions between Kupffer cells and platelets have not been well characterized during LPS exposure *in vivo*.

IV. CONCLUSION

This introductory chapter has focused on the roles of inflammatory mediators, including neutrophils, Kupffer cells, the coagulation system and lipid derivatives, in the pathogenesis of LPS-mediated hepatotoxicity. In addition, emphasis has been placed on platelets and their possible contributions to the development of tissue injury. The main focus of this dissertation is to examine the role of platelets in LPS-induced liver injury, focusing on the potential interactions between platelets and other cellular and soluble mediators of inflammation.

The global hypothesis is that platelets contribute to LPS-induced hepatotoxicity by mechanisms involving their interactions with other cellular and soluble inflammatory mediators.

V. RESEARCH GOALS

1. A main goal of this research was to determine if platelets accumulate in the liver after exposure to LPS. In addition, time course studies were conducted to describe the temporal relationships between hepatic platelet accumulation and thrombocytopenia relative to hepatic neutrophil accumulation, activation of the

coagulation system and onset of hepatocellular injury. Platelet depletion experiments were performed to ascertain whether platelets are critical for the pathogenesis of LPS-induced liver injury. These studies also evaluated the effect of platelet depletion on hepatic neutrophil accumulation, production of plasma TNF-α activity and activation of the coagulation system.

2. Since both neutrophils and Kupffer cells contribute to LPS-induced liver injury and both of these cells can interact with platelets, the roles of Kupffer cells and neutrophils in the hepatic platelet accumulation and thrombocytopenia after exposure to LPS were evaluated.

3. Exposure to LPS activates the coagulation system, and this activation is critical for the development of hepatocellular damage. The coagulation system, most notably thrombin, can stimulate platelet aggregation. Studies were conducted with heparin and the thrombin inhibitor, hirudin, to evaluate the role of the coagulation system in the hepatic platelet accumulation and thrombocytopenia during exposure to LPS. In addition, to determine whether the coagulation system acts as a distal mediator (i.e. acts shortly before the onset of liver injury), experiments were performed in which heparin and hirudin were administered at various times after LPS exposure and their effects on the development of liver injury were evaluated.

4. Numerous inflammatory cells, including platelets, neutrophils and Kupffer

cells, can release and be stimulated by PAF and leukotrienes. To address the roles of these lipid mediators in the thrombocytopenia, activation of the coagulation system and liver injury mediated by LPS, studies were conducted with a PAF receptor antagonist and a 5-lipoxygenase inhibitor.

5. Cyclooxygenase metabolites, in particular thromboxane, are produced from platelets and other inflammatory cells and are critical for the development of numerous LPS-mediated pathophysiologic effects. A study was conducted to determine the temporal relationship between the production of thromboxane relative to other LPS-mediated alterations. To address the role of cyclooxygenase metabolites and thromboxane in the pathogenesis of LPS-induced thrombocytopenia, activation of the coagulation system, hepatic platelet accumulation and liver injury, experiments with aspirin and the thromboxane synthase inhibitor, dazmegrel were performed.

CHAPTER 2

ROLE OF PLATELETS IN LPS-INDUCED LIVER INJURY

2. A. Abstract

After LPS injection into rats, plasma fibrinogen concentration and numbers of blood platelets and leukocytes decrease. Results of our studies, using immunocytochemistry for the detection of neutrophils and ^{111}indium-labeling to identify platelets, indicate that both neutrophils and platelets accumulate within the liver early after administration of LPS to rats. The accumulation of platelets in the liver prior to the onset of injury suggested that platelets contribute to the manifestation of LPS-induced hepatotoxicity. To test this hypothesis, the number of circulating blood platelets was decreased by the administration of an anti-rat platelet serum (APS) prior to LPS administration. The consequent thrombocytopenia by APS administration was associated with an attenuation of both LPS-induced liver injury and the activation of the coagulation system. However, the APS treatment did not prevent the hepatic neutrophil accumulation. These results suggest that platelets contribute to the pathogenesis of liver injury after LPS administration, perhaps through their integral role in coagulation and/or interaction with neutrophils, but they do not appear to contribute to hepatic neutrophil accumulation.

2.B. Introduction

Several studies suggest a role for platelets in the pathogenesis of some LPS-induced responses. Platelet sequestration in both the lung and liver has been reported in experimental models of endotoxin shock (Christenson *et al.*, 1987; Sostman *et al.*, 1983). In addition, antibody-induced thrombocytopenia protects rabbits (Kramer *et al.*, 1977) and rats (Ito *et al.*, 1990) from endotoxin-induced disseminated intravascular coagulation (DIC). Furthermore, drugs that prevent synthesis or action of thromboxane A_2, which is produced by activated platelets, provide protection in animal models of LPS-induced DIC and liver injury (Ito *et al.*, 1990; Wise *et al.*, 1980). A recent study revealed a correlation between the severity of LPS-induced liver injury and thrombocytopenia (Shibayama *et al.*, 1995). Taken together, these observations suggest a relationship between platelets and liver injury. However, studies to test the hypothesis that platelets actually contribute to the pathogenesis of liver injury have not been reported.

To elucidate the role of platelets in LPS-induced liver injury, the temporal relationships between the onset of liver injury and changes in plasma fibrinogen concentration, plasma TNF-α activity, and the numbers of platelets and neutrophils in blood and liver were evaluated in the same group of rats after intravenous injection of *E. coli* LPS. To test the hypothesis that platelets contribute to the manifestation of LPS-induced hepatotoxicity, the influence of thrombocytopenia on liver injury was examined. Finally, to investigate potential

interactions between platelets and other mediators *in vivo*, the effects of platelet depletion on LPS-induced changes in fibrinogen concentration and TNF-α activity in plasma and neutrophil accumulation in liver were evaluated.

2. C. Materials and methods

2. C. 1. Materials

Lipopolysaccharide (*Escherichia coli*, serotype 128:B12), Kit 605-D for measurement of bilirubin concentration and Kit 59 for determination of alanine aminotransferase activity (ALT) were purchased from Sigma Chemical Company (St. Louis, MO). Dilution and lysis of erythrocytes for platelet enumeration were performed in platelet Unopettes® (Baxter Scientific Products, McGaw Park, IL). Fibrinogen concentration was measured in a BBL® Fibrometer (Becton, Dickinson and Company, Hunt Valley, MD) using Data-Fi® fibrinogen determination kit (Baxter Scientific Products, McGaw Park, IL). All immunocytochemical reagents, except rabbit anti-rat neutrophil immunoglobulin, were purchased from Vector Laboratories (Burlingame, CA). ^{111}Indium-oxine was purchased from Medi-Physics Division of Amersham (Arlington Heights, IL). Prostaglandin E_1 was purchased from Calbiochem (San Diego, CA).

2. C. 2. Animals

To be consistent with previous studies with this model, female, Sprague-Dawley rats (Crl:CD BR (SD) VAF/plus, Charles River, Portage, MI) weighing 200-250g were used in all studies. The animals were maintained on a 12 hr light/dark cycle under controlled temperature (18-21° C) and humidity (55 ± 5%). Food (Rat chow, Teklad, Madison, WI) and water were allowed *ad libitum*. All procedures on animals were carried out according to the guidelines of the American Association of Laboratory Animal Sciences and the University Laboratory Animal Research facilities of Michigan State University.

2. C. 3. Anti-rat platelet serum

Polyclonal, anti-rat platelet serum was collected from a Nubian goat as described previously (White *et al.*, 1989). Complement was inactivated by incubation of serum at 56° C for 45 minutes. Anti-platelet serum (APS) was adsorbed with washed rat red blood cells. Control serum (CS) was similarly prepared from a goat that had not been immunized. Both APS and CS were stored in 2 ml aliquots at -20^0 C until use.

2. C. 4. Time course studies

Initial studies were performed to assess the temporal effects of lipopolysaccharide (LPS) administration on blood constituents and the liver. Rats were treated either with LPS (4 mg/kg) or with its saline (SAL) vehicle in a volume of 4 ml/kg as a single injection in the tail vein. This dose of LPS

results in reproducible liver injury within 6 hours without significant mortality. At various times after LPS administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn into sodium citrate (0.38% final concentration) from the descending aorta, and liver sections were fixed in Histochoice® fixative for histologic evaluation and immunocytochemistry. This treatment regimen resulted in no mortality within 6 hrs after LPS administration.

Platelets were counted in a hemocytometer after erythrocyte lysis and dilution in platelet Unopettes. Total leukocyte (WBC) counts were performed using a Coulter counter (Coulter Electronics Limited, Luton, England). Differential counts were performed on blood smears stained with buffered, modified differential Wright-Giemsa stain to obtain the fraction of neutrophils and lymphocytes in each blood sample. The total number of WBCs was multiplied by this fraction to obtain absolute numbers of neutrophils and lymphocytes.

Plasma fibrinogen concentration was determined from the thrombin clotting times of diluted plasma samples measured in a fibrometer. Plasma alanine aminotransferase activity (ALT) was used as a marker of hepatic parenchymal cell injury and was determined spectrophotometrically using Sigma Kit 59. Total plasma bilirubin was used as a marker of cholestatic liver injury and was determined spectrophotometrically using Sigma Kit 605-D. Plasma tumor necrosis factor *alpha* (TNF-α) was measured by the lysis of the fibrosarcoma cell line, WEHI 164, clone 13 as described previously (Hewett *et*

al., 1993). Human recombinant TNF-α was used to establish a standard curve (R and D Systems, Minneapolis, MN).

2. C. 5. Neutrophil immunocytochemistry

To quantify hepatic neutrophil numbers, an immunocytochemical stain for neutrophils was employed. Liver sections were collected, preserved in Histochoice® fixative, embedded in paraffin and sectioned at 5 microns. Paraffin was removed from tissue sections with xylene prior to immunocytochemical staining. The immunocytochemical procedure was performed on an automated immunostainer (Leica Histostainer Ig, Model V2.01, Australian Biomedical Corporation Ltd., Mount Waverly, Australia) using Vectastain ABC Immunohistochemical Kit (Vector, Burlingame, CA). All incubations were performed at room temperature. After each incubation, slides were rinsed twice with Tris-phosphate buffered saline solution (0.75% Trizma-HCl, 0.9% NaCl, pH 7.6). To eliminate nonspecific binding, tissue sections were incubated with normal goat serum (30 minutes), avidin blocking reagent (15 minutes) and biotin blocking reagent (15 minutes) as described in the Vectastain ABC Immunocytochemical kit. Sections were then incubated with rabbit anti-rat neutrophil polyclonal immunoglobulin for 1 hour. This immunoglobulin was isolated from the serum of rabbits immunized with rat neutrophils as described by Hewett and Roth (Hewett *et al.*, 1992). To maximize neutrophil staining, an avidin/biotin labeling procedure was used. Tissue sections were incubated with biotinylated goat anti-rabbit IgG (20

minutes). Incubation with an avidin-conjugated alkaline phosphatase (30 minutes) and Vector Red substrate (8 minutes) resulted in red-staining of neutrophils within the liver sections. Tissues were then counterstained with Lerner 2 Hematoxylin for 2 minutes. The numbers of sinusoidal hepatic neutrophils were determined with microscopy by enumerating neutrophils in 20 fields spaced equally throughout the length of each tissue section and was expressed as average cells/mm^2 of liver.

2. C. 6. Platelet Sequestration Studies

To assess hepatic platelet sequestration, platelets were radiolabeled with ^{111}indium-oxine. Briefly, blood was collected by cardiac puncture from donor, female, Sprague-Dawley rats in 3.8% sodium citrate (9:1, blood:sodium citrate). Platelet rich plasma (PRP) was isolated by spinning whole blood for 15 min at 250*g* in a centrifuge. The PRP was spun at 800*g* for 20 min to isolate platelets from platelet poor plasma (PPP). The platelet pellet was washed and resuspended in 2 ml Tyrode's buffer (pH 6.5) containing 137 mM NaCl, 2.6 mM KCl, 11.9 mM $NaHCO_3$, 0.32 mM NaH_2PO_4, 2 mM $MgCl_2$, 5.6 mM D-glucose, 25,000 U/L sodium heparin and 300 ng/ml prostaglandin E_1. Platelets were labelled with 200 μCi ^{111}In-oxine for 10 min at 37° C. After labeled platelets were pelleted at 400*g* for 20 min, the supernatant was removed and platelets were washed and resuspended in 2 ml Tyrode's buffer. This centrifugation and wash were repeated. Finally, platelets were resuspended in 2 ml PPP. From this suspension, they were enumerated in a hemacytometer as described above.

The final volume of the injected ^{111}In-oxine labeled platelet solution was adjusted with PPP such that each rat received 0.3 ml solution containing 3-5 x 10^9 radiolabeled platelets. An aliquot of this injection solution was removed for determination of injected radioactivity. This procedure has been utilized extensively and has been shown not to injure platelets, as assessed by transmission electron microscopy and platelet aggregometry (White *et al.*, 1989).

In the hepatic platelet sequestration studies, each rat received a single bolus injection into the tail vein of 0.3 ml ^{111}In-oxine-labeled platelets suspended in plasma. Two hours after the administration of radiolabeled platelets, animals were treated with LPS (4 mg/kg, iv) or SAL vehicle. At various times after the administration of LPS or SAL vehicle, animals were anesthetized as described above. A blood sample was collected and the liver, spleen, lungs and left kidney were excised. Each organ was weighed and the radioactivity of a fraction of the organ (liver) or the entire organ (kidney, spleen, and lungs) was measured in a gamma counter (TM Analytic GammaTrac 1193, TM Analytic, Elk Grove Village, IL). Total organ radioactivity was calculated. No correction for blood content was made when calculating organ radioactivity. Blood (1 ml) and platelet poor plasma (0.2 ml) samples were evaluated for radioactivity. Data are expressed as percent of total injected radioactivity in individual organs or per ml blood or plasma.

2. C. 7. Platelet depletion studies

To assess the role of platelets in LPS-induced liver injury, anti-rat platelet serum (APS, 0.5 ml/rat, ip) or control serum (CS) was administered to rats 12 hrs prior to the administration of LPS. This treatment regimen reduced blood platelet numbers to 10-20% of normal throughout the duration of the study (data not shown, (White *et al.*, 1989)). Six hours after the administration of LPS (4 mg/kg, iv, bolus) or its saline vehicle, animals were anesthetized as described above, and citrated blood and liver sections were collected. Blood leukocytes and platelets, ALT activity and bilirubin concentration in plasma and plasma fibrinogen concentration were determined as described above.

2. C. 8. Morphometric analysis.

In the platelet depletion studies, samples of liver were fixed in 10% neutral buffered formalin and processed for histopathologic evaluation. Paraffin-embedded sections were cut at 6 μm and stained with hematoxylin and eosin. Slides were coded, randomized, and evaluated 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) and severe necrosis with marked hemorrhage (5).

Morphometric assessment was performed on coded liver sections using 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 hepatic tissue examined, the area of individual lesions, the number of segmented neutrophils per lesion and the number of neutrophils per area of tissue were determined. The area of liver with lesions was calculated and expressed as percentage of liver affected, and the number of lesions/area of liver was determined. The injury index was defined as the product of the mean lesion area (cm^2) and the mean lesion severity score. The number of neutrophils per lesion was determined by direct microscopic examination of tissue and was expressed as cells/lesion. The number of neutrophils/area of lesion was calculated. The number of neutrophils/area of liver was determined by enumerating neutrophils in 20 fields spaced equally throughout the length of each tissue section and was expressed as cells/mm^2 of liver.

2. C. 9. TNF-α activity.

To determine the effect of platelet depletion on plasma TNF-α activity, animals were treated with APS or CS 12 hr prior to bolus administration of LPS (4 mg/kg, iv) or its SAL vehicle (as described above). One and a half hours after the administration of LPS, animals were anesthetized, and plasma samples were collected and frozen at -20^0 C for later measurement of plasma TNF-α activity as described above.

2. C. 10. Data analysis

A one-way , completely random analysis of variance (ANOVA) was used to analyze time-dependent alterations in plasma ALT and TNF-α activities and

fibrinogen concentration, blood platelet numbers, hepatic neutrophil numbers and hepatic platelet accumulation after LPS administration. Data with nonhomogeneous variances were square root- or log-transformed prior to analysis. Comparisons among groups were performed using Tukey's omega test (Steel *et al.*, 1980). A Kruskal-Wallis one-way ANOVA on ranks was used to analyze plasma bilirubin concentrations and white cell numbers, as variances were nonhomogeneous.

In the platelet depletion studies, a 2 X 2 multifactorial, completely random ANOVA was used to analyze plasma ALT activity, plasma fibrinogen and TNF-α concentrations, and blood platelet, total WBC and neutrophil numbers. Data with nonhomogeneous variances were square root- or log-transformed prior to analysis. Comparisons between pairs of treatment groups were performed with Tukey's omega test for plasma ALT and TNF activity, fibrinogen concentration and blood cell numbers. Comparisons of plasma bilirubin concentrations were performed with the Kruskal-Wallis rank sums test, as the variances were not homogeneous (Steel *et al.*, 1980). Results are expressed as mean ± SEM.

Morphometric data were analyzed using the Kruskal-Wallis nonparametric test. Comparisons between pairs of treatment groups were performed using the rank sum test (Steel *et al.*, 1980). The criterion for significance was $p \leq 0.05$ for all studies.

2. D. Results

2.D.1. Time course of LPS-induced alterations in blood and liver.

Decreases in plasma fibrinogen concentration and blood platelet numbers *in vivo* can reflect activation of the coagulation system and platelet activation, respectively. Figure 2.1 shows the development of effects of LPS administration on plasma fibrinogen concentration and blood platelet numbers relative to events occurring in livers of the same animals. A pronounced decrease in both circulating fibrinogen concentration and blood platelet numbers began between 2 and 4 hrs after LPS administration. Plasma TNF-α activity increased within 1 hr and peaked about 1.5 hrs after LPS injection (Figure 2.1). This time course is consistent with previous reports on plasma TNF-α levels after LPS exposure (Hewett *et al.*, 1993). These changes occurred prior to the onset of liver injury, as estimated by elevations in plasma ALT activity. This marker of liver injury was marginally elevated at 4 hours and more markedly elevated by 6 hours after LPS administration. The increased plasma ALT activity was associated with hepatic parenchymal cell necrosis as revealed by histopathologic evaluation (see below).

Figure 2.1 also shows the pronounced hepatic neutrophil accumulation that began within 15 min after LPS administration and continued until 3 hours. The numbers of neutrophils within the liver sinusoids were not different at 3

and 6 hours after LPS administration. Blood neutrophils and lymphocytes decreased rapidly after LPS administration (Figure 2.2). Animals remained lymphopenic throughout the 6 hrs after LPS administration. By contrast, blood neutrophil numbers began to increase by 2 hrs and returned to control levels within 6 hr after LPS administration. The increase in blood neutrophils was characterized by increases in immature, band neutrophils as well as mature, segmented neutrophils (a regenerative left shift, Figure 2.2).

To assess hepatic platelet accumulation, ^{111}In-oxine-labeled platelets were used to trace platelets *in vivo* after LPS administration. Radioactivity accumulated in the liver within 1 hr after LPS administration (Figure 2.1). Hepatic radioactivity continually rose throughout the 6 hr after LPS administration. LPS administration did not significantly alter ^{111}In accumulation in the spleen, kidneys or lungs (Figure 2.3) as compared to SAL-treated rats. However, similar to studies with blood platelet numbers (Figure 2.1), ^{111}In-oxine-labeled platelets in blood decreased significantly 6 hr after LPS administration (Figure 2.3). Less than 1% of injected radioactivity was detected in plasma and less than 0.001% of total injected radioactivity was detected in the bile from either SAL or LPS treated animals (data not shown).

2. D. 2. Verification of platelet depletion

Since blood platelet numbers decreased prior to the onset of liver injury in this model and since ^{111}In-oxine-labeled platelets accumulated in the liver

after LPS administration (Figure 2.1), platelet depletion studies were conducted to test the hypothesis that platelets may be important in the manifestation of LPS-induced liver injury. The administration of an anti-rat platelet serum (APS) 12 hr prior to the administration of LPS reduced blood platelet numbers to less than 20% of control (Figure 2.4). Consistent with the results in Figure 2.1, blood platelet numbers were significantly reduced 6 hrs after the administration of LPS to CS pretreated rats.

2. D. 3. The effect of platelet depletion on liver injury

Platelet depletion was associated with a marked attenuation of LPS-induced liver injury, as measured by elevations in plasma ALT activity and bilirubin concentration (Figure 2.5) and by morphometric analysis of liver sections (Figure 2.6, Table 2.1). Livers from SAL-treated controls pretreated with CS or APS had no evidence of inflammation (Figure 2.6). The number of neutrophils per area of liver in SAL-treated rats was quite small and was unaffected by APS treatment (1 ± 0/mm^2 for APS pretreated rats and 2 ± 1/mm^2 for CS pretreated rats). Six hours after LPS administration, livers from CS pretreated rats had multifocal, acute, moderate to marked hepatocellular necrosis (neutrophilic hepatitis) (Figure 2.6). The hepatic sinusoids contained many neutrophils (Table 2.1), few plump Kupffer cells and small amounts of an eosinophilic, proteinaceous precipitate. There were multifocal, irregularly shaped areas of midzonal hepatocellular necrosis. These lesions were

characterized by hypereosinophilic parenchymal cells with small, pyknotic nuclei or pale parenchymal cells with indistinct or absent nuclei and indistinct cytoplasmic borders. The necrotic foci contained a moderate number of degenerate neutrophils, and some had small amounts of hemorrhage. The number of neutrophils in lesions was greater than that in unaffected tissue (Table 2.1). There were also few single necrotic cells scattered within the liver sections. Six hours after LPS administration, livers from rats that were platelet depleted with APS had lesions that were similar in character but much fewer and smaller compared to livers from rats pretreated with CS (Figure 2.6, Table 2.1). However, there was no difference in the severity of lesions or the number of neutrophils observed in each lesion, per area of the lesion, or per unit of area liver between LPS-treated rats pretreated with APS and those treated with CS.

2. D. 4. Effect of platelet depletion on the coagulation system

Pretreatment with APS had no effect on plasma fibrinogen concentration in SAL- treated animals, however platelet depletion prevented the LPS-induced decrease in plasma fibrinogen concentration (Figure 2.7).

2. D. 5. Effect of platelet depletion on plasma TNF activity

In animals pretreated with either CS or APS, plasma TNF-α activity was below the level of detection (less than 1 ng/ml) 1.5 hr after the administration of SAL vehicle (Figure 2.8). Plasma TNF-α activity was elevated 1.5 hr after the administration of LPS in CS pretreated rats. In platelet depleted rats, this elevation in plasma TNF-α was even more pronounced.

2. D. 6. Effect of platelet depletion on total WBC counts

Six hours after LPS administration, animals pretreated with either CS or APS had significantly reduced total WBC counts, which was due to a reduction in the numbers of lymphocytes (Figure 2.9). Blood neutrophil numbers were not significantly altered 6 hr after LPS administration. This finding is consistent with time course studies (Figure 2.2), which showed that blood neutrophil numbers return to normal by 6 hr after LPS administration.

Figure 2.1. Temporal effects of LPS administration on blood (top) and liver (bottom) markers. LPS (4 mg/kg) or SAL vehicle was administered iv at 0 hr. Since values from SAL vehicle-treated animals did not change significantly with time, these were combined and expressed as a time-zero control. Samples were collected and evaluated as described in "Methods." Results are normalized to 1.0, where a value of 1.0 (± weighted standard error of differences) corresponded to the following: 160 ± 9.8 mg/dl for plasma fibrinogen concentration; 861,000 ± 71,000 cells/μl blood for platelets; 95.6 ± 13.0 ng/ml for plasma TNF concentration; 388 ± 30 cells/mm^2 for neutrophils per area liver; 56.8 ± 3.7 percent of injected radioactivity for hepatic platelet accumulation and 1253 ± 106.2 U/L for plasma ALT. N = 6-14 for each group. Filled circle denotes significant difference from vehicle control (i.e. 0 hr).

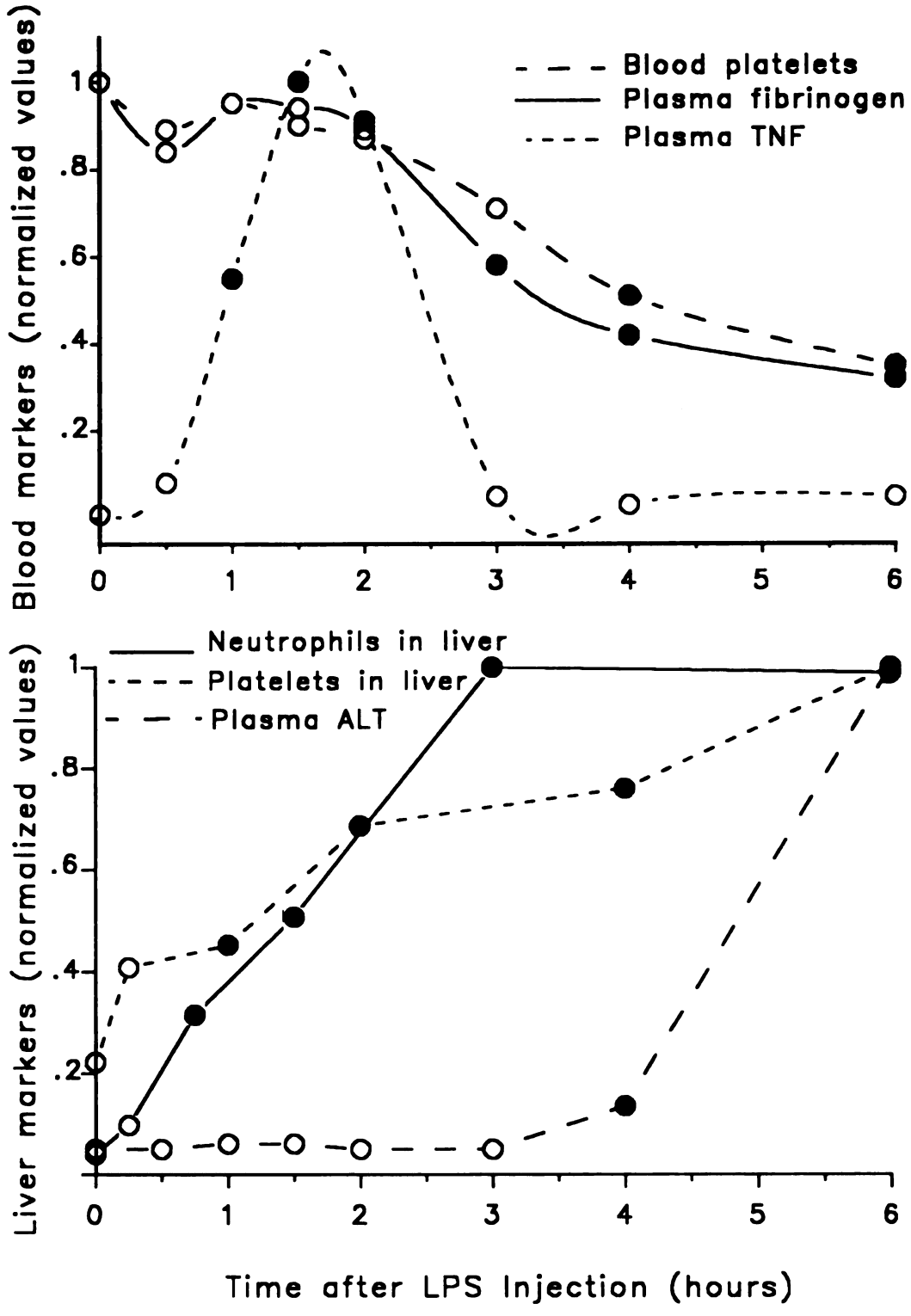

Blood platelets
Plasma fibrinogen
Plasma TNF
Blood markers (normalized values)
1
.8
.6
.4
.2
0
1
2
3
4
5
6
Neutrophils in liver
Platelets in liver
Plasma ALT
Liver markers (normalized values)
1
.8
.6
.4
.2
0
1
2
3
4
5
6
Time after LPS Injection (hours)

Figure 2.1

Figure 2.2. Effects of LPS administration on circulating white blood cells. LPS (4 mg/kg) or its SAL vehicle was administered iv at 0 hr. Cells in blood were enumerated as described in "Methods." Since values for SAL vehicle-treated rats did not change significantly with time, these were combined and expressed as vehicle control (i.e. 0 hr).

(A) Total white blood cell numbers (WBCs), neutrophils and lymphocytes.

a, total WBCs are signifcantly different from SAL vehicle (i.e., 0 hr)

b, lymphocytes are signifcantly different from SAL vehicle (i.e., 0 hr)

c, neutrophils are signifcantly different from SAL vehicle (i.e., 0 hr)

(B) Total blood neutrophils, segmented neutrophils and banded neutrophils.

a, segmented neutrophils are signifcantly different from SAL vehicle (i.e., 0 hr)

b, band neutrophils are significantly different from SAL vehicle (i.e., 0 hr)

c, total neutrophils are significantly different from SAL vehicle (i.e., 0 hr)

Results are expressed as mean ± SEM, N = 6-14.

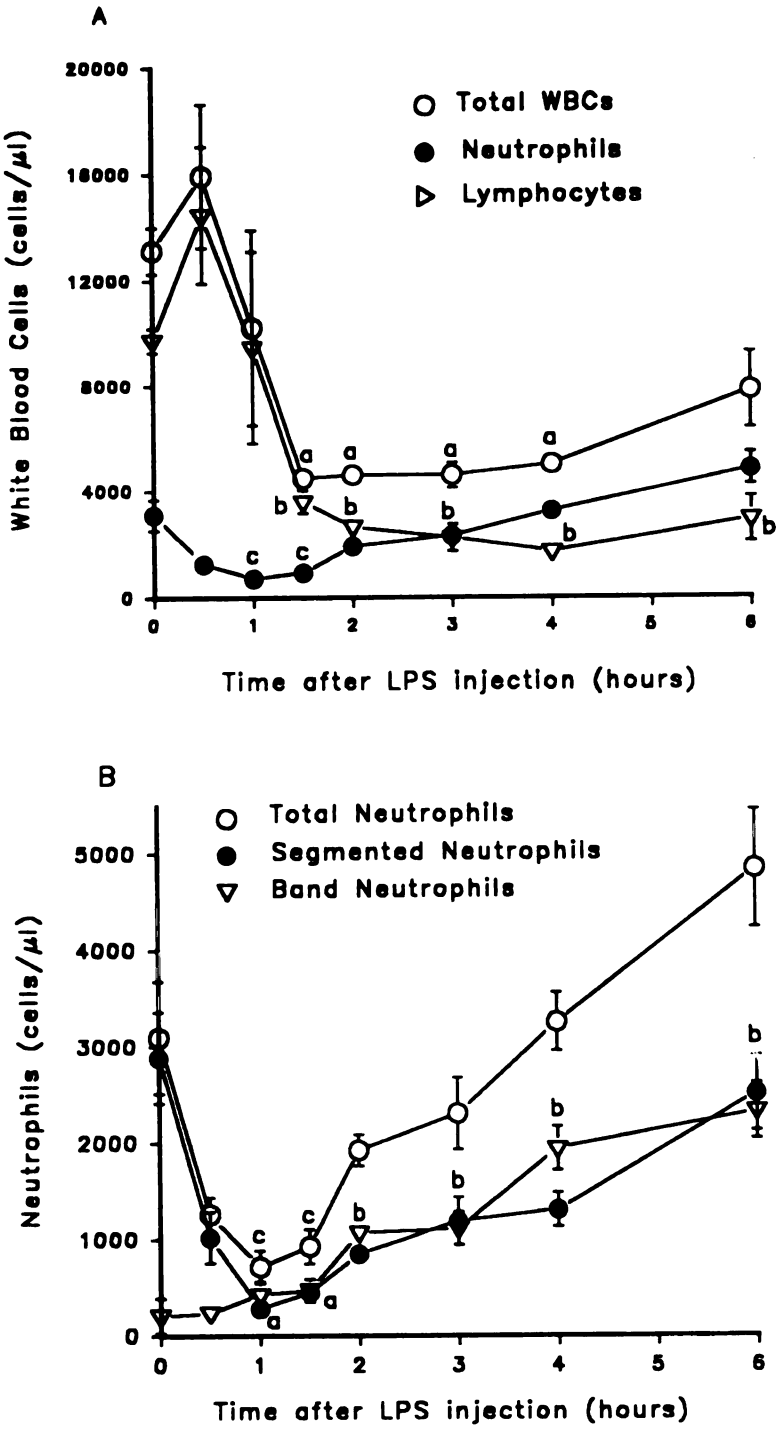

A
20000
16000
12000
8000
4000
0
White Blood Cells (cells/μl)
Total WBCs
Neutrophils
Lymphocytes
0
1
2
3
4
5
6
Time after LPS injection (hours)
B
5000
4000
3000
2000
1000
0
Neutrophils (cells/μl)
Total Neutrophils
Segmented Neutrophils
Band Neutrophils
0
1
2
3
4
5
6
Time after LPS injection (hours)

FIGURE 2.2

Figure 2.3. The effect of LPS on blood and organ platelet distribution. Platelet accumulation was measured as changes in tissue radioactivity after the administration of ^{111}indium-oxine-labeled platelets. Results are expressed as the percent of total injected radioactivity present in 1 ml blood (A), spleen (B), kidneys (C), and lungs (D), as described in "Methods." Results are expressed as mean ± SEM, N = 6-8.

a, significantly different from SAL vehicle (i.e., 0 hr).

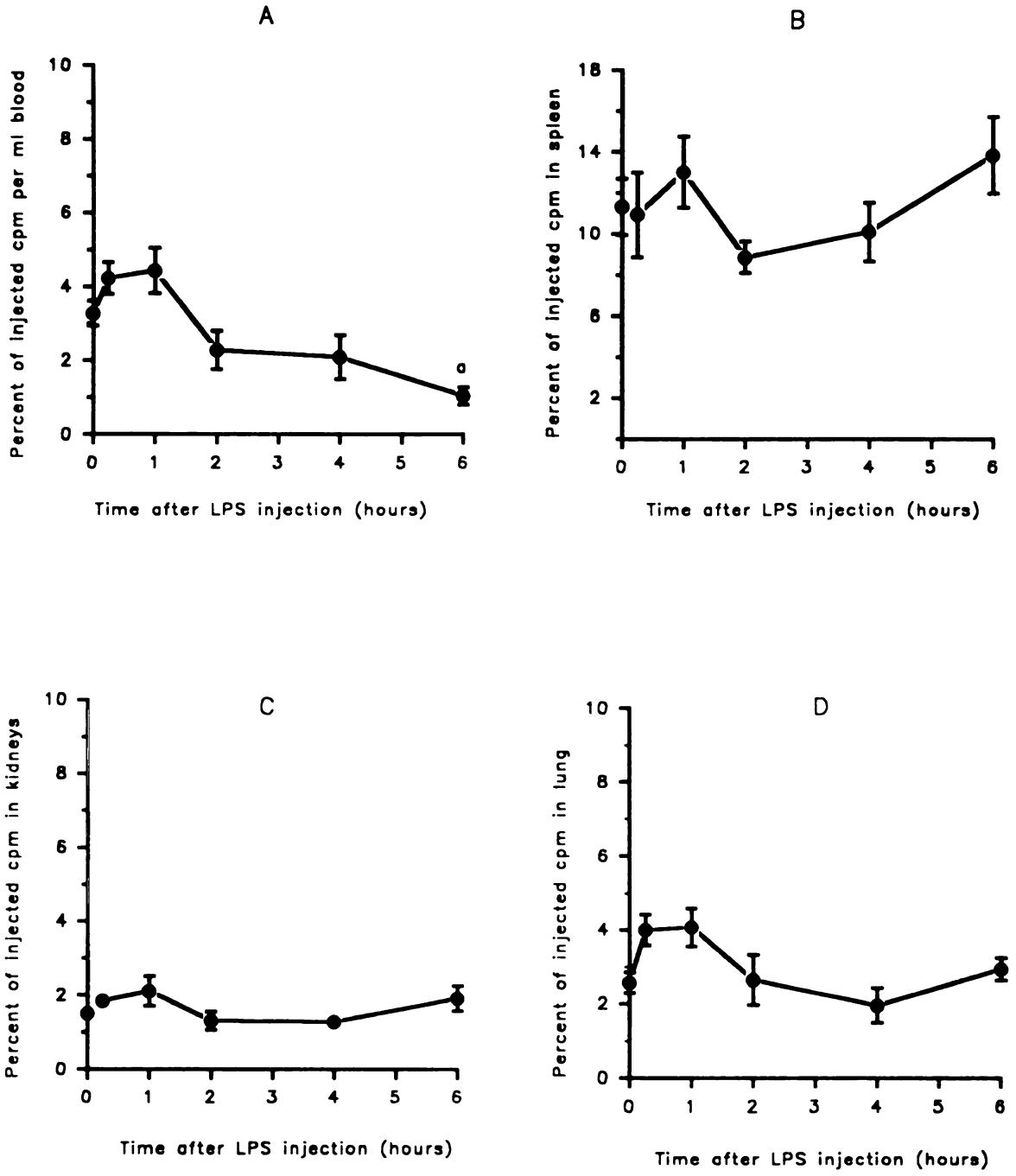

A
Percent of injected cpm per ml blood
Time after LPS injection (hours)
a
B
Percent of injected cpm in spleen
Time after LPS injection (hours)
C
Percent of injected cpm in kidneys
Time after LPS injection (hours)
D
Percent of injected cpm in lung
Time after LPS injection (hours)

FIGURE 2.3

Figure 2.4. Effects of platelet depletion and LPS on blood platelet numbers. Rats were pretreated with either control serum (CS) or anti-platelet serum (APS) 12 hours before the iv administration of LPS (4 mg/kg) or SAL vehicle. 6 hrs after the administration of LPS or SAL, blood samples were collected for platelet enumeration as described in "Methods". Results are expressed as mean ± SEM, N = 8.

a, significantly different from respective CS group.

b, significantly different from respective SAL group.

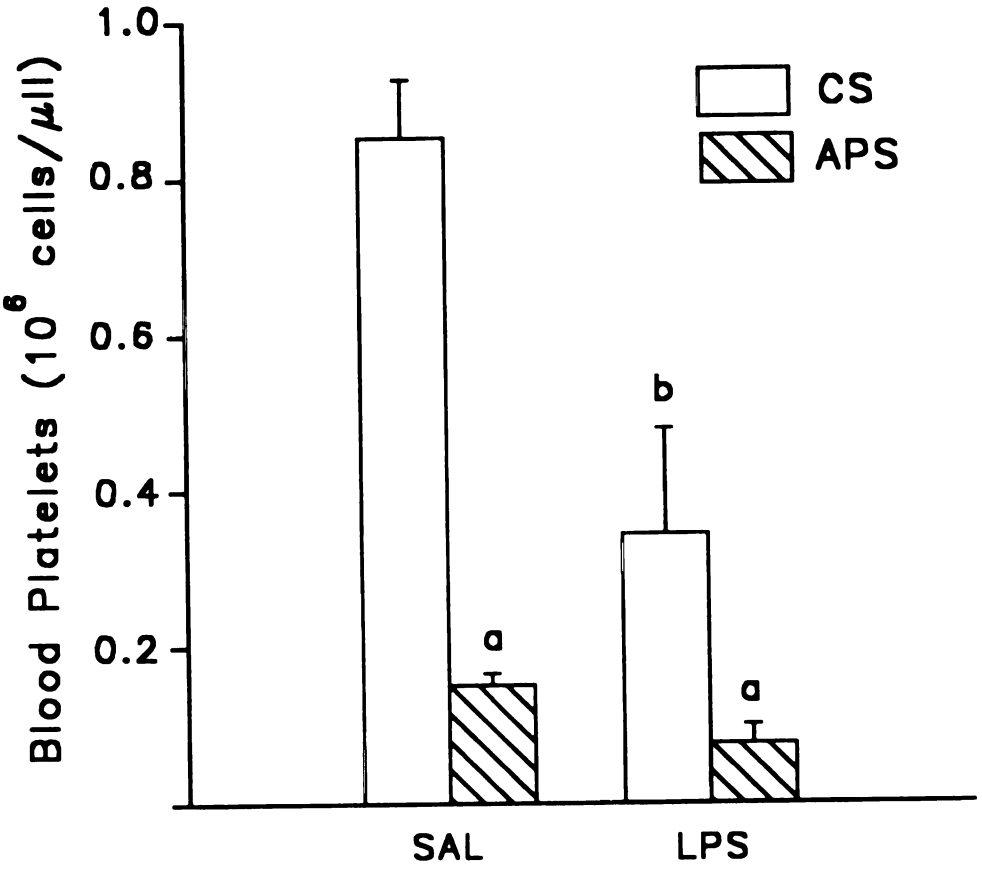


FIGURE 2.4

Figure 2.5. The effect of platelet depletion on LPS-induced liver injury. Animals were pretreated either with control serum (CS) or anti-platelet serum (APS) 12 hours before the administration of LPS (4 mg/kg, iv) or saline vehicle. 6 hrs after the administration of LPS or SAL, blood samples were collected for measurement of plasma alanine aminotransferase (ALT) activity (A) and total plasma bilirubin (B), as described in "Methods". Results are expressed as mean ± SEM, N = 8.

a, significantly different from respective SAL group.

b, significantly different from respective CS group.

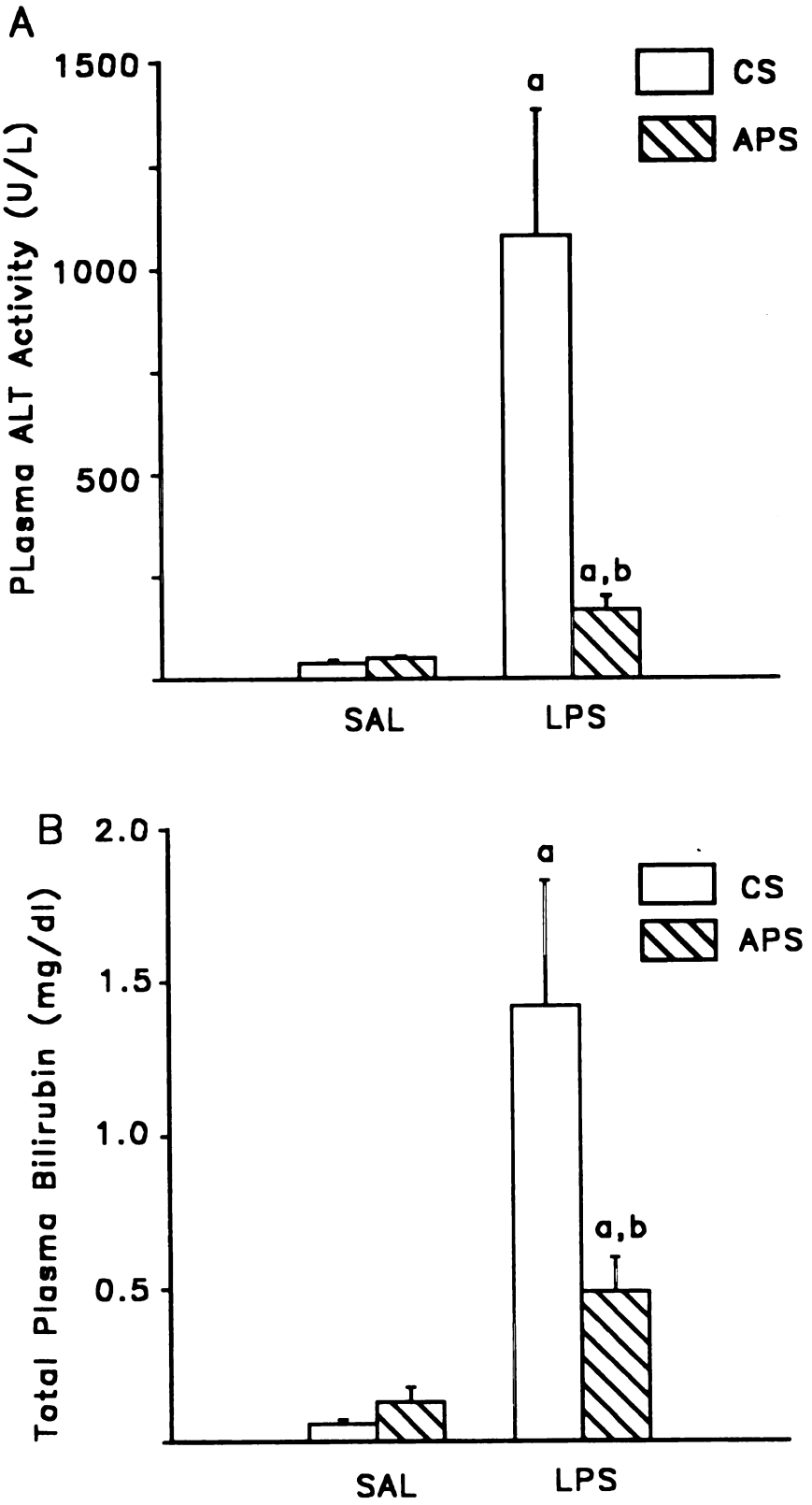
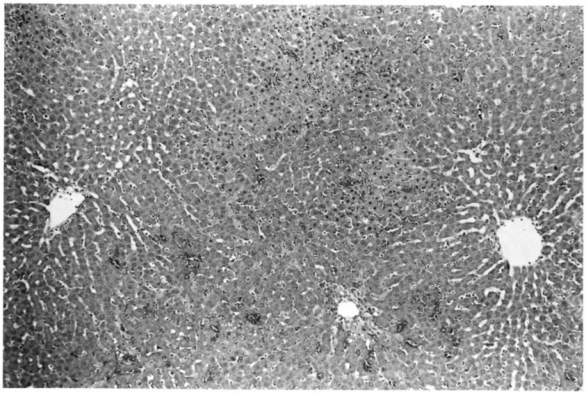

A
1500
1000
500
PLasma ALT Activity (U/L)
a
a,b
CS
APS
SAL
LPS
B
2.0
1.5
1.0
0.5
Total Plasma Bilirubin (mg/dl)
a
a,b
CS
APS
SAL
LPS

FIGURE 2.5

Figure 2.6. Photomicrographs of sections of liver from rats treated with CS/LPS (top) and APS/LPS (bottom). Top: Livers from CS/LPS treated rats had lesions were characterized by multifocal, large, irregularly shaped foci of midzonal hepatotcellular necrosis (area between arrows) with sinusoidal neutrophilia. Bottom: In the livers from animals treated with APS/LPS, the foci of midzonal hepatocellular necrosis (area between arrows) were fewer and much smaller. Sinusoidal neutrophilia was not changed by pretreatment with APS. Hematoxylin and eosin stains. 100X magnification, bar = 200 μm.



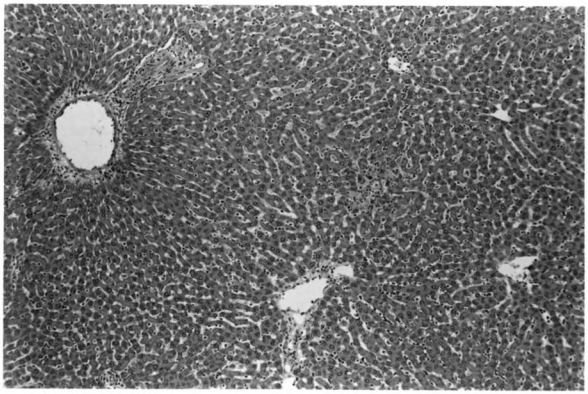


TABLE 2.1

MORPHOMETRIC ANALYSIS OF LIVERS FROM RATS TREATED WITH LPS[A]

	TREATMENT	
	CS + LPS	**APS + LPS**
% Liver Affected	6.7 ± 2.9	0.2 ± 0.1*
Lesions/Area Liver (lesions/cm^2)	67 ± 23	11 ± 4*
Mean Lesion Area (mm^2)	0.063 ± 0.018	0.023 ± 0.005*
Lesion Severity	1.80 ± 0.17	2.03 ± 0.22
Injury Index[C]	0.135 ± 0.043	0.038 ± 0.009*
Neutrophils/Lesion	17 ± 3	17 ± 3
Neutrophils/Lesion Area (cells/mm^2)	528 ± 163	980 ± 187
Neutrophils/Area Liver (cells/mm^2)	124 ± 14	109 ± 9

[A]Animals were pretreated either with control serum (CS) or anti-platelet antiserum (APS) 12 hours before the administration of LPS. 6 hr after the administration of LPS (4 mg/kg, i.v.) or saline vehicle, liver sections were collected for morphometric analysis, as described in "Methods." Results are expressed as means ± SEM, N=8-10. Results shown in this table consist only of LPS treated animals. None of the animals treated with saline vehicle had liver lesions, and all tabularized values were significantly different from their respective control.

[B]Determined as lesioned area/total area examined for each rat multiplied by 100.
[C]Determined as the product of the severity score (0-5) and lesion area for each rat.
*Significantly different from CS group.

Figure 2.7. The effect of platelet depletion on the LPS-induced change in plasma fibrinogen. Animals were pretreated with either control serum (CS) or anti-platelet antiserum (APS) 12 hours before the administration of LPS (4 mg/kg, iv) or SAL vehicle. 6 hrs after the administration of LPS or SAL vehicle, blood samples were collected for measurement of plasma fibrinogen as described in "Methods". Results are expressed as mean ± SEM, N=8.

a, significantly different from respective SAL group.

b, significantly different from respective CS group.

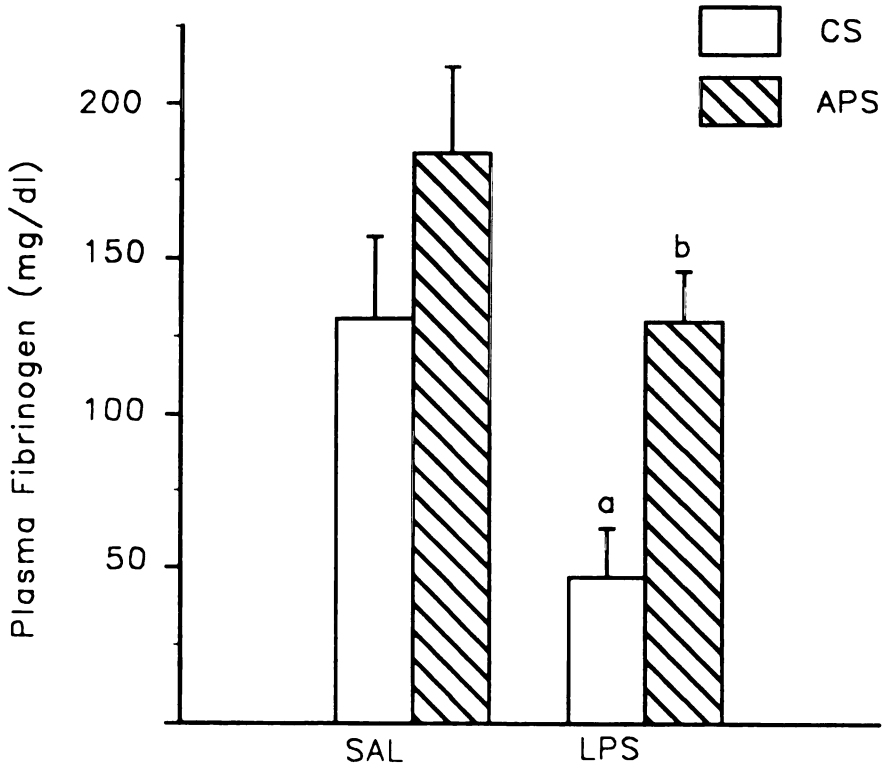


FIGURE 2.7

Figure 2.8. The effect of platelet depletion on LPS-induced elevation in plasma TNF-α activity. Animals were pretreated with either control serum (CS) or anti-platelet antiserum (APS) 12 hours before the administration of LPS (4 mg/kg, iv) or SAL vehicle. 1.5 hrs after the administration of LPS or SAL, blood samples were collected for measurement of plasma TNF-α activity, as described in "Methods". Results are expressed as mean ± SEM, N = 3-8.

N.D. (not detectable), values less than 1 ng/ml.

a, significantly different from respective SAL group.

b, significantly different from respective CS group.

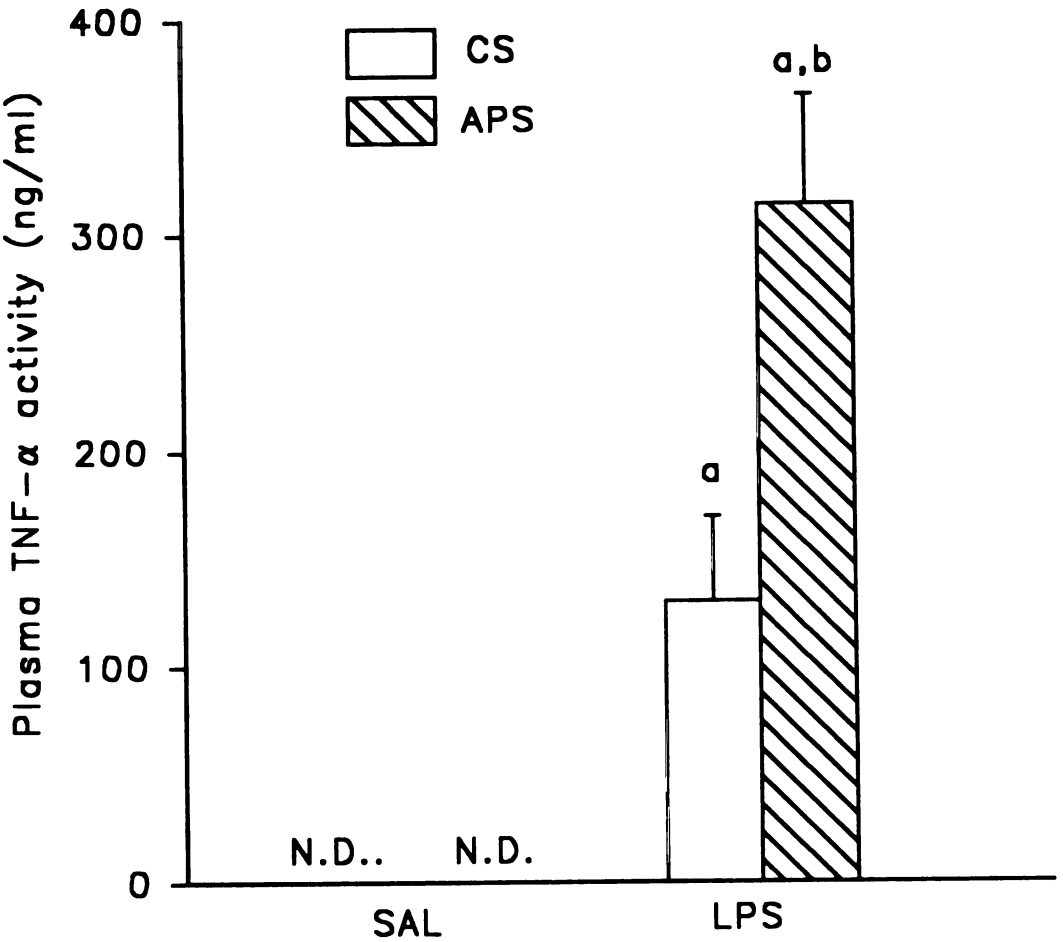

400
300
200
100
0
Plasma TNF-α activity (ng/ml)
CS
APS
a,b
a
N.D..
N.D.
SAL
LPS

FIGURE 2.8

Figure 2.9. The effect of platelet depletion on LPS-induced alterations in white blood cell numbers. Animals were pretreated with either control serum (CS) or anti-platelet antiserum (APS) 12 hours before the administration of LPS (4 mg/kg, iv) or SAL vehicle. 6 hrs after the administration of LPS or SAL, blood samples were collected for measurement of total white cells (TOTAL WBC), neutrophils and lymphocytes as described in "Methods". Results are expressed as mean ± SEM, N = 8.

a, significantly different from respective SAL group.

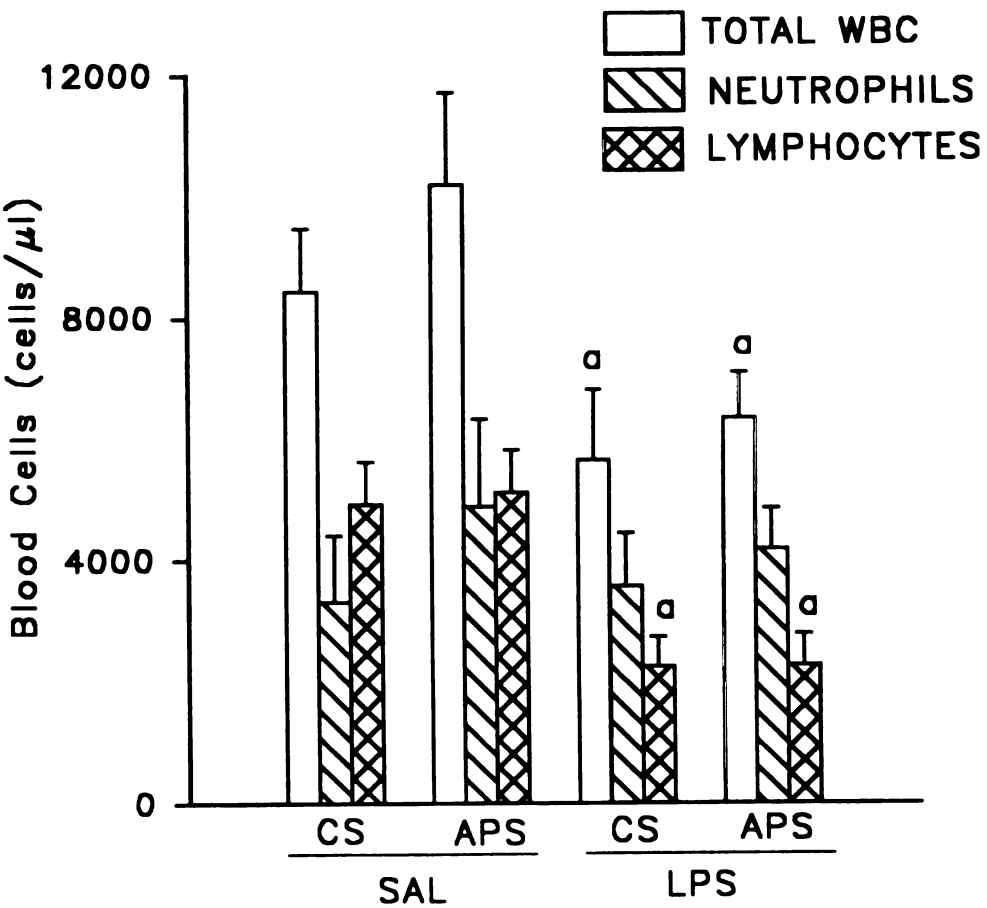

TOTAL WBC
NEUTROPHILS
LYMPHOCYTES
12000
8000
4000
0
Blood Cells (cells/μl)
a
a
a
a
CS
APS
CS
APS
SAL
LPS

FIGURE 2.9

2. E. Discussion

Evidence from this study suggests that platelets are involved in the manifestation of LPS-induced liver injury. We found that the numbers of blood platelets decreased prior to the onset of liver injury, confirming observations of others (Shibayama *et al.*, 1995). Secondly, radiolabeled-platelets accumulated in the liver prior to the development of liver injury (Figure 2.1). Finally, platelet depletion attenuated LPS-induced liver injury, as measured by plasma ALT activity, bilirubin concentration and morphometric analysis of liver sections, which revealed a pronounced reduction in the size and number of lesions in livers of LPS-treated animals (Figure 2.6, Table 2.1).

Administration of polyclonal or monoclonal antibodies against platelets has been used to explore the role of these cells in several other LPS-induced pathophysiologic alterations (Ito *et al.*, 1990; Kramer *et al.*, 1977; Piguet *et al.*, 1993) supporting the usefulness of this method *in vivo*. We used a polyclonal, anti-rat platelet antiserum (APS) to induce thrombocytopenia prior to LPS administration. The APS was selective for platelets, since the numbers of no other circulating blood cells were altered (Figure 2.9). In addition, the APS alone had no apparent affect on the liver as assessed by morphometric analysis and ALT activity and bilirubin concentration in plasma.

The use of a polyclonal antiserum to deplete platelets raises the possibility that development of antibody-antigen complexes and consequent complement activation may have contributed to the hepatoprotective effects of the APS.

Previous studies have shown that the administration of an antibody to deplete platelets results in clearance of the platelets by the Kupffer and endothelial cells of the liver. The possibility exists that Kupffer cell function may be altered by these phagocytosed platelets. Inasmuch as Kupffer cells have been shown to mediate LPS-induced hepatotoxicity (Iimuro *et al.*, 1994), alteration of Kupffer cell function with platelet-antibody complexes might contribute to the hepatoprotective effects of the APS. However, the use of a polyclonal anti-lymphocyte serum to reduce blood lymphocyte numbers was not protective in this model (Hewett *et al.*, 1992), which supports the contention that the APS afforded protection by depleting platelets rather than by the generation of immune complexes. In addition, it appears that such immune complexes may have actually stimulated rather than inhibited Kupffer cells since plasma TNF-α activity is enhanced in platelet depleted animals given LPS (Figure 2.8). This finding is similar to previous results in which an antibody against neutrophils resulted in elevated plasma TNF-α activity after LPS administration compared to animals treated with LPS and control serum (Hewett *et al.*, 1992; Hewett *et al.*, 1993). TNF-α has been implicated as a requirement for liver injury in this model (Hewett *et al.*, 1993). The finding that depletion of neutrophils and platelets resulted in enhanced plasma TNF-α concentration in LPS-treated rats yet prevented hepatic injury suggests that the critical action of TNF-α in liver injury depends upon the presence of neutrophils and platelets.

Support for the hypothesis that platelets contribute to LPS-induced liver injury was provided by studies in which ^{111}In-oxine-labeled platelets were used

to assess hepatic platelet accumulation. Platelets accumulated in the liver prior to the onset of LPS-induced liver injury. Radiolabeled platelets have been widely used to measure platelet deposition in tissues. The radiolabeling procedure does not alter platelet function (Sostman *et al.*,1983; White *et al.*,1989). The time course of hepatic platelet deposition in this study was similar to that reported by others using serotonin (5-HT) as a marker of hepatic platelet accumulation after LPS administration (Endo *et al.*,1993). We considered the possiblity that the increase in hepatic radioactivity was due to free ^{111}Indium that dissociated from platelets and was cleared into the bile. This is unlikely, since bile from either SAL- or LPS-treated rats contained very little radioactivity (data not shown). In addition, plasma samples had little radioactivity, whereas blood samples contained considerable radioactivity, suggesting that the ^{111}In remains associated with the platelets. Selective clearance of the radiolabeled, but not unlabeled platelets by the liver might have contributed to the increased hepatic radioactivity in LPS-treated rats. However, preliminary studies indicated that radiolabeled platelets in control rats were cleared equally from both the spleen and liver, and the kinetics of this clearance corresponded to the circulating half-life of rat platelets (data not shown, (White *et al.*,1989)). Moreover, LPS administration resulted in an increase only in hepatic radioactivity, without any significant alterations in splenic radioactivity. The increase in hepatic ^{111}In was not a result of pooling of blood in liver tissue since liver weights were not significantly elevated after LPS administration as compared to SAL-treated animals (data not shown). Thus, LPS administration

appears to cause selective accumulation of platelets in liver. This finding is consistent with and may explain the correlation between the severity of LPS-induced thrombocytopenia and liver injury (Shibayama *et al.*, 1995). The stimulus for this platelet accumulation and the basis for the liver selectivity remain unclear.

The hepatic platelet accumulation and thrombocytopenia suggest that platelets are activated during LPS exposure. The mechanisms of platelet activation in this model are unknown, although in other species LPS may activate platelets by direct or indirect mechanisms. Evidence from this study suggests that platelets are not directly activated by LPS *in vivo* since blood platelets did not decrease until nearly 3 hrs after LPS administration and the hepatic platelet accumulation was not significant until 1 hr after LPS administration and continued through at least 5 hours thereafter (Figure 2.1). Accordingly, available evidence suggests that accumulation and activation of platelets within the livers of LPS-treated rats occur by indirect mechanisms. For example, injury to endothelial cells (Harlan *et al.*, 1983b; Hewett *et al.*, 1992) and consequent exposure of the subendothelium might activate the coagulation cascade and prompt platelet adherence and aggregation.

The findings that either neutrophil depletion (Hewett *et al.*, 1992) or platelet depletion provides protection from LPS-induced hepatotoxicity indicates that neither platelets nor neutrophils are sufficient to produce injury by themselves. It may be that either cooperation between these two cell types or the sequential activation of them is required for the expression of liver injury.

In this study, both platelets and neutrophils were present in the liver prior to the onset of liver injury. Interestingly, platelet depletion attenuated liver injury but did not alter the numbers of neutrophils within the sinusoids. Thus, although LPS-induced liver injury is neutrophil dependent, the presence of neutrophils within the liver is not sufficient to produce liver injury in the absence of platelets, suggesting that platelets interact with neutrophils in the pathogenesis. In this model, neutrophils are present throughout the entire liver, not solely within necrotic foci. It would be of interest to know whether platelets are associated generally with neutrophils in the livers of LPS-treated rats or whether they tend to associate preferentially with neutrophils in areas undergoing hepatocellular necrosis.

It is possible that platelets are activated indirectly in this model by an effect of LPS on neutrophils. LPS as well as soluble mediators released from cells during LPS exposure can stimulate neutrophils to release proteases such as cathepsin G. This protein causes platelet aggregation, calcium mobilization and the release of thromboxane A_2 and serotonin *in vitro* (del Maschio *et al.*, 1990; Ferrer-Lopez *et al.*, 1990; Selak *et al.*, 1988). Furthermore, priming of neutrophils with TNF-α increases neutrophil-induced platelet aggregation by increasing the amount of cathepsin G released from neutrophils (Renesto *et al.*, 1991). Although these studies have been conducted *in vitro*, it is possible that similar events occur *in vivo*. Not only do neutrophils and platelets accumulate in the livers of LPS-treated rats, but significant amounts of TNF-α are present in the plasma prior to the onset of liver injury (Figure 2.1).

Accordingly, the release of cathepsin G may be a mechanism by which platelets, neutrophils and TNF-α interact in the genesis of liver injury after LPS exposure.

Platelets play an important role in thrombosis and hemostasis, and LPS may influence platelets by activating the coagulation system. LPS activates both the intrinsic and extrinsic pathways of coagulation *in vitro*. Platelets can also activate the coagulation system, and it is possible that such an interaction is involved in the hepatocellular injury that occurs during LPS exposure. In this study, thrombocytopenia and hypofibrinogenemia occurred between 2-3 hours after LPS injection (Figure 2.1), suggesting a consumptive coagulopathy. Results of a previous study suggest a role for thrombin in the genesis of LPS-induced liver injury and that its critical action is independent of its ability to form insoluble fibrin clots (Hewett *et al.*, 1995). Thrombin can activate platelets and may provide the link between platelets and coagulation in this model. In this study, platelet depletion prevented LPS-induced activation of the coagulation system as well as liver injury. This suggests that platelets contribute to liver injury through a mechanism that involves activation of the coagulation system and resultant formation of thrombin.

Activated platelets can release many inflammatory mediators, including serotonin, platelet activating factor (PAF) and thromboxane A_2. As mentioned previously, liver serotonin increases after LPS administration (Endo *et al.*, 1993). PAF promotes neutrophil chemotaxis, aggregation, granule secretion, superoxide production and the release of arachidonic acid metabolites

(Montrucchio *et al.*, 1993; Hosford *et al.*, 1993). Treatment of rats with a PAF receptor antagonist affords protection from LPS-induced liver injury (Imura *et al.*, 1986). Thromboxane A_2 may contribute to the development of liver injury, since plasma thromboxane is elevated after LPS administration and drugs which inhibit the synthesis or action of thromboxane A_2 protect rats from LPS-induced liver injury (Wise *et al.*, 1980). Thus, platelets may contribute to LPS-induced liver injury by releasing one or more of these chemical mediators.

In summary, platelets and neutrophils accumulate within the liver prior to the onset of liver injury. Platelets apparently contribute to LPS-induced liver injury, inasmuch as platelet depletion attenuates hepatocellular necrosis. Neutrophils and TNF-α are critical mediators of liver pathogenesis during LPS exposure, however, platelets mediate neither the increase in plasma TNF-α concentration nor the hepatic accumulation of neutrophils. It is possible that platelets contribute to neutrophil activation. They play an important role in activation of the coagulation system since platelet depletion prevents LPS-induced hypofibrinogenemia. A complete understanding of the critical role of platelets in LPS-induced liver injury is likely to require elucidation of their interactions with other cellular and soluble inflammatory mediators.

CHAPTER 3

INTERDEPENDENCE AMONG NEUTROPHILS, KUPFFER CELLS AND PLATELETS DURING LPS EXPOSURE

3. A. Abstract

Exposure to lipopolysaccharide (LPS) from gram-negative bacteria leads to an array of pathophysiologic changes, including disseminated intravascular coagulation, multiple organ failure and liver injury. The development of liver injury entails contributions from both cellular and soluble mediators, including neutrophils, platelets, Kupffer cells and components of the coagulation system. Much remains unknown about the interactions among these mediators in the pathogenesis of liver injury *in vivo*. Accordingly, studies were conducted in neutrophil-depleted animals to evaluate the role of neutrophils in LPS-induced thrombocytopenia and hepatic platelet accumulation. As shown previously, neutrophil depletion with an anti-neutrophil immunoglobulin attenuated liver injury, as marked by elevations in plasma alanine aminotransferase activity. In addition, neutrophil depletion the decrease in blood platelet numbers but did not inhibit the hepatic platelet accumulation observed during LPS exposure. To evaluate the role of Kupffer cells in these LPS-mediated events, studies were conducted with gadolinium chloride, an agent that inhibits Kupffer cell function and prevents LPS-induced liver injury. Female, Sprague-Dawley rats were pretreated with $GdCl_3$-$6H_2O$ (10 mg/kg, iv) or saline vehicle 24 hrs prior to the administration of LPS (4 mg/kg, iv) or saline vehicle. They were then treated with ^{111}indium-labeled platelets 2 hrs before LPS administration to quantify platelet deposition in various tissues. As shown previously, pretreatment with $GdCl_3$ attenuated liver injury. Pretreatment with $GdCl_3$ attenuated

thrombocytopenia and hepatic platelet accumulation observed after LPS exposure, but it did not alter the activation of the coagulation system, as marked by a decrease in plasma fibrinogen concentration. These results suggest that Kupffer cels but not neutrophils contribute to the accumulation of platelets in the liver after LPS administration and raise the possibility that protection against LPS-induced hepatic injury by Kupffer cell inactivation may be due to decreased deposition of platelets within the liver. Results from these studies also suggest that the presence of platelets within the liver is insufficient to result in liver injury and suggest that an interdependence between neutrophils and platelets may be required for the manifestation of LPS-induced liver injury in the rat.

3. B. Introduction

Neutrophils and platelets accumulate in the liver within 1 hour after LPS administration. Neutrophils can stimulate platelet aggregation by a mechanism involving the release of cathepsin G. This lysosomal protease stimulates platelet aggregation and the subsequent release of serotonin and TxA_2 (Evangelista *et al.*, 1992). Cathepsin G also stimulates the expression of platelet glycoprotein IIb/IIIa, an action that would promote platelet aggregation (LaRosa *et al.*, 1994). Thereby, neutrophils might mediate LPS-induced hepatic platelet accumulation and thrombocytopenia.

Kupffer cells, the resident macrophages of the liver, rapidly remove LPS from the circulation and release numerous inflammatory mediators in response to LPS exposure. Gadolinium chloride ($GdCl_3$), an agent that inhibits Kupffer cell phagocytosis, protects rats from LPS-induced liver injury (Iimuro *et al.*,1994; Brown *et al.*, 1995). The specific role of Kupffer cells in the development of liver pathogenesis is unclear, but it is possible that Kupffer cells interact with other critical inflammatory cells such as platelets during the development of liver injury.

Electron micrographs of murine livers after LPS exposure have revealed platelets in the liver sinusoids and the spaces of Disse and within Kupffer cells (Endo *et al.*,1993), but the mechanisms by which platelets accumulate in the liver after LPS administration have not been elucidated. LPS-stimulated Kupffer cells release numerous inflammatory mediators such as thromboxane A_2, which is a potent activator of platelets that might contribute to the deposition of these cells (Decker,1990; Cook *et al.*,1981; Rodriguez de Turco *et al.*,1990). Moreover, the reticuloendothelial system of the liver and spleen might contribute to the removal of platelets from the blood as it does in other pathophysiologic conditions such as immune thrombocytopenia purpura (du P Heyns *et al.*,1986). Taken together, these findings raise the possibility that stimulated Kupffer cells contribute to the decrease in blood platelets and the accumulation of platelets within the liver after LPS administration.

To evaluate potential interactions of neutrophils and Kupffer cells with platelets or the coagulation system, separate studies were conducted using

either neutrophil-depleted or Kupffer cell-inactivated animals. Studies were conducted to evaluate the effects of neutrophil depletion or Kupffer cell inhibition on thrombocytopenia, activation of the coagulation system and sequestration of platelets in liver after LPS administration to rats.

3. C. Materials and methods

3. C. 1.

See Chapter 2, section C.1.

3. C. 2. Animals

See Chapter 2, section C.2.

3. C. 3. Platelet distribution in tissues

See Chapter 2, section C. 6.

3. C. 4. Preparation of anti-neutrophil immunoglobulin

Rabbits were immunized against rat neutrophils by injecting neutrophils suspended in Freund's complete adjuvent into the foot pads, as described

previously (Hewett *et al.*, 1992). This was followed by intramuscular booster injections of neutrophils. One week after the second neutrophil booster injection, blood was drawn from the central ear artery and allowed to clot. The total immunoglobulin (Ig) fraction of serum from rabbits was isolated by ammonium sulfate precipitation (Hewett *et al.*, 1992) and stored at -20^0 C prior to use. Total Ig from untreated rabbits was isolated in a similar manner and used as a control (control Ig).

3. C. 5. Neutrophil depletion studies

Rats were treated with anti-PMN Ig or control Ig (1 ml, iv) 18 and 6 hours before the administration of LPS. This procedure has been used previously and the administration of anti-PMN Ig reduces blood neutrophils numbers to $<5\%$ of those from rats treated with control Ig (Hewett *et al.*, 1992). The numbers of red blood cells, other leukocytes and platelets are not significantly altered by the administration of the anti-PMN Ig. Two hours before LPS (4 mg/kg, iv) or saline vehicle administration, animals were treated with ^{111}In-labeled platelets, as described above. Six hours after the administration of LPS, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn from the descending aorta into sodium citrate (0.38% final concentration). The liver, spleen, kidneys and lungs were excised for determination of radioactivity (see above). Blood platelet concentration was determined in a hemocytometer after erythrocyte lysis and dilution in platelet Unopettes. Total white blood cell counts were performed in

a Coulter Counter Model ZM. Differential counts were performed on blood smears stained with buffered, differential Wright-Giemsa stain to obtain the fraction of neutrophils and lymphocytes in each blood sample. The total number of white blood cells was multiplied by this fraction to obtain numbers of neutrophils (cells/μl blood). Plasma samples were assayed for ALT activity.

3. C. 6. Gadolinium chloride studies

Rats were pretreated with $GdCl_3$-$6H_2O$ (10 mg/kg, iv) or saline vehicle (2 ml/kg) via the tail vein 24 hrs before LPS administration. This treatment regimen inhibits Kupffer cell phagocytosis as measured by clearance of colloidal carbon from blood (Iimuro *et al.*, 1994; Brown *et al.*, 1995). Two hours before LPS (4 mg/kg, iv) or saline vehicle administration, animals were treated with ^{111}In-labeled platelets, as described above. Six hours after the administration of LPS, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn from the descending aorta into sodium citrate (0.38% final concentration). The liver, spleen, kidneys and lungs were excised for determination of radioactivity (see above). Blood platelet concentrations were determined in a hemocytometer after erythrocyte lysis and dilution in platelet Unopettes. Plasma samples were assayed for plasma fibrinogen concentration in a BBL Fibrometer (Becton, Dickinson and Company, Hunt Valley, MD) and for ALT activity.

3. C. 7. Data analysis

A 2 X 2 multifactorial, completely random analysis of variance (ANOVA) was used to evaluate all data. Data with nonhomogeneous variances were square root- or log-transformed prior to analysis. Comparisons between groups were performed with Tukey's omega test (Steel and Torrie, 1980). The criterion for significance for all studies was $p \leq 0.05$. Results are expressed as mean ± standard error of the mean (SEM). For all results presented, N represents the number of individual animals used in the study.

3. D. Results

3. D. 1. Neutrophil depletion studies

To address the contribution of neutrophils to hepatotoxicity, thrombocytopenia and hepatic platelet accumulation caused by LPS exposure, blood neutrophil concentration was reduced by the administration of anti-neutrophil Ig. The administration of LPS resulted in a significant decrease in blood neutrophils (Figure 3.1). As shown previously (Hewett *et al.*, 1992), anti-neutrophil Ig markedly reduced the numbers of neutrophils in blood. Consistent with previous results (Hewett *et al.*, 1992; Jaeschke *et al.*, 1991b), the reduction in blood neutrophil numbers was associated with an attenuation of LPS-induced liver injury, as marked by elevated ALT activity (Figure 3.2).

model, we evaluated the effect of neutrophil depletion on LPS-induced hepatic platelet accumulation and thrombocytopenia. The administration of anti-neutrophil Ig increased the accumulation of radiolabeled platelets within the liver in saline-treated animals (Figure 3.3). The administration of LPS increased hepatic platelet accumulation, and this effect was enhanced in neutrophil-depleted animals (Figure 3.3). The administration of LPS produced a significant reduction in blood platelet concentration (Figure 3.4), a finding consistent with previous studies showing that LPS administration results in thrombocytopenia (Pearson *et al.*, 1995). Neutrophil-depletion with anti-PMN Ig resulted in a slightly less pronounced decrease in total blood platelet concentration after LPS administration (Figure 3.4). Consistent with previous studies, LPS exposure did not alter the distribution of platelets in the lungs, kidneys or spleen. Similar to results with blood platelet concentration, it did not reduce blood radioactivity (Table 3.1). Neutrophil depletion did not alter radiolabeled plateletss in blood and other organs.

3. D. 2. $GdCl_3$ Studies

Six hours after the administration of LPS, liver injury was evaluated. Plasma ALT activity was elevated after the administration of LPS (Figure 3.5), and this was consistent with histologic findings (data not shown). As reported previously (Iimuro *et al.*, 1994; Brown *et al.*, 1995), pretreatment with $GdCl_3$ significantly reduced this LPS-induced liver injury.

The administration of LPS resulted in a significant accumulation of ^{111}In-

radiolabeled platelets within the liver, as measured by increased hepatic radioactivity (Figure 3.6). Pretreatment with $GdCl_3$ attenuated this LPS-induced hepatic platelet accumulation by 46%. Consistent with previous studies (Pearson *et al.* 1995), LPS administration did not alter the distribution of radiolabeled platelets in the lungs, spleen and kidneys (Table 3.1). However, the administration of LPS to animals pretreated with $GdCl_3$ resulted in a significant increase in radiolabeled platelets in the lungs of rats treated with LPS.

LPS exposure produced a significant reduction in both radiolabeled platelets in blood (Figure 3.7) and blood total platelet concentration (Figure 3.8), a finding consistent with previous studies showing that LPS administration results in thrombocytopenia (Pearson *et al.*, 1995). Pretreatment with $GdCl_3$ resulted in a less pronounced decrease in radiolabeled platelets in blood after LPS administration (Figure 3.7), but it did not significantly alter blood total platelet concentration after LPS exposure (Figure 3.8).

LPS administration resulted in activation of the coagulation system, as demonstrated by a decrease in plasma fibrinogen concentration (Figure 3.9). This decrease was not affected by pretreatment with $GdCl_3$.

Figure 3.1. The effects of anti-neutrophil Ig and LPS on blood neutrophils. Animals were treated with control Ig or anti-neutrophil Ig 18 and 6 hours before the administration of LPS. Six hours after the administration of LPS (4 mg/kg, iv) or its saline (SAL) vehicle, blood samples were collected for the enumeration of total white blood cell counts and differential counts as described in Methods. Results are expressed as mean ± SEM, N = 4-7 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of anti-neutrophil Ig

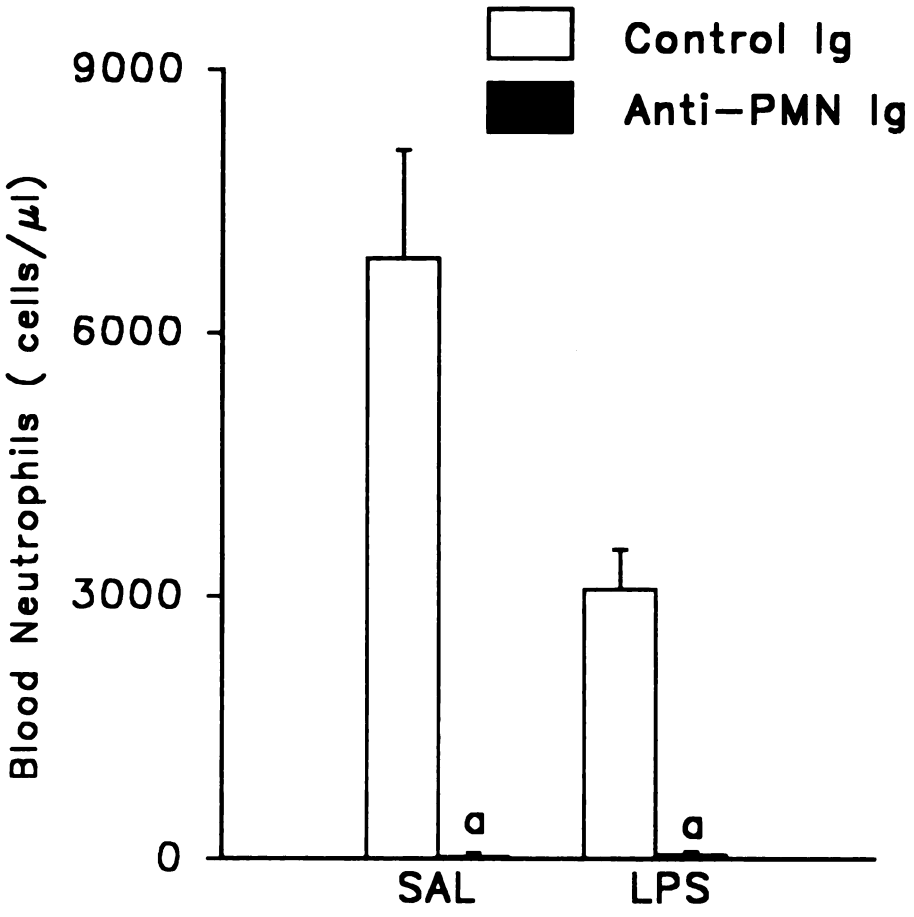

Control Ig
Anti-PMN Ig
9000
6000
3000
0
Blood Neutrophils (cells/μl)
a
a
SAL
LPS

Figure 3.1

Figure 3.2. The effects of neutrophil depletion and LPS on plasma ALT activity. Animals were treated with anti-neutrophil Ig or control Ig (1ml, iv) 18 and 6 hours before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 4-7 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of anti-neutrophil Ig

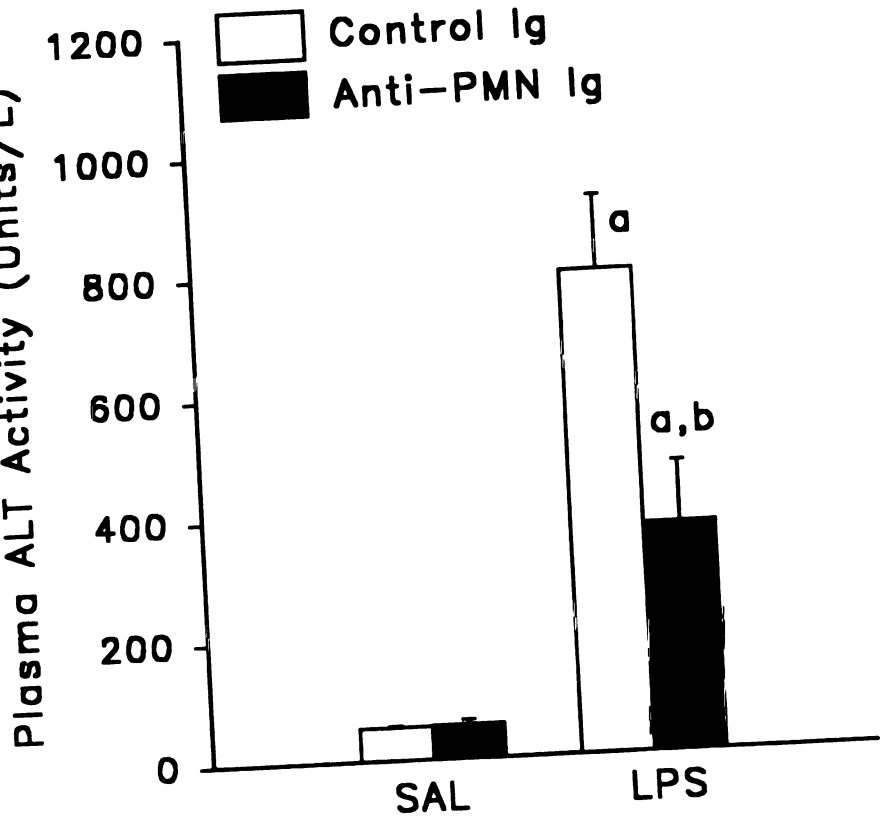

Control Ig
Anti-PMN Ig
Plasma ALT Activity (Units/L)
1200
1000
800
600
400
200
0
a
a,b
SAL
LPS

Figure 3.2

Figure 3.3. The effects of neutrophil depletion and LPS on hepatic platelet sequestration. All animals were treated with ^{111}In-labeled platelets (iv) 2 hrs before the administration of LPS. Animals were treated with anti-neutrophil Ig or control Ig (1 ml, iv) 18 and 6 hours before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, liver sections were collected for measurement of radioactivity in a gamma counter as described in Methods. Results are expressed as mean ± SEM, N=4-7 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of anti-neutrophil Ig

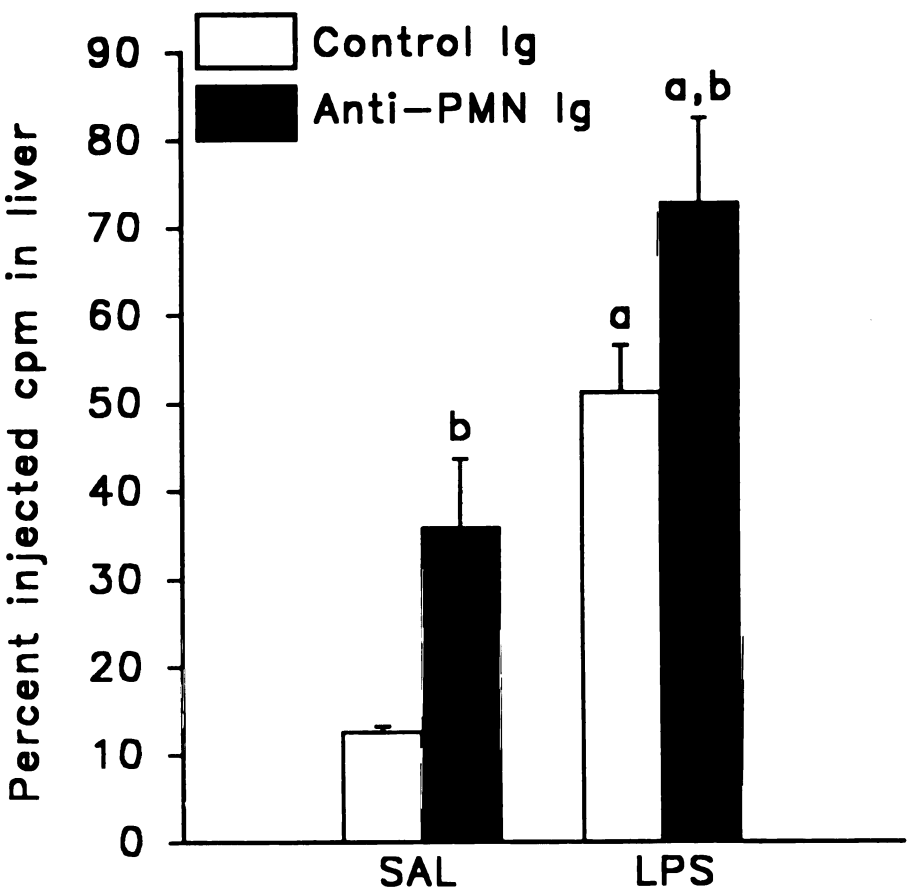

Control Ig
Anti-PMN Ig
Percent injected cpm in liver
90
80
70
60
50
40
30
20
10
0
a,b
a
b
SAL
LPS

Figure 3.3

Figure 3.4. The effects of neutrophil depletion and LPS on blood platelet concentration. Animals were treated with anti-neutrophil Ig or control Ig (1 ml, iv) 18 and 6 hours before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for enumeration of blood platelets as described in Methods. Results are expressed as mean ± SEM, N = 4-7 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of anti-neutrophil Ig

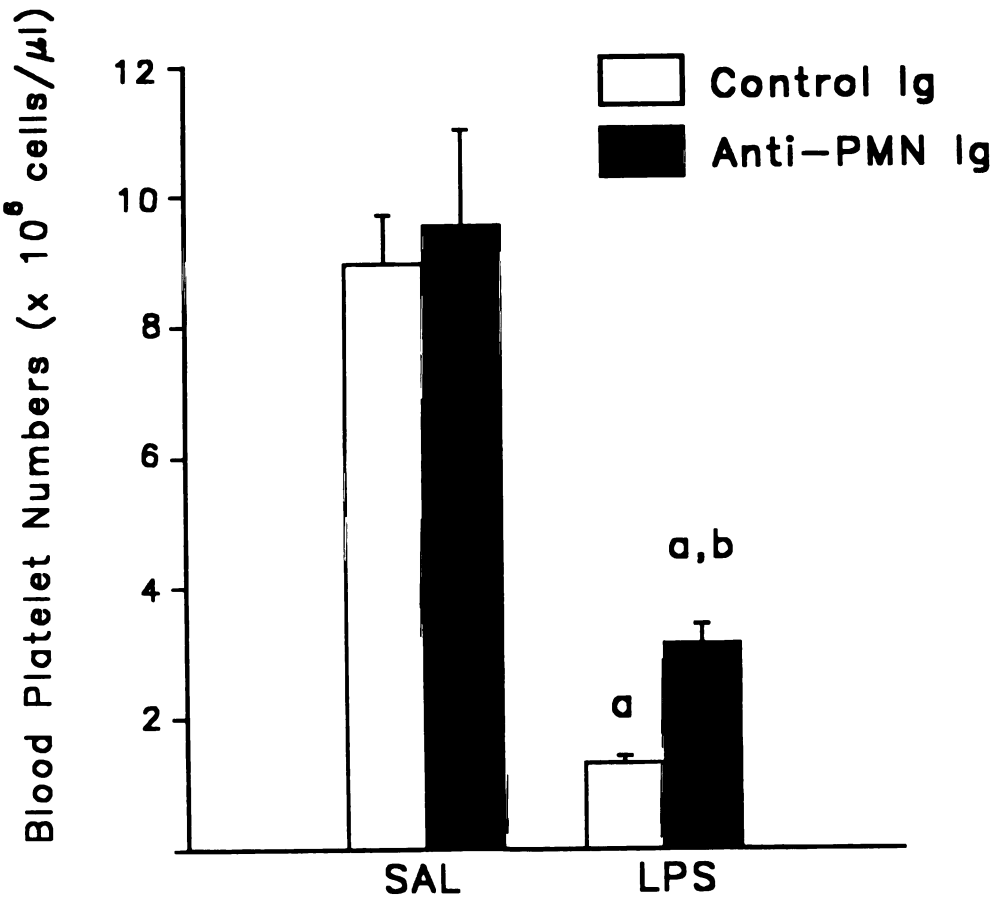

Blood Platelet Numbers (x 10⁶ cells/μl)
12
10
8
6
4
2
Control Ig
Anti-PMN Ig
a,b
a
SAL
LPS

FIGURE 3.4

TABLE 3.1

Effects of Neutrophil Depletion and LPS on Platelet Distribution in Blood, Spleen, Kidneys and Lungs

TISSUE	Con+SAL	PMN+SAL	Con+LPS	PMN+LPS
BLOOD	4.9±0.2	4.2±0.3	1.0±0.1*	1.3±0.1*
SPLEEN	18.5±1.4	22.9±1.9	12.8±2.7	15.9±2.2
KIDNEY	1.7±0.2	1.7±0.2	2.4±0.3	2.3±0.2
LUNGS	3.2±0.2	3.5±0.4	3.0±0.5	2.3±0.4

All animals were treated with ^{111}In-labeled platelets (iv) 2 hrs before the administration of LPS. Animals were treated with anti-neutrophil Ig (PMN) or control Ig (Con)18 and 6 hours before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood and organs were collected for measurement of radioactivity in a gamma counter as described in Methods. Results are expressed as percent of injected radioactivity in 1 ml blood or entire organ (mean ± SEM), N=4-7 per group.

*significantly different from respective SAL group

Figure 3.5. The effects of LPS and $GdCl_3$ on plasma ALT activity. Animals were treated with $GdCl_3$ (10 mg/kg, iv) or its saline vehicle (SAL) 24 hrs before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 6-10 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of $GdCl_3$

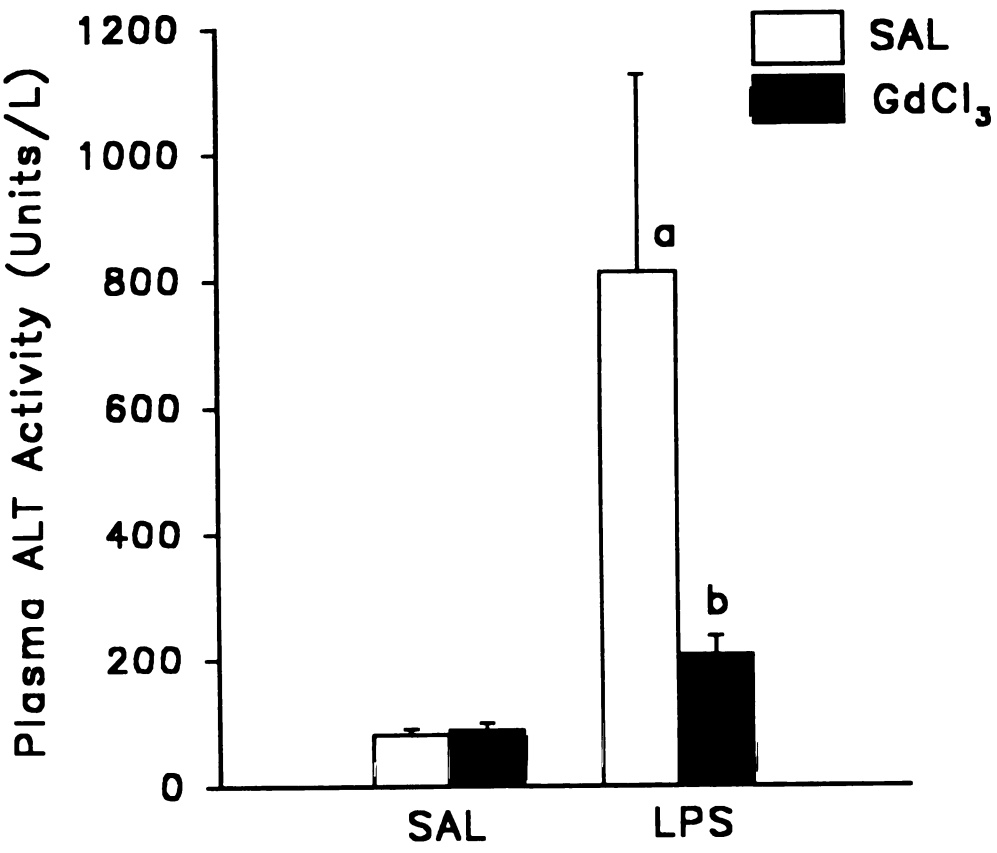

1200
1000
800
600
400
200
0
Plasma ALT Activity (Units/L)
SAL
GdCl3
a
b
SAL
LPS

FIGURE 3.5

Figure 3.6. The effects of LPS and $GdCl_3$ on hepatic platelet sequestration. All animals were treated with ^{111}In-labeled platelets (iv) 2 hrs before the administration of LPS. Animals were treated with $GdCl_3$ (10 mg/kg, iv) or its saline vehicle (SAL) 24 hrs before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, liver sections were collected for measurement of radioactivity in a gamma counter as described in Methods. Results are expressed as mean ± SEM, N = 6-10 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of $GdCl_3$

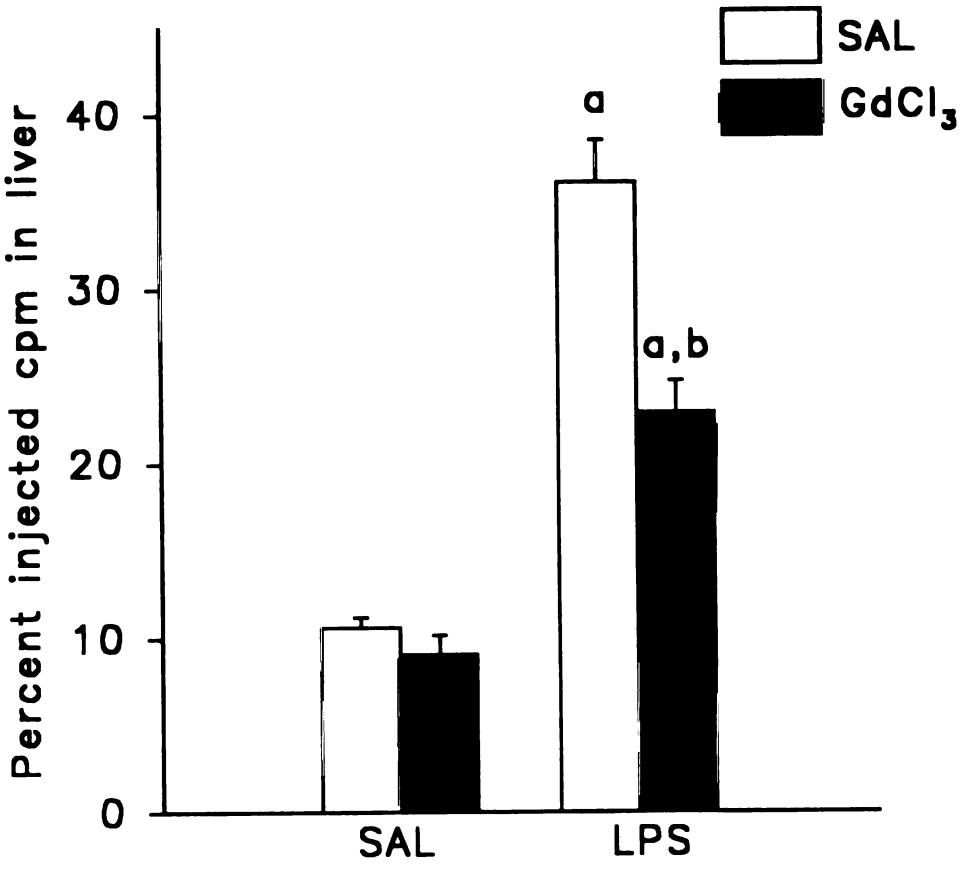

SAL
GdCl3
Percent injected cpm in liver
40
30
20
10
0
a
a,b
SAL
LPS

Figure 3.6

TABLE 3.2

Effects of $GdCl_3$ and LPS on Platelet Distribution in the Spleen, Kidneys and Lungs

TISSUE	SAL + SAL	$GdCl_3$ + SAL	SAL + LPS	$GdCl_3$ + LPS
SPLEEN	13.6 ± 0.7	11.5 ± 1.5	11.5 ± 0.8	14.6 ± 0.8
KIDNEYS	2.4 ± 2.0	2.1 ± 1.6	4.5 ± 1.1	4.4 ± 1.1
LUNGS	2.5 ± 0.2	2.7 ± 0.2	2.0 ± 0.2	3.5 ± 0.1[a,b]

Rats were pretreated with $GdCl_3$ (10 mg/kg, iv) or its saline vehicle (SAL) 24 hrs before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. They were given ^{111}In-labeled platelets 2 hrs before the administration of LPS or its SAL vehicle. Six hours after the administration of LPS, blood and organs were collected for measurement of radioactivity as described in Methods. Results are expressed as percent of injected radioactivity in entire organ (mean ± SEM), N = 6-10 per group.

[a]significantly different from respective value in the absence of LPS

[b]significantly different from respective value in the absence of $GdCl_3$

Figure 3.7. The effects of LPS and $GdCl_3$ on blood radioactivity. Animals were pretreated with $GdCl_3$ (10 mg/kg, iv) or its saline vehicle (SAL) 24 hrs before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. To measure radiolabeled platelet distribution in blood and organs, rats were treated with ^{111}In-labeled platelets (iv) 2 hrs before the administration of LPS. Six hours after LPS administration, blood samples were collected for measurement of radioactivity in a gamma counter as described in Methods. Results are expressed as mean ± SEM, N = 6-10 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of $GdCl_3$

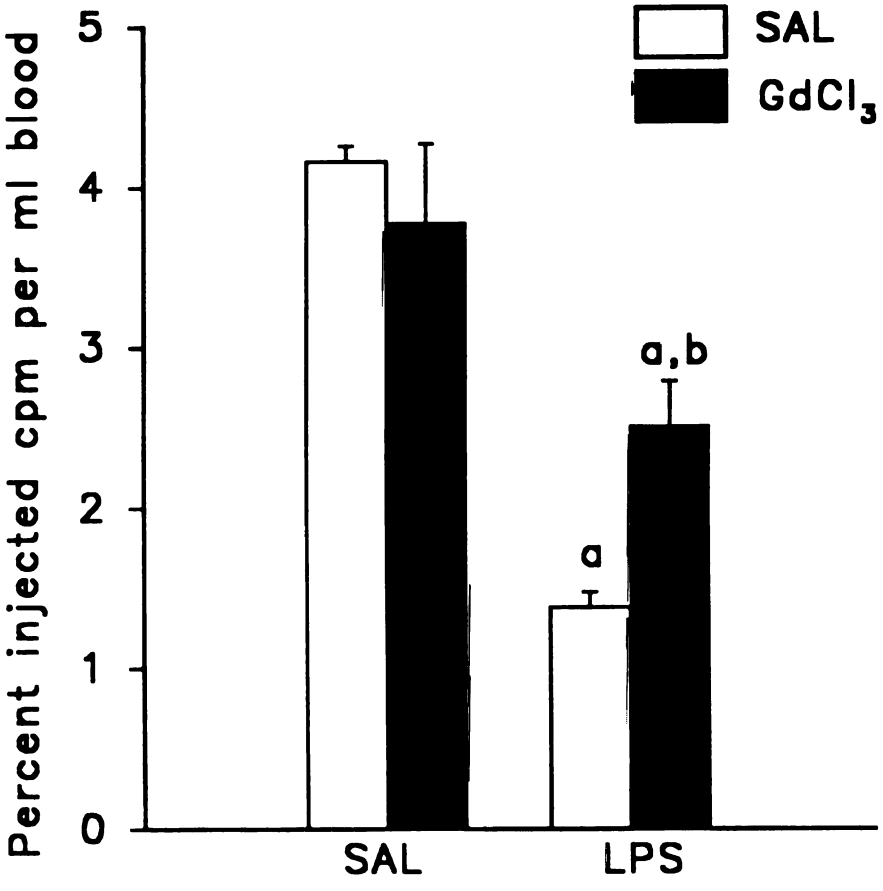

Percent injected cpm per ml blood
5
4
3
2
1
0
SAL
GdCl3
a,b
a
SAL
LPS

Figure 3.7

Figure 3.8. The effects of LPS and $GdCl_3$ on blood platelet concentration. Animals were treated with $GdCl_3$ (10 mg/kg, iv) or its saline vehicle (SAL) 24 hrs before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for enumeration of blood platelets as described in Methods. Results are expressed as mean ± SEM, N = 6-10 per group.

a, significantly different from respective value in the absence of LPS

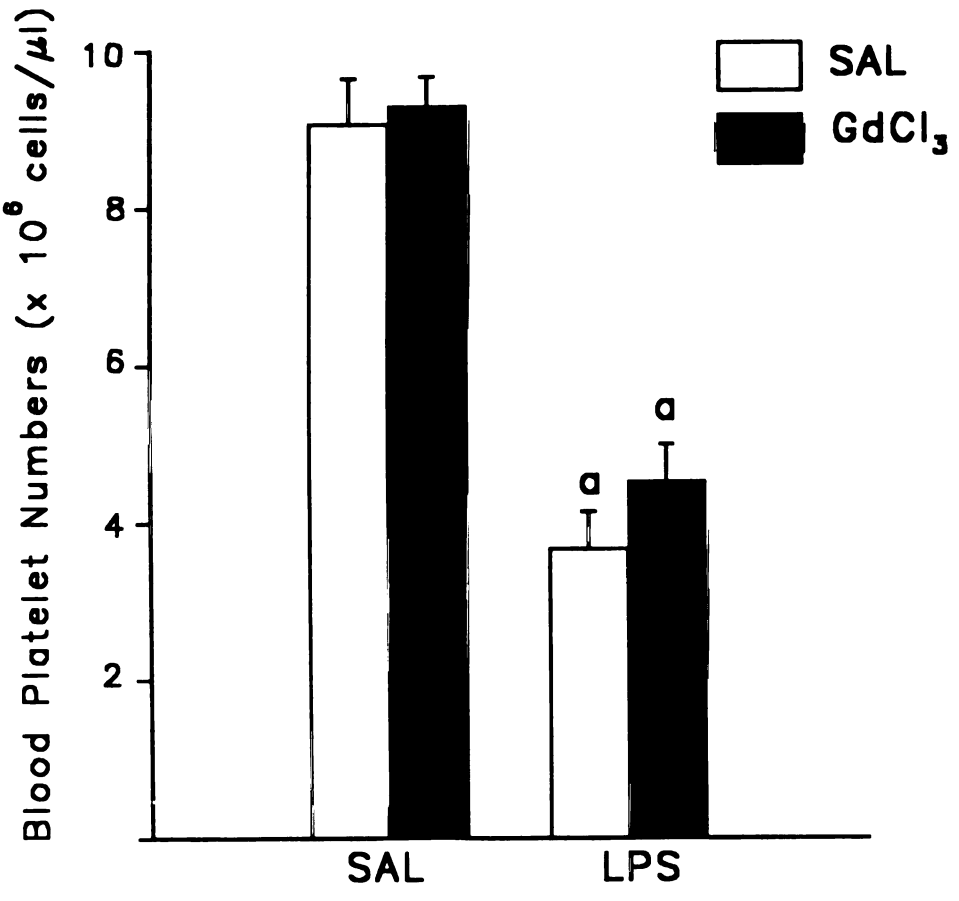

Blood Platelet Numbers (x 10^6 cells/μl)
10
8
6
4
2
SAL
$GdCl_3$
a
a
SAL
LPS

Figure 3.8

Figure 3.9. The effects of LPS and $GdCl_3$ on plasma fibrinogen concentration. Animals were treated with $GdCl_3$ (10 mg/kg, iv) or its saline vehicle (SAL) 24 hrs before the administration of LPS (4 mg/kg, iv) or its SAL vehicle.. Six hours after the administration of LPS, blood was collected for measurement of plasma fibrinogen concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-10 per group.

a, significantly different from respective value in the absence of LPS

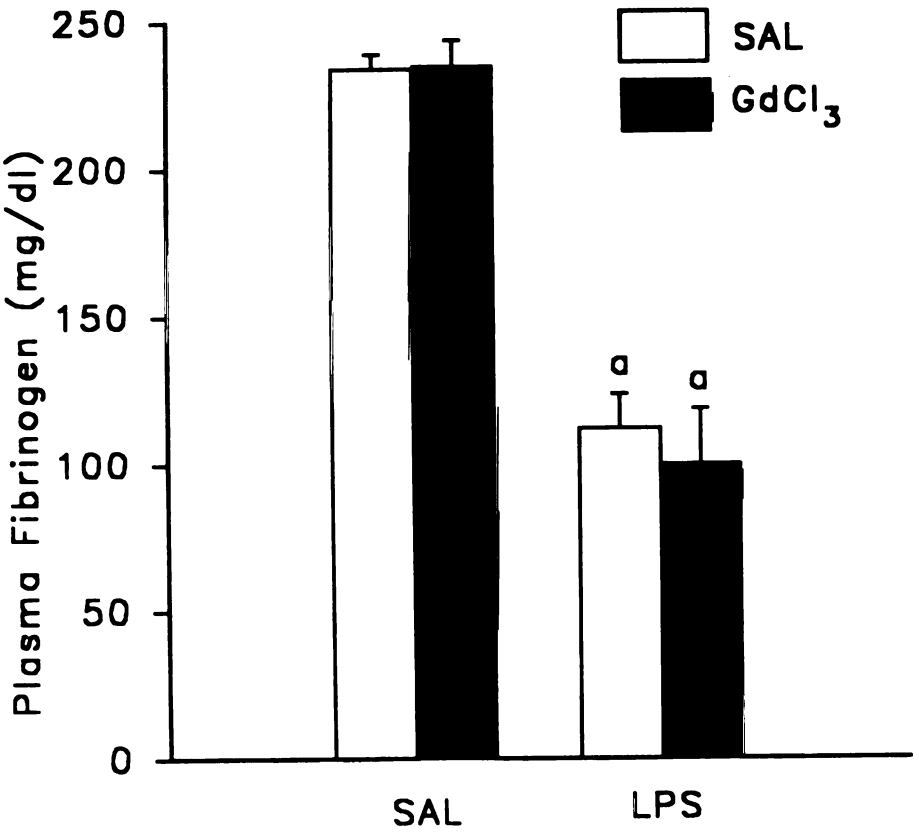

250
200
150
100
50
0
Plasma Fibrinogen (mg/dl)
SAL
GdCl3
a
a
SAL
LPS

Figure 3.9

3. E. Discussion

In this study, rats exposed to LPS developed hepatocellular injury, as measured by elevations in plasma ALT activity and histologic evidence of necrosis. The administration of LPS also resulted in activation of the coagulation system and thrombocytopenia. The loss of platelets in the blood was reflected in a significant and selective accumulation of platelets within the liver. The distribution of radiolabeled platelets in the lungs, kidneys and spleen was not altered by exposure to LPS. These results confirm the effects of LPS exposure reported previously (Pearson *et al.*, 1995).

Both neutrophils and platelets accumulate in the livers of LPS-treated animals (Hewett *et al.* 1992; Levy *et al.* 1968b; Endo *et al.* 1992). To uncover interactions between these inflammatory cells in LPS-mediated alterations *in vivo*, animals were rendered neutropenic by the administration of an anti-neutrophil Ig. As shown previously (Hewett *et al.*, 1992; Jaeschke *et al.*, 1991b), neutrophil-depleted animals were less sensitive to the hepatotoxic effects of LPS. However, despite the capacity of activated neutrophils to adhere to and stimulate platelets and cause them to express glycoprotein IIb/IIIa *in vitro* (Evangelista *et al.*, 1992; LaRosa *et al.*, 1994), neutrophils apparently do not contribute to LPS-induced hepatic platelet accumulation, since hepatic radioactivity remained elevated in neutropenic animals.

In this study, the role of Kupffer cells in LPS-mediated pathophysiology was evaluated by the pretreatment with $GdCl_3$. $GdCl_3$ forms insoluble

carbonate and phosphate precipitates in blood which are phagocytosed by Kupffer cells, resulting in their inactivation (Dean *et al.*,1988; Hardonk *et al.*,1992). The $GdCl_3$ treatment regimen we used inhibits Kupffer cell phagocytosis of carbon particles *in vivo*, as measured by carbon clearance and histologic and morphologic analysis (Hardonk *et al.*,1992; Ganey *et al.*, 1993). Consistent with other studies (Iimuro *et al.*,1994; Brown *et al.*, 1995), pretreatment with $GdCl_3$ attenuated the hepatotoxicity observed after LPS administration. The mechanisms by which Kupffer cell inhibition with $GdCl_3$ leads to protection from liver injury remains unclear. In a previous study, $GdCl_3$ administration did not reduce hepatic neutrophil accumulation observed after LPS administration (Iimuro *et al.*,1994; Brown *et al.*, 1995), suggesting that Kupffer cells are not needed for the accumulation of neutrophils in the liver. These results suggested that Kupffer cells contribute to some other critical event required for the expression of hepatic injury after LPS exposure.

The inhibition or depletion of Kupffer cells and platelets, respectively, protects completely against the hepatotoxic effects of LPS, suggesting that these cells might be linked in the pathogenesis. To uncover an interaction between Kupffer cells and platelets during LPS-induced liver injury, we evaluated the effect of pretreatment with $GdCl_3$ on hepatic accumulation of ^{111}In-labeled platelets. Pretreatment with $GdCl_3$ reduced hepatic platelet accumulation during LPS exposure (Figure 3.6), suggesting that Kupffer cells contribute in part to the hepatic platelet sequestration. The exact mechanism by which Kupffer cells mediate hepatic platelet accumulation is not fully

understood. The accumulation of radiolabeled platelets within the liver is not likely due to the removal of platelets injured by the radiolabeling procedure, since numerous studies have demonstrated that this technique does not alter platelet function (White *et al.*,1988; Christenson *et al.*,1987; Sostman *et al.*,1983). The finding that Kupffer cells contribute to hepatic platelet sequestration is consistent with electron micrographic studies which have shown that LPS exposure results in the presence of platelets both within Kupffer cells and within the sinusoids and the spaces of Disse (Endo *et al.*,1993). Using electron microscopy, we have confirmed in this model the presence of platelets in both the Kupffer cells and sinusoids 6 hrs after LPS administration (unpublished results).

Kupffer cells could contribute to hepatic platelet accumulation by several mechanisms. The hepatic reticuloendothelial system, consisting of Kupffer cells and endothelial cells, can clear senescent platelets as well as platelet-immune complexes, as observed during immune thrombocytopenia purpura (du P Heyns *et al.*,1986). The clearance of platelets by Kupffer cells may be mediated by the C1q receptor located on Kupffer cells (Toth *et al.*,1992). This receptor complex contributes to the clearance of numerous cells, coagulation factors, bacteria and cellular debris after opsonization of the particulate matter with fibronectin, an abundant serum constituent. Stimulated Kupffer cells release fibronectin, and LPS administration increases serum fibronectin concentration (Toth *et al.*,1992; Richards *et al.*,1985). Moreover, enhanced Kupffer cell phagocytic activity observed after challenge with a small dose of LPS correlates

with elevated plasma fibronectin concentration (Richards *et al.*, 1985). Accordingly, the Kupffer cells may contribute to hepatic platelet accumulation by a mechanism involving LPS-mediated changes in fibronectin concentration.

The administration of the anti-neutrophil Ig could stimulate Kupffer cells by the formation of antibody-antigen complexes. Enhanced Kupffer cell activity could stimulate phagocytosis of platelets. Such a mechanism would explain the finding that anti-neutrophil Ig administration increased hepatic platelet accumulation, even in the absence of LPS.

Kupffer cells could also contribute to hepatic platelet sequestration by releasing soluble mediators that activate platelets. For example, *in vitro,* LPS-stimulated Kupffer cells release thromboxane A_2, a potent platelet activator (Rodriguez de Turco *et al.*, 1990; Ishiguro *et al.*, 1994). Plasma concentration of thromboxane B_2, the stable metabolite of thromboxane A_2, is elevated after LPS administration *in vivo* (Wise *et al.*, 1980). However, the cellular source of this thromboxane and the role of thromboxane in LPS-induced hepatic platelet accumulation are not known. Kupffer cells and neutrophils release oxygen radicals in response to LPS exposure (Liu *et al.*, 1995). This respiratory burst could directly influence platelets, inasmuch as large concentrations of hydrogen peroxide stimulate platelet aggregation (Salvemini *et al.*, 1993). Alternatively, activated Kupffer cells, through the release of lipoxygenase metabolites and oxygen radicals, can directly injure the sinosoidal endothelium (Deaciuc *et al.*, 1993a; Deaciuc *et al.*, 1994; Arai *et al.*, 1993; Liu *et al.*, 1994), an event that promotes platelet aggregation. Moreover, oxygen radical-mediated injury to the

endothelium, through changes in the production of platelet inhibitors such as prostacyclin and nitric oxide, may alter the activity of platelets (Salvemini *et al.*, 1993). Since treatment with $GdCl_3$ inhibits superoxide production from Kupffer cells (Iimuro *et al.*, 1994), it is possible that these resident macrophages contribute to hepatic platelet accumulation by a mechanism involving oxygen radical-mediated endothelial cell injury. These potential interactions between Kupffer cells, the endothelium and platelets have not been well characterized during LPS exposure *in vivo.*

As shown previously (Pearson *et al.*, 1995), the administration of LPS resulted in a pronounced thrombocytopenia. Decreases in total platelets (Figure 3.8) as well as radiolabeled platelets in blood (Figure 3.7) followed LPS injection. Pretreatment with $GdCl_3$ attenuated the decrease in blood radioactivity; however, it did not significantly alter total blood platelet concentration after LPS administration. These contrasting results may be explained by platelet dynamics *in vivo*. The concentration of endogenous platelets is determined by rates of both removal and replenishment. Accordingly, total blood platelet concentration is influenced by both of these factors, whereas the number of injected, radiolabeled platelets is influenced only by processes of removal. The removal of platelets from the circulation stimulates a rapid replenishment of them from splenic megakaryocytes (du P Heyns *et al.*, 1985). Accordingly, when LPS exposure results in thrombocytopenia, it is likely that the megakaryocytic pool responds by releasing platelets into the blood. This would tend to increase total platelet

concentration in blood but would obviously not contribute to the numbers of radiolabeled platelets. The attenuation by $GdCl_3$ of the LPS-induced decrease in ^{111}In-labeled platelets (Figure 3.7) indicates that $GdCl_3$ pretreatment reduced the removal of platelets from the circulation. This would be expected also to reduce the stimulus for platelet release from storage pools. Accordingly, the consequent reduction in the compensatory release of platelets from megakaryocytes into blood may have resulted in no net effect of $GdCl_3$ pretreatment on LPS-induced thrombocytopenia (Figure 3.8) despite its effect in decreasing platelet removal from blood (Figure 3.7). Thus, platelets newly released from endogenous storage pools likely represent a greater percentage of total blood platelets in rats treated with only LPS compared to those that also received $GdCl_3$.

Exposure to LPS resulted in a redistribution of platelets, since blood platelet concentration decreased and platelets accumulated in the liver. By contrast, LPS administration did not alter the distribution of radiolabeled platelets in the spleen, kidneys or lungs. In contrast to platelets, neutrophils accumulate in large numbers in alveolar walls after LPS administration (Frevert *et al.*, 1992; Brown *et al.*, 1995). This difference suggests that tissue neutrophil accumulation does not necessarily promote accumulation of platelets, a conclusion consistent with the results in liver (see above). $GdCl_3$ treatment results in a modest increase in pulmonary platelets in animals given LPS. This suggests that reduced Kupffer cell activity results in an increase in pulmonary platelets. This makes sense, if, as the results in liver suggest, Kupffer cells are

responsible for phagocytosing a population of circulating platelets altered during LPS exposure. Inhibition of Kupffer cell phagocytosis of such platelets would allow access of them to other tissues, where the altered platelets might tend to accumulate. Since the pulmonary vasculature represents the first capillary bed downstream from the liver, it is reasonable that the lungs would be a site of accumulation in this circumstance.

In summary, neutrophil depletion was associated with an attenuation of LPS-induced liver injury and thrombocytopenia, but had no effect on hepatic platelet accumulation. These results suggest that neutrophils contribute to liver injury by a mechanism that is independent of hepatic platelet accumulation. $GdCl_3$ given at a dose that inhibits Kupffer cell phagocytosis protected rats against liver injury and attenuated both the removal of radiolabeled platelets from the blood and their accumulation in the liver. By contrast, Kupffer cell inhibition did not affect the LPS-induced activation of the coagulation system. These results suggest that Kupffer cells contribute to the hepatic platelet accumulation during LPS exposure by a mechansim that is independent of the the coagulation system.

THE ROLE OF PLATELETS IN LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY

Volume II

by

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1996

CHAPTER 4

THE ROLE OF THROMBIN IN LPS-MEDIATED LIVER INJURY

4. A. Abstract

The intravenous administration of lipopolysaccharide (LPS) from gram-negative bacteria to rats results in hepatic parenchymal cell injury within 6 hrs. The coagulation system and platelets are critical to the pathogenesis since pretreatment with the anticoagulants, warfarin or heparin, or platelet depletion attenuated LPS-induced liver injury. Prior depletion of fibrinogen with ancrod did not prevent liver injury, suggesting that thrombin may be a key mediator of liver injury in this model. However, any interaction between platelets and the coagulation system as it relates to liver injury *in vivo* is not fully understood. Accordingly, we tested the effect of pretreating animals with the anticoagulant, heparin, and the thrombin inhibitor, hirudin, on LPS-induced liver injury, thrombocytopenia and hepatic platelet accumulation. Pretreatment with heparin or hirudin effectively inhibited coagulation, as evidenced by prolonged activated partial thromboplastin time (APTT) and by maintenance of plasma fibrinogen concentration in LPS-treated rats. Treatment with heparin or hirudin prevented LPS-induced liver injury, assessed by plasma alanine aminotransferase activity and histologic evidence of hepatocellular necrosis. Pretreatment with either heparin or hirudin did not alter LPS-induced hepatic platelet accumulation. Furthermore, pretreatment with hirudin, but not heparin, partially attenuated the decrease in blood platelet concentration that accompanied LPS administration. These findings suggested that the coagulation system, in particular thrombin, is involved in LPS-induced liver injury by a mechanism which is independent of

hepatic platelet accumulation. To elucidate further the role of the coagulation system in the mechanism of liver injury in this model, we administered heparin or hirudin at various times after LPS administration. When heparin or hirudin was administered 2.5 hours after the administration of LPS, a time when platelets have accumulated within the liver, hepatic injury was prevented. These findings suggest that thrombin is a critical and distal mediator of LPS-induced liver injury.

4. B. Introduction

Evidence for activation of the coagulation system during LPS exposure includes a pronounced decrease in plasma fibrinogen (Prager *et al.*, 1979), an increase in the prothrombin and partial thromboplastin times (Yoshikawa *et al.*, 1981) and elevations of plasma fibrin degradation products (Gomez *et al.*, 1989). These alterations in the coagulation system begin 2-3 hrs after the intravenous administration of LPS and occur prior to the onset of liver injury (Hewett *et al.*, 1995; Pearson *et al.*, 1995). The coagulation system appears to contribute to LPS-induced hepatocellular damage, since pretreatment with anticoagulants such as warfarin or heparin at doses large enough to reduce thrombin activity attenuates liver injury (Margaretten *et al.*, 1967; Hewett *et al.*, 1995). The actual mechanism by which the coagulation system contributes

to hepatic parenchymal cell injury remains unclear, but evidence suggests that the liver injury is independent of fibrinogen and formation of insoluble fibrin clots (Hewett *et al.*, 1995). Taken together, these findings implicate thrombin as a possible mediator of LPS-induced hepatocellular injury.

LPS exposure also results in a decrease in blood platelet concentration within 2-3 hrs after LPS administration to rats (Pearson *et al.*, 1995). Studies with ^{111}indium-labeled platelets show that platelets begin to accumulate in the liver within 1 hr after LPS administration and prior to the onset of liver injury (Pearson *et al.*, 1995). Furthermore, platelets appear to contribute to liver injury since depletion of platelets prior to LPS administration prevents hepatocellular injury (Pearson *et al.*, 1995). The mechanism by which platelets contribute to liver injury is not known, however platelet depletion studies suggest that platelets are critical for the LPS-induced activation of the coagulation cascade (Pearson *et al.*, 1995). These findings support the hypothesis that platelets interact with the coagulation system in the development of LPS-induced liver injury *in vivo*.

To our knowledge, there are no reports describing how platelets and the coagulation system interact to contribute to liver injury *in vivo*. To increase understanding of interactions between these inflammatory components during the genesis of LPS-induced liver injury *in vivo*, rats were pretreated with heparin or the highly selective thrombin inhibitor, hirudin, to determine the role of the coagulation system and thrombin in LPS-induced hepatic platelet sequestration. In addition, in an attempt to understand when the coagulation system

contributes to hepatocellular injury, heparin or hirudin was administered at various times after the administration of LPS.

4. C. Materials and methods

4. C. 1. Materials

See Chapter 2, section C. 1.

Reagents for measurement of activated partial thromboplastin times (APTTs) were purchased from Sigma Chemical Company (St. Louis, MO). APTTs were measured in a BBL Fibrometer (Becton, Dickinson and Company, Hunt Valley, MD). Recombinant hirudin (HBW-023) was a generous gift from Behring (Behringwerke Aktiengesellschaft, Marburg, Germany).

4. C. 2. Animals

See Chapter 2, Section C.2

4. C. 3. Quantification of hepatic platelet accumulation

See Chapter 2, Section C.2.

4. D. 4. Heparin pretreatment studies

Rats were given radiolabeled platelets 2 hrs before the administration of LPS (time 0 hr) as described above. One hour prior to the administration of

LPS, animals were treated with heparin (2000 U/kg, iv) or its saline vehicle. Six hours after the administration of LPS (4 mg/kg, iv) or its saline vehicle, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn into sodium citrate (0.38% final concentration) from the descending aorta and organs were collected for the determination of radioactivity (see above). Liver sections were fixed in 10% neutral buffered formalin and processed for histologic evaluation. Blood platelets were determined in a hemacytometer after red cell lysis and dilution in platelet Unopettes. Plasma samples were assayed for plasma fibrinogen concentration and APTT in a BBL Fibrometer (Becton, Dickinson and Company, Hunt Valley, MD) and for ALT activity.

4. C. 5. Hirudin pretreatment studies

To assess the role of thrombin in LPS-induced liver injury, animals were pretreated with recombinant hirudin. Preliminary studies were conducted to determine a dose and route of administration of hirudin that would effectively inhibit thrombin as measured by elevations in activated partial thromboplastin times (APTTs). All animals were given radiolabeled platelets to evaluate hepatic platelet sequestration. Two hours after the administration of radiolabeled platelets, animals were treated with LPS (5 mg/kg, iv) or saline vehicle (CONTROL). They were then treated with recombinant hirudin (36,000 units/kg, sc) or its saline (SAL) vehicle 0.5, 2.5 and 4.5 hr after the administration of LPS. Six hours after the administration of LPS, animals were

anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn into sodium citrate (0.38% final concentration) from the descending aorta, and organs were collected for determination of radioactivity (see above). Liver sections were fixed in 10% neutral buffered formalin and processed for histologic evaluation. Blood platelet concentrations were determined in a hemocytometer after erythrocyte lysis and dilution in platelet Unopettes. Plasma samples were assayed for APTT, fibrinogen concentration (Hewett *et al.*, 1995; Margaretten *et al.*, 1967) and ALT activity.

4. C. 6. Quantification of hepatic neutrophil accumulation

See Chapter 2, section 3.

4. C. 7. Heparin posttreatment studies.

To elucidate when the coagulation system contributes to the pathogenesis of LPS-induced liver injury, animals were treated with LPS (4 mg/kg, iv) or its saline vehicle. Animals were also given heparin (2000 U/kg, iv) or its saline vehicle either 1 hr before or 1.5 or 2.5 hrs after LPS administration. Six hours after the administration of LPS, animals were anesthetized as described above, and blood and liver sections were collected for the evaluation of liver injury, as measured by plasma ALT activity and histologic evaluation.

4. C. 8. Hirudin posttreatment studies

Preliminary studies were conducted to determine a dose and route of administration of hirudin that would effectively inhibit thrombin as measured by elevations in activated partial thromboplastin times (APTT, data not shown). These results showed that a subcutaneous administration of recombinant hirudin (HBW-023; 36,000 U/kg) produced a three-fold elevation in plasma APTT that lasted for 2 hrs. To test the hypothesis that thrombin is a distal mediator of liver injury, animals were treated with LPS (4 mg/kg, iv) or its saline vehicle. Animals were subsequently given r-hirudin (36,000 U/kg, sc) or its saline vehicle 2.5 and 4.5 hrs after LPS administration. Six hours after the administration of LPS, animals were anesthetized as described above, and blood and liver sections were collected for the determination of liver injury, as measured by plasma ALT activity and histologic evaluation.

4. C. 10. Histopathologic evaluation

Sections of liver were fixed in 10% neutral buffered formalin and processed for histopathologic evaluation. Paraffin-embedded sections were cut at 6 μm and stained with hematoxylin and eosin. Slides were coded, randomized and evaluated by a pathologist. The severity of each lesion was graded as follows: 0 = no evidence of inflammation or necrosis; 1 = sinusoidal neutrophilia only; 2 = sinusoidal neutrophilia with multifocal, single cell necrosis; 3 = multifocal, acute, mild hepatocellular necrosis with sinusoidal neutrophilia; 4 = multifocal, acute, moderate hepatocellular necrosis with

sinusoidal neutrophilia; 5 = multifocal, acute, marked hepatocellular necrosis with sinusoidal neutrophilia.

4. C. 11. Data analysis

A 2 X 2 multifactorial, completely random analysis of variance (ANOVA) was used to evaluate heparin and hirudin pretreatment studies. Data with nonhomogeneous variances were square root- or log-transformed prior to analysis. Comparisons among groups were performed with the LSD test for APTT values. Comparisons among groups were performed with Tukey's omega test for all other data (Steel and Torrie, 1980). In the heparin and hirudin posttreatment studies, comparisons between groups were performed with the Student's t test (Steel and Torrie, 1980). The criterion for significance for all studies was $p \leq 0.05$. Data are expressed as mean ± SEM.

4. D. Results

4. D. 1. Heparin pretreatment studies

To test the hypothesis that the coagulation system contributes to LPS-induced hepatic platelet accumulation and liver injury, animals were pretreated with the anticoagulant, heparin, prior to LPS administration. Figure 4.1 shows the effects of LPS and heparin on activation of the coagulation system, as measured by changes in plasma fibrinogen concentration. As reported

previously (Hewett *et al.*, 1995; Pearson *et al.*, 1995), the administration of LPS resulted in a marked decrease in plasma fibrinogen. Pretreatment with heparin prevented the LPS-induced decrease in plasma fibrinogen, a finding that verified the anticoagulant effect of heparin treatment. Consistent with previous findings (Hewett *et al.*, 1995), pretreatment with heparin attenuated LPS-induced liver injury, as detected by elevations in plasma alanine aminotransferase activity (Figure 4.2) and morphologic evaluation of liver sections (Table 4.1). Livers from LPS-treated rats had mild to marked hepatocellular necrosis with a marked sinusoidal neutrophilia. Pretreatment with heparin attenuated the LPS-induced hepatocellular necrosis but did not prevent the LPS-induced sinusoidal neutrophilia (Table 4.1).

To evaluate whether an activated coagulation system contributes to the pathogenesis of LPS-induced liver injury by altering platelet kinetics, we determined the effect of pretreatment with heparin on LPS-induced thrombocytopenia and hepatic platelet accumulation. The administration of LPS resulted in a pronounced decrease in blood platelet concentration (Figure 4.3). This was not altered by treatment with heparin. LPS administration caused a significant accumulation of platelets within the liver as measured by elevation in radioactivity in the liver (Figure 4.4). Administration of heparin did not significantly alter the hepatic platelet accumulation. Neither LPS nor heparin administration affected the distribution of radiolabeled platelets in the spleen, kidneys or lungs (Table 4.2). In contrast, LPS produced a significant decrease in radiolabeled platelets in blood radioactivity, consistent with the decrease in

blood platelet concentrations. Heparin treatment did not significantly alter this decrease in blood radioactivity (Table 4.2).

4. D. 2. Hirudin pretreatment studies

To evaluate the role of thrombin in LPS-induced liver injury, we used the specific thrombin inhibitor, hirudin. Figure 4.5 confirms the effectiveness of hirudin in inhibiting thrombin, as indicated by increases in APTT. Treatment with hirudin significantly prolonged APTT as compared to animals administered saline vehicle. Treatment with LPS also tended to prolong APTT, although this was not statistically significant. Treatment with LPS resulted in activation of the coagulation system, as marked by decreased plasma fibrinogen concentrations (Figure 4.6). The administration of hirudin prevented this decrease, confirming that the hirudin dosing regimen effectively inhibited the actions of thrombin.

Figure 4.7 shows the effect of hirudin on the LPS-induced loss of hepatic parenchymal cell integrity, as estimated by increased plasma ALT activity. The administration of LPS produced hepatocellular injury within 6 hrs, and treatment with hirudin prevented liver damage. This finding is consistent with histologic analysis of liver sections (Figure 4.8, Table 4.3). Livers from LPS-treated animals had mild to marked hepatocellular necrosis with sinusoidal neutrophilia. Treatment with hirudin attenuated LPS-induced hepatocellular necrosis but did not prevent infiltration of neutrophils within the liver sinusoids (Figure 4.9; Table 4.3).

To evaluate the mechanism by which thrombin contributes to LPS-induced liver injury, we evaluated the effect of hirudin on LPS-induced thrombocytopenia and hepatic platelet accumulation, as measured by blood platelet enumeration and radiolabeled platelet sequestration in the liver, respectively. The administration of LPS produced a pronounced decrease in blood platelet concentration (Figure 4.10). This LPS-induced thrombocytopenia was more modest in animals treated with hirudin. Similar to the findings with total blood platelet concentrations, LPS produced a significant decrease in radiolabeled platelets in the blood, and treatment with hirudin slightly attenuated this decrease in blood radioactivity (Figure 4.11).

As reported previously, LPS administration caused hepatic platelet accumulation as measured by the elevation in hepatic ^{111}In (Figure 4.12). Administration of hirudin did not significantly alter the hepatic platelet accumulation. The administration of LPS, with or without hirudin, did not alter the distribution of radiolabeled platelets in the spleen, kidneys or lungs (Table 4.4).

4. D. 3. Heparin and hirudin posttreatment studies

Results from these studies suggested that an activated coagulation system contributes to the pathogenesis of LPS-induced liver injury but does not mediate the LPS-induced hepatic neutrophil and platelet sequestration. This raised the possibility that the coagulation system plays a role in liver injury after these inflammatory cells have already accumulated within the liver. To test this

hypothesis, heparin or its saline vehicle was administered either 1 hr prior to or 1.5 or 2.5 hrs after the administration of LPS, and liver injury was evaluated at 6 hr. Pretreatment with heparin attenuated LPS-induced liver injury, as measured by plasma ALT activity (Figure 4.13). The administration of heparin 1.5 or 2.5 hrs after LPS similarly reduced liver injury. The administration of heparin 3.5 hrs after LPS did not significantly alter liver injury observed at 6 hrs (data not shown).

These findings suggested that an activated coagulation system is a distal mediator of LPS-induced liver injury. To understand further the mechanism by which the coagulation system contributes to hepatocellular damage, we used the specific thrombin inhibitor, hirudin, to evaluate whether thrombin contributes to tissue injury. Hirudin was administered 2.5 and 4.5 hrs after the administration of LPS, and liver injury was assessed by plasma ALT activity at 6 hr. Similar to the findings with heparin, the administration of hirudin beginning 2.5 hrs after LPS reduced liver injury (Figure 4.14).

Figure 4.1. The effects of LPS and heparin on plasma fibrinogen concentration. Animals were treated with heparin (HEP, 2000 U/kg, iv) or its saline vehicle (SAL) 1 hr prior to the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma fibrinogen concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-12 per group.

a, significantly different from SAL/SAL group

b, significantly different from SAL/LPS group

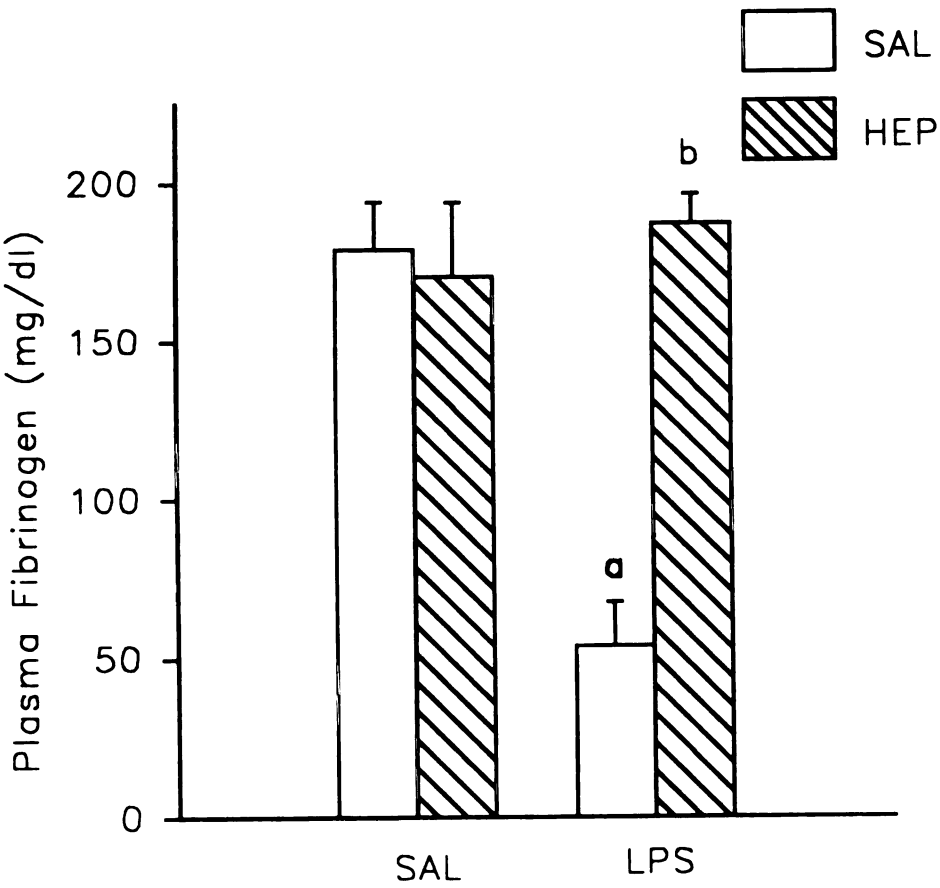


Figure 4.1

Figure 4.2. The effects of LPS and heparin on liver injury (plasma ALT activity). Animals were treated with heparin (HEP, 2000 U/kg, iv) or its saline vehicle (SAL) 1 hr prior to the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for measurement of liver injury as measured by plasma ALT activity. Results are expressed as mean ± SEM, N = 6-12 per group.

a, significantly different from SAL/SAL group

b, significantly different from SAL/LPS group

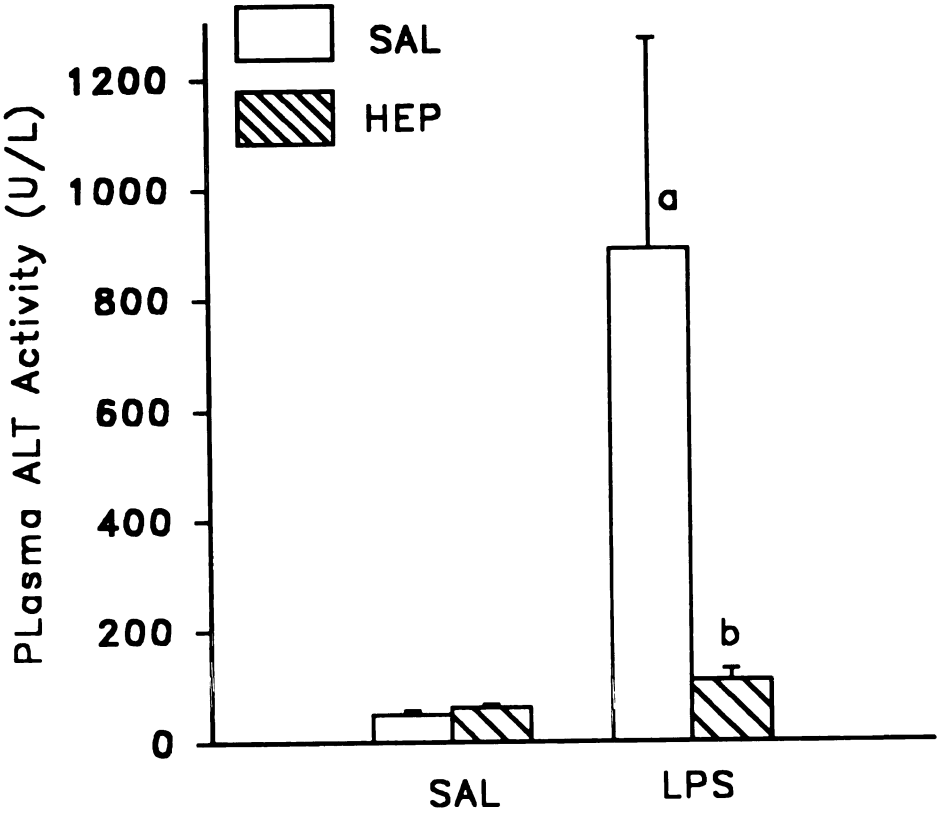

SAL
HEP
1200
1000
800
600
400
200
0
PLasma ALT Activity (U/L)
a
b
SAL
LPS

Figure 4.2

TABLE 4.1

Protection from LPS-Induced Hepatotoxicity by Administration of Heparin

Treatment	Percent of Animals with Histopathologic Score					
	0	1	2	3	4	5
Saline/Saline	100	-	-	-	-	-
Heparin/Saline	100	-	-	-	-	-
Saline/LPS	-	17	8	41	17	17
Heparin/LPS	-	75	25	-	-	-

Heparin (2000 U/kg, iv) or its saline vehicle was administered to rats 1 hr prior to the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after the administration of LPS, liver sections were collected and fixed in 10% neutral buffered formalin and prepared for analysis by light microscopy. The severity of hepatic injury was graded on a scale of 0 - 5 reflecting the frequency and size of the lesions. 0 = no evidence of inflammation; 1 = sinusoidal neutrophilia only; 2 = sinusoidal neutrophilia with multifocal single cell necrosis; 3 = multifocal, acute, mild hepatocellular necrosis with sinusoidal neutrophilia; 4 = multifocal, acute, moderate hepatocellular necrosis with sinusoidal neutrophilia; 5 = multifocal, acute marked hepatocellular necrosis with sinusoidal neutrophilia. Values indicate the percent of animals presenting each score.

Figure 4.3. The effects of LPS and heparin on blood platelet concentration. Animals were treated with heparin (HEP, 2000 U/kg, iv) or its saline vehicle (SAL) 1 hr prior to the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for enumeration of blood platelets as described in Methods. Results are expressed as mean ± SEM, N = 6-12 per group.

a, significantly different from respective SAL group

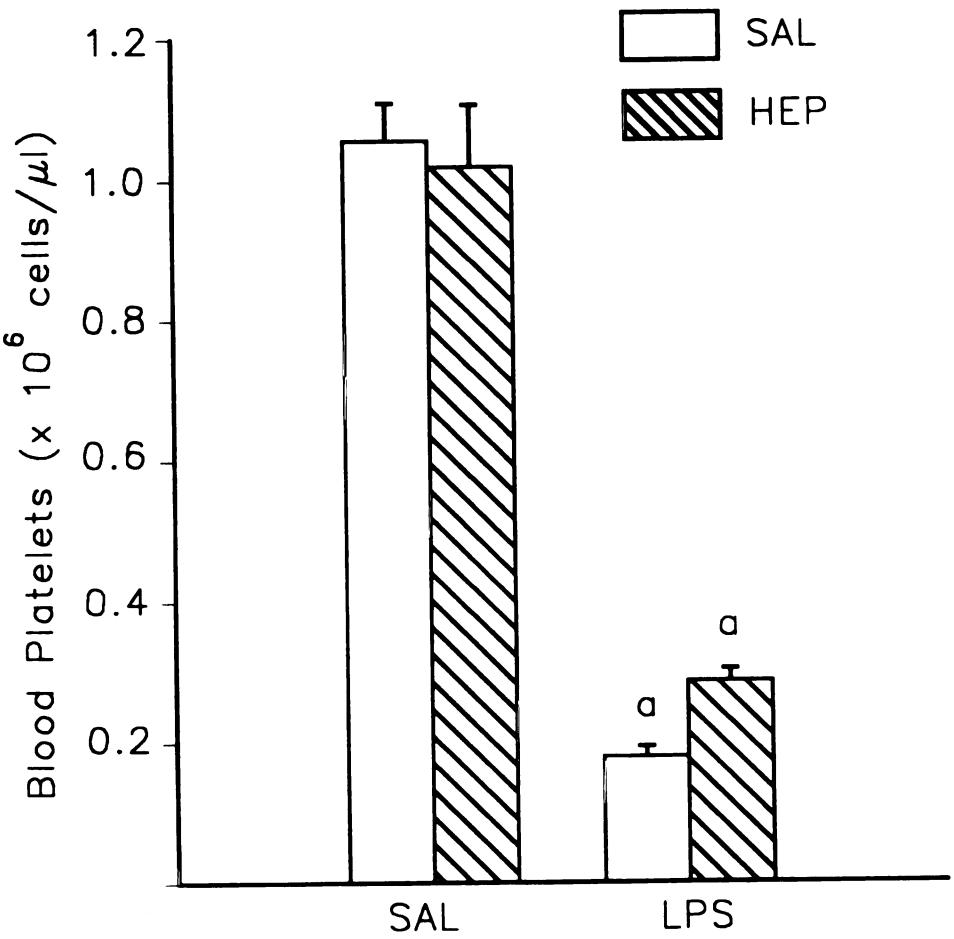

SAL
HEP
1.2
1.0
0.8
0.6
0.4
0.2
Blood Platelets (x 10^6 cells/μl)
a
a
SAL
LPS

Figure 4.3

Figure 4.4. The effects of LPS and heparin on hepatic platelet sequestration. To measure hepatic platelet sequestration, all animals were treated with ^{111}In-oxine radiolabeled platelets (iv) 2 hrs prior to the administration of LPS. Animals were treated with heparin (HEP, 2000 U/kg, iv) or its saline vehicle (SAL) 1 hr prior to the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, liver sections were collected for measurement of radioactivity in a gamma counter as described in Methods. Results are expressed as mean ± SEM, N = 6-12 per group.

a, significantly different from respective SAL group

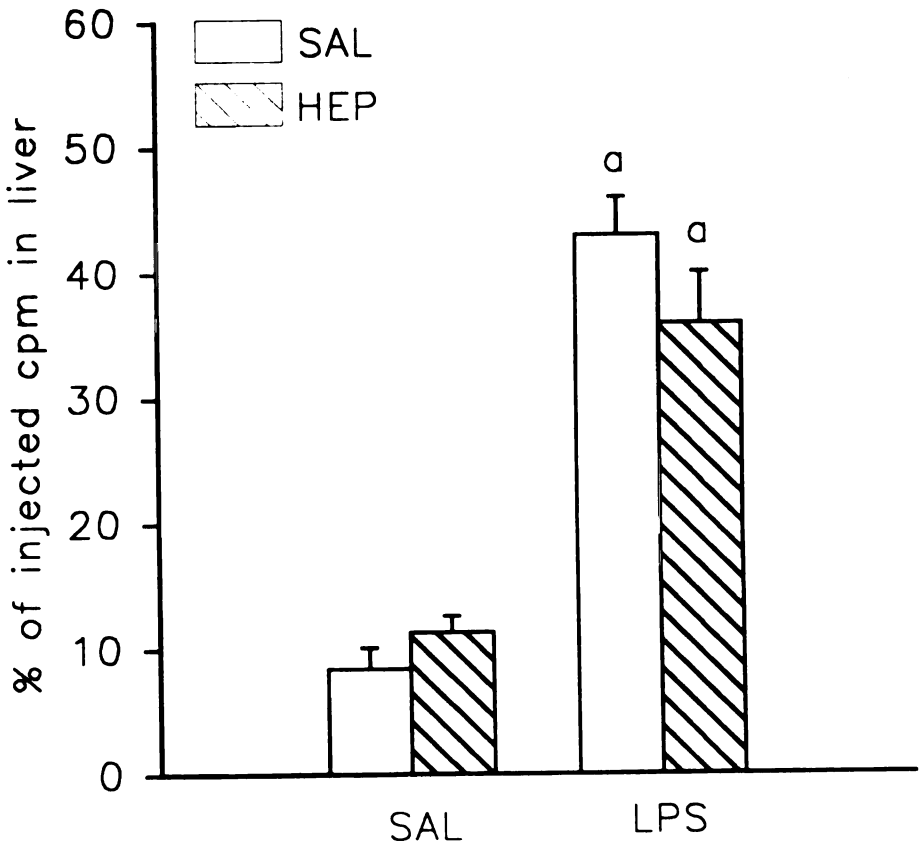


Figure 4.4

TABLE 4.2

Effects of Heparin and LPS on Platelet Distribution in Blood, Spleen, Kidneys and Lungs

TISSUE	SAL + SAL	HEP + SAL	SAL + LPS	HEP + LPS
BLOOD	3.2 ± 0.2	4.0 ± 0.6	0.9 ± 0.4*	1.8 ± 0.4*
SPLEEN	10.6 ± 2.8	14.0 ± 1.0	12.2 ± 1.3	14.4 ± 0.8
KIDNEYS	1.6 ± 0.2	1.7 ± 0.3	2.6 ± 0.3	1.7 ± 0.6
LUNGS	2.1 ± 0.6	2.5 ± 0.3	2.8 ± 0.4	3.9 ± 0.4

All animals were given ^{111}In-oxine radiolabeled platelets 2 hrs prior to the administration of LPS or its saline vehicle. Rats were treated with heparin (HEP, 2000 U/kg, iv) or its saline vehicle (SAL) 1 hr prior to the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after the administration of LPS, blood and organs were collected for measurement of radioactivity as described in Methods. Results are expressed as percent of injected radioactivity in 1 ml blood or entire organ (mean ± SEM), N = 8-14 per group.

*significantly different from respective SAL group in the absence of LPS

Figure 4.5. The effects of LPS and hirudin on activated partial thromboplastin time (APTT). LPS (5 mg/kg) or its saline vehicle was administered in the tail vein. Animals were treated with hirudin (36,000 U/kg, sc) or its saline vehicle 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hrs after LPS administration, blood samples were collected for measurement of plasma APTT as described in Methods. Results are expressed as mean ± SEM, N = 6-13 per group.

a, significantly different from respective CONTROL group

b, significantly different from SAL/LPS group

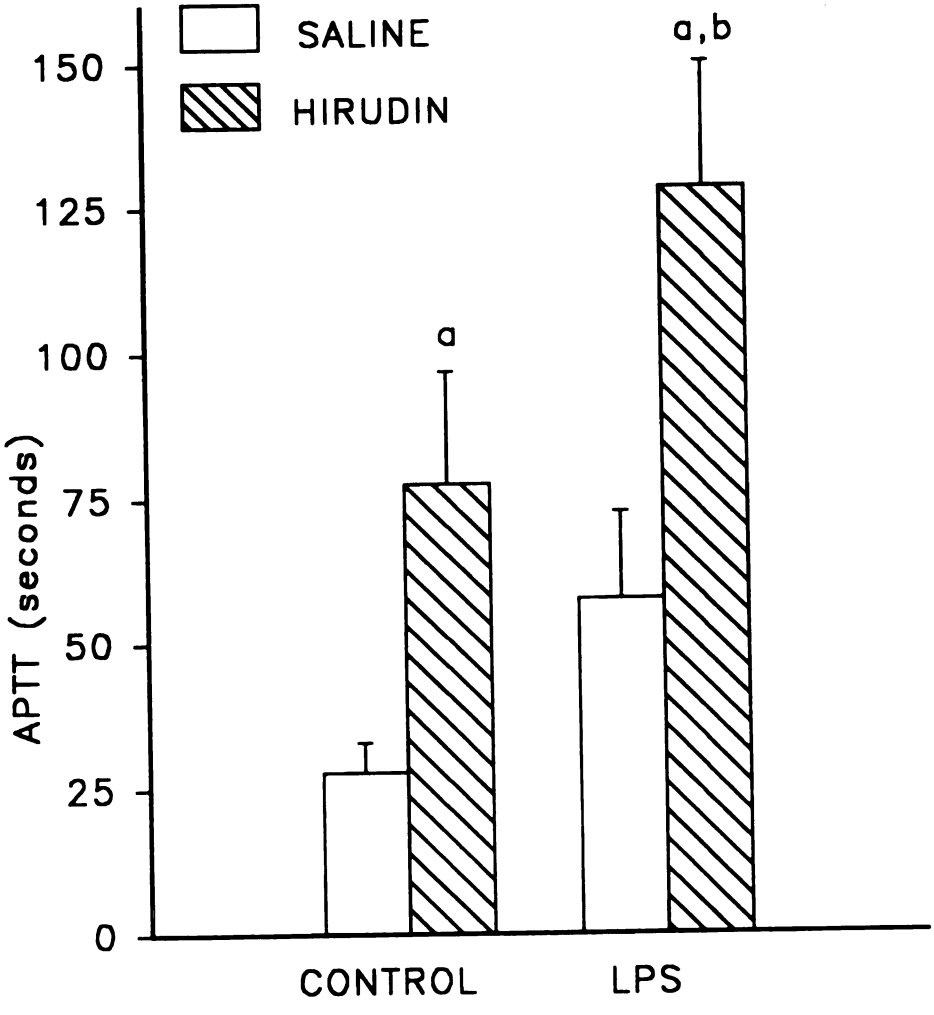

SALINE
HIRUDIN
a,b
a
150
125
100
75
50
25
0
APTT (seconds)
CONTROL
LPS

Figure 4.5

Figure 4.6. The effects of LPS and hirudin on plasma fibrinogen concentration. LPS (5 mg/kg) or its saline vehicle was administered in the tail vein. Animals were treated with hirudin (36,000 U/kg, sc) or its saline vehicle 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hrs after LPS administration, blood samples were collected for measurement of plasma fibrinogen concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-13 per group.

a, significantly different from respective CONTROL group

b, significantly different from SAL/LPS group

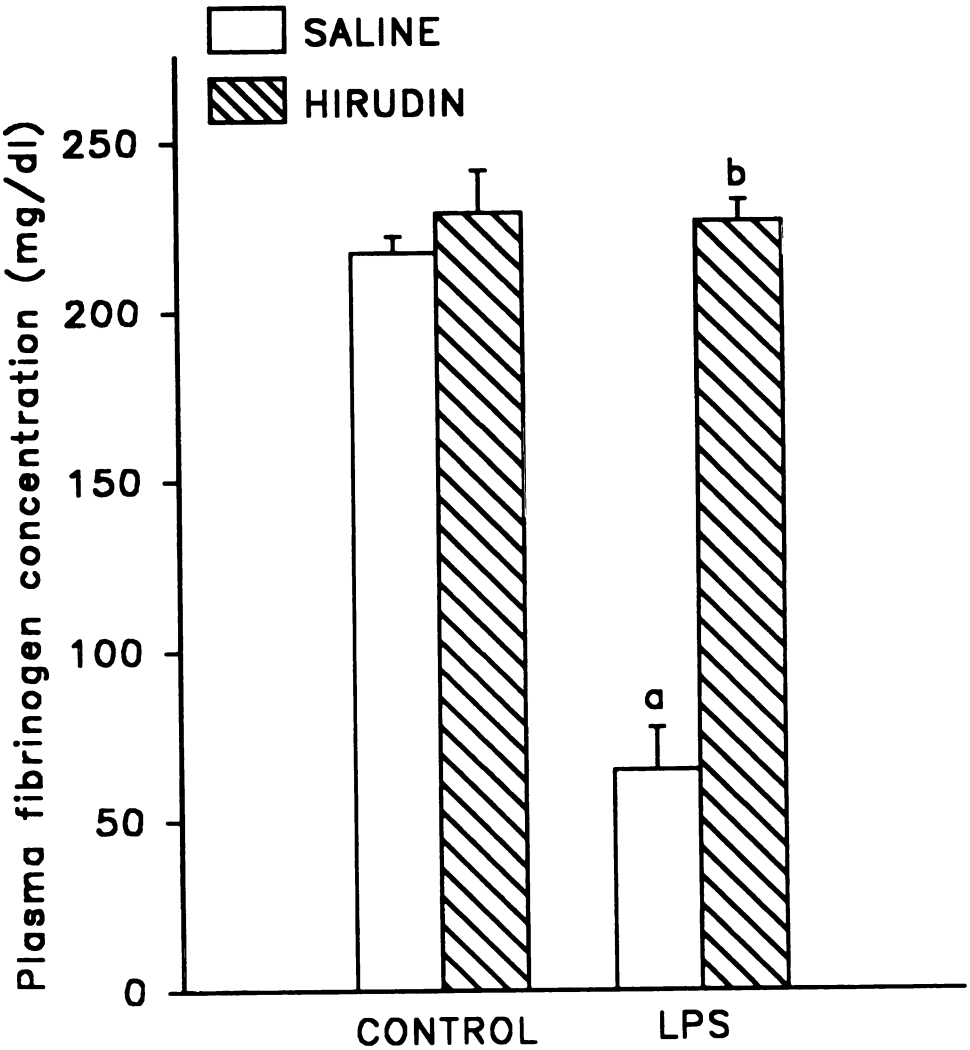

SALINE
HIRUDIN
Plasma fibrinogen concentration (mg/dl)
250
200
150
100
50
0
a
b
CONTROL
LPS

Figure 4.6

Figure 4.7. The effects of LPS and hirudin on plasma alanine aminotransferase (ALT) activity. LPS (5 mg/kg) or its saline vehicle was administered in the tail vein. Animals were treated with hirudin (36,000 U/kg, sc) or its saline vehicle 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hrs after LPS administration, blood samples were collected for measurement of plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 6-13 per group.

a, significantly different from respective CONTROL group

b, significantly different from respective SAL/LPS group

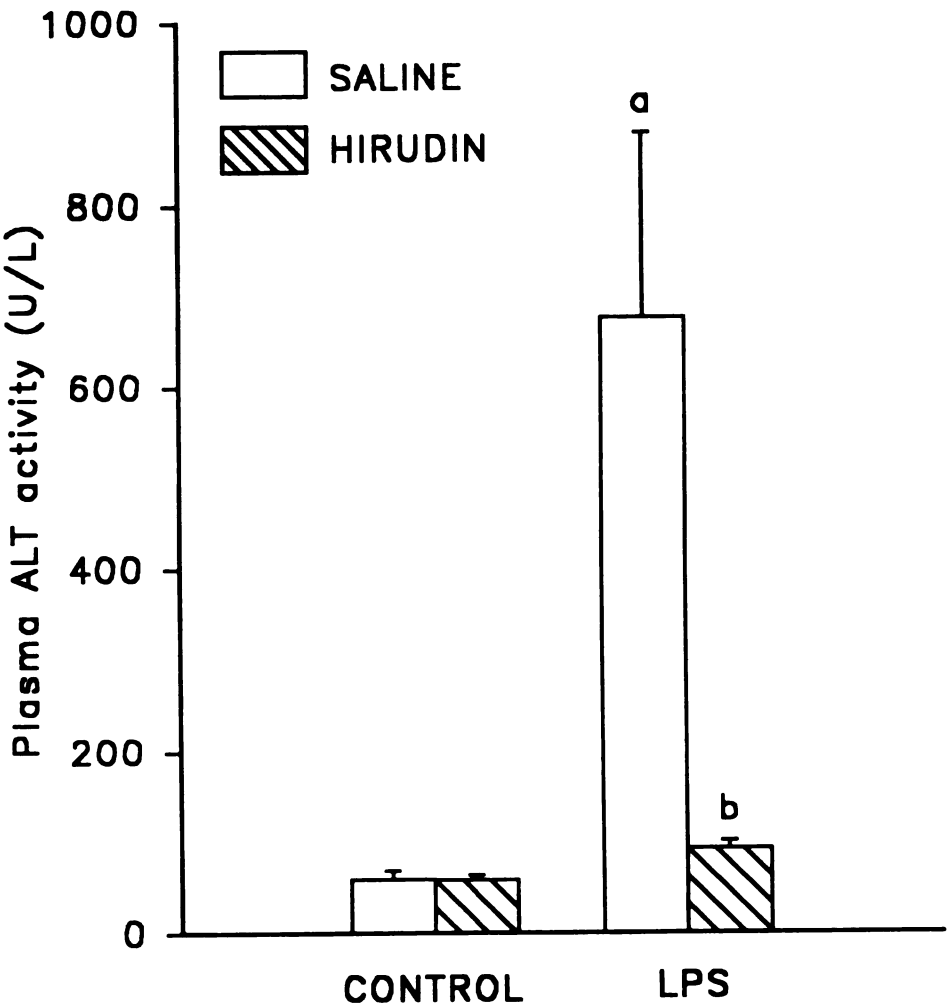
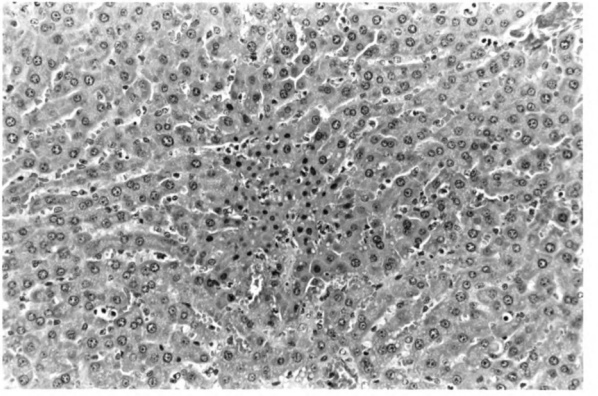

1000
800
600
400
200
0
Plasma ALT activity (U/L)
SALINE
HIRUDIN
a
b
CONTROL
LPS

Figure 4.7

Figure 4.8. Photomicrographs of sections of liver from rats treated with LPS alone (top) or with LPS and hirudin (bottom). Top: LPS-induced lesions are characterized by multifocal, large, irregularly shaped foci of midzonal hepatocellular necrosis with sinusoidal neutrophilia. Bottom: Treatment with LPS and hirudin resulted in smaller foci of midzonal hepatocellular necrosis but neutrophils were still present within the liver sinusoids.



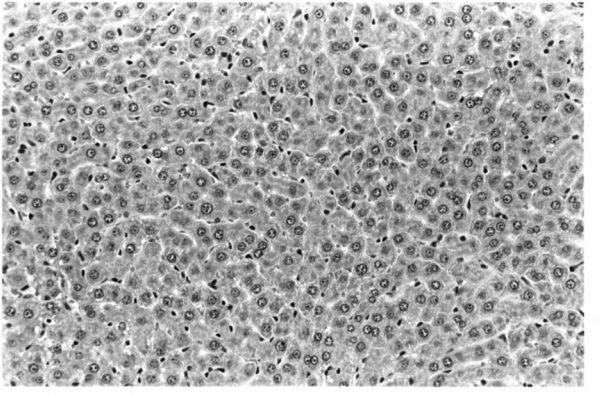


TABLE 4.3

Protection from LPS-Induced Hepatotoxicity by Administration of Hirudin

Treatment	Percent of Animals with Histopathologic Score					
	0	1	2	3	4	5
Control/SAL	100	-	-	-	-	-
Control/Hirudin	100	-	-	-	-	-
LPS/SAL	-	8	8	42	25	17
LPS/Hirudin	-	33	33	33	-	-

Rats were treated with LPS (5 mg/kg, iv) or its saline vehicle (CONTROL). Hirudin (36,000 U/kg, sc) or its saline vehicle (SAL) was administered 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hours after the administration of LPS, liver sections were collected and fixed in 10% neutral buffered formalin and prepared for analysis by light microscopy. The severity of hepatic injury was graded on a scale of 0 - 5 reflecting the frequency and size of the lesions. 0 = no evidence of inflammation or necrosis; 1 = sinusoidal neutrophilia only; 2 = sinusoidal neutrophilia with multifocal, single cell necrosis; 3 = multifocal, acute, mild hepatocellular necrosis with sinusoidal neutrophilia; 4 = multifocal, acute, moderate hepatocellular necrosis with sinusoidal neutrophilia; 5 = multifocal, acute, marked hepatocellular necrosis with sinusoidal neutrophilia. Values indicate the percentage of animals presenting each score. N=6-12 per group.

Figure 4.9. The effects of LPS and hirudin on hepatic neutrophil accumulation. LPS (5 mg/kg) or its saline vehicle was administered in the tail vein. Subsequently, animals were treated with hirudin (36,000 U/kg, sc) or its saline vehicle 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hrs after LPS administration, liver sections were collected and prepared for immunocytochemistry for neutrophils as described in Methods. Neutrophils were counted in 20 500X fields and averaged for each animal. Results are expressed as mean ± SEM. N = 6-13 per group.

a, significantly different from respective CONTROL group.

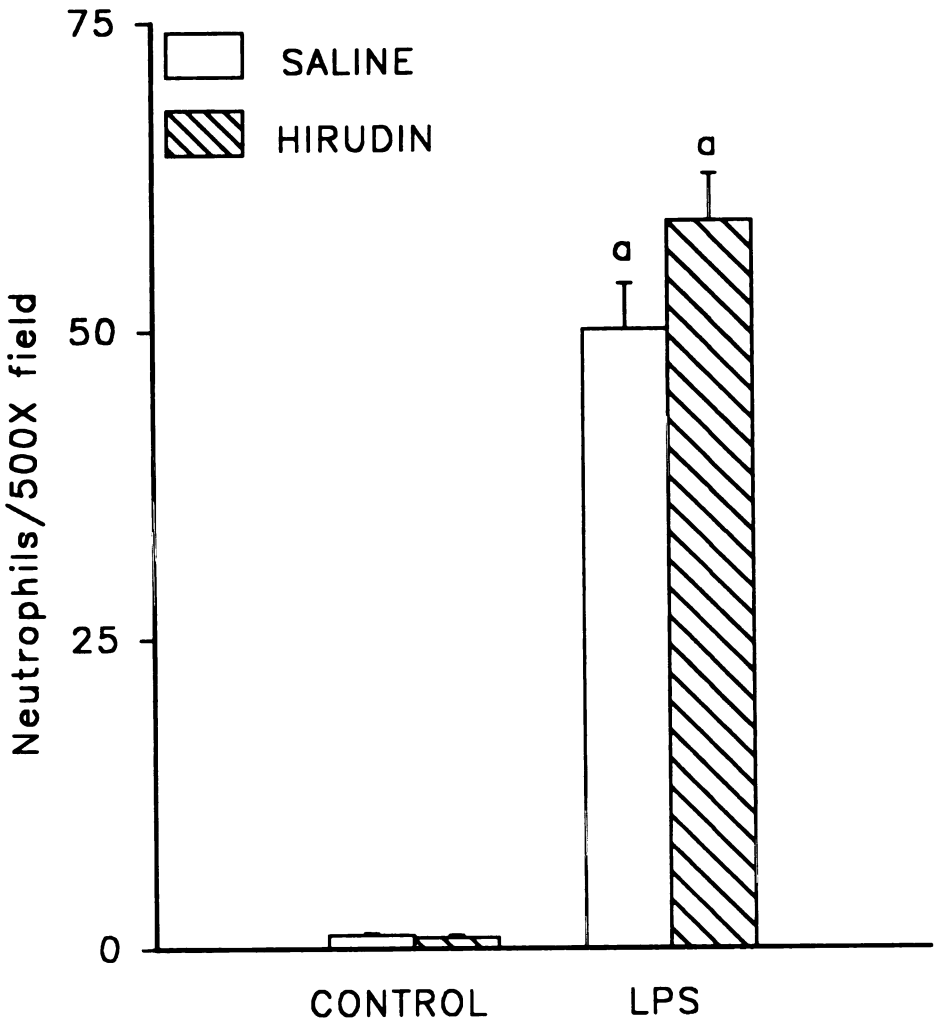

75
50
25
0
Neutrophils/500X field
SALINE
HIRUDIN
a
a
CONTROL
LPS

Figure 4.9

Figure 4.10. The effects of LPS and hirudin on blood platelet concentrations. LPS (5 mg/kg) or its saline vehicle was administered in the tail vein. Subsequently, animals were treated with hirudin (36,000 U/kg, sc) or its saline vehicle 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hrs after LPS administration, blood samples were collected for enumeration of blood platelet numbers as described in Methods. Results are expressed as mean ± SEM, N = 6-13 per group.

a, significantly different from respective CONTROL group

b, significantly different from SAL/LPS group

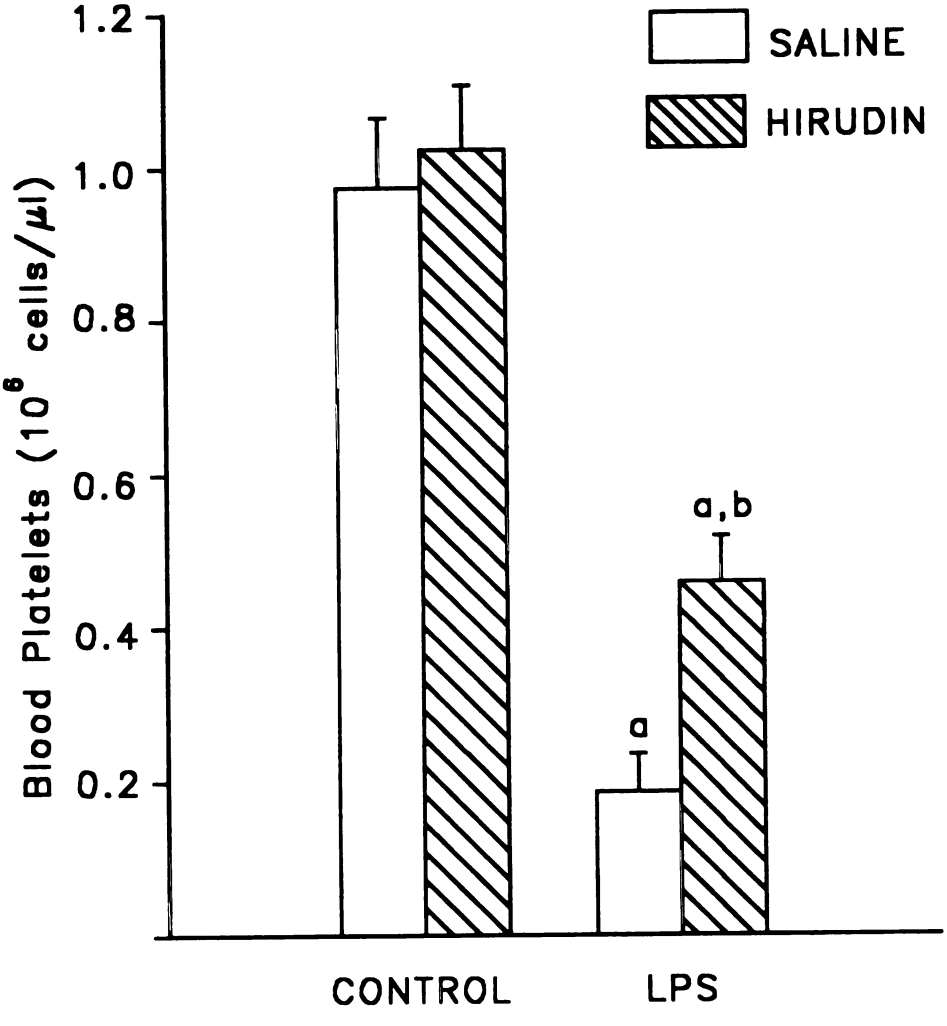

1.2
1.0
0.8
0.6
0.4
0.2
Blood Platelets (10^6 cells/μl)
SALINE
HIRUDIN
a
a,b
CONTROL
LPS

Figure 4.10

Figure 4.11. The effects of LPS and hirudin on numbers of radiolabeled platelets in blood. Two hours prior to the administration of LPS (5 mg/kg, iv) or its saline vehicle, ^{111}In-radiolabeld platelets were administered to rats. Animals were treated with hirudin (36,000 U/kg, sc) or its saline vehicle 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hrs after LPS administration, blood samples were collected for measurement of blood radioactivity as described in Methods. Results are expressed as mean ± SEM, N = 6-13 per group.

a, significantly different from respective CONTROL group

b, significantly different from SAL/LPS group

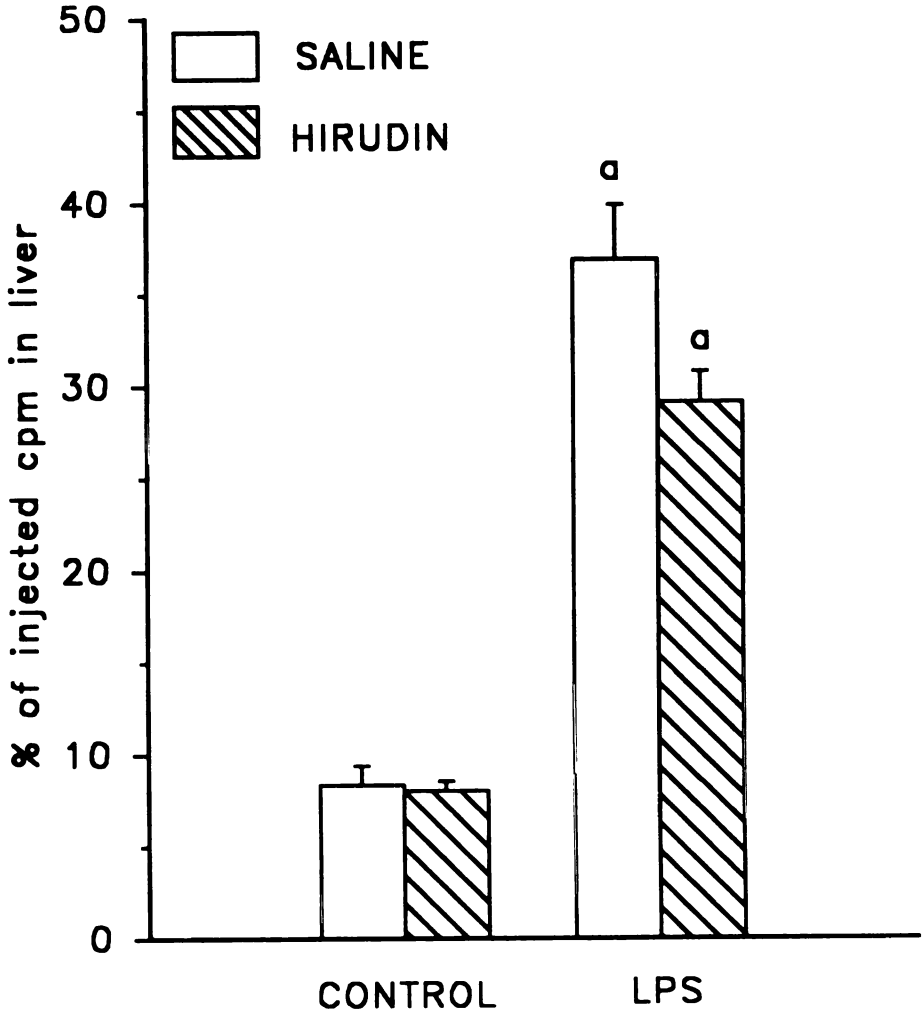

50
40
30
20
10
0
% of injected cpm in liver
SALINE
HIRUDIN
a
a
CONTROL
LPS

Figure 4.11

TABLE 4.4

Effects of LPS and Hirudin on Platelet Accumulation in Spleen, Kidneys and Lungs

TREATMENT	SPLEEN	KIDNEYS	LUNGS
CONTROL/SAL	14.6 ± 0.5	1.2 ± 0.1	3.0 ± 0.2
CONTROL/HIR	13.8 ± 0.5	1.6 ± 0.3	2.6 ± 0.1
LPS/SAL	11.3 ± 1.3	1.4 ± 0.2	2.4 ± 0.3
LPS/HIR	14.6 ± 0.7	1.9 ± 0.2	3.5 ± 0.3

Each animal received ^{111}indium-radiolabeled platelets as described in Methods to determine the distribution of platelets. Two hours after the administration of radiolabeled platelets, LPS (5 mg/kg, iv) or its saline vehicle (CONTROL) was administered. Hirudin (HIR, 36,000 U/kg, sc) or its saline vehicle (SAL) was administered 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hours after the administration of LPS, organs were collected and radioactivity was measured as a marker of platelet accumulation. Data are expressed as percent of injected radioactivity in each organ ± SEM. N=6-12.

Figure 4.12. The effects of LPS and hirudin on hepatic platelet accumulation. Rats were given ^{111}In-radiolabeled platelets i.v. as described in Methods. LPS (5 mg/kg) or its saline vehicle was administered in the tail vein. Animals were treated with hirudin (36,000 U/kg, sc) or its saline vehicle 0.5, 2.5 and 4.5 hrs after the administration of LPS. Six hrs after LPS administration, liver samples were collected for measurement of hepatic radioactivity. Results are expressed as mean ± SEM, N = 6-13 per group.

a, significantly different from respective CONTROL group

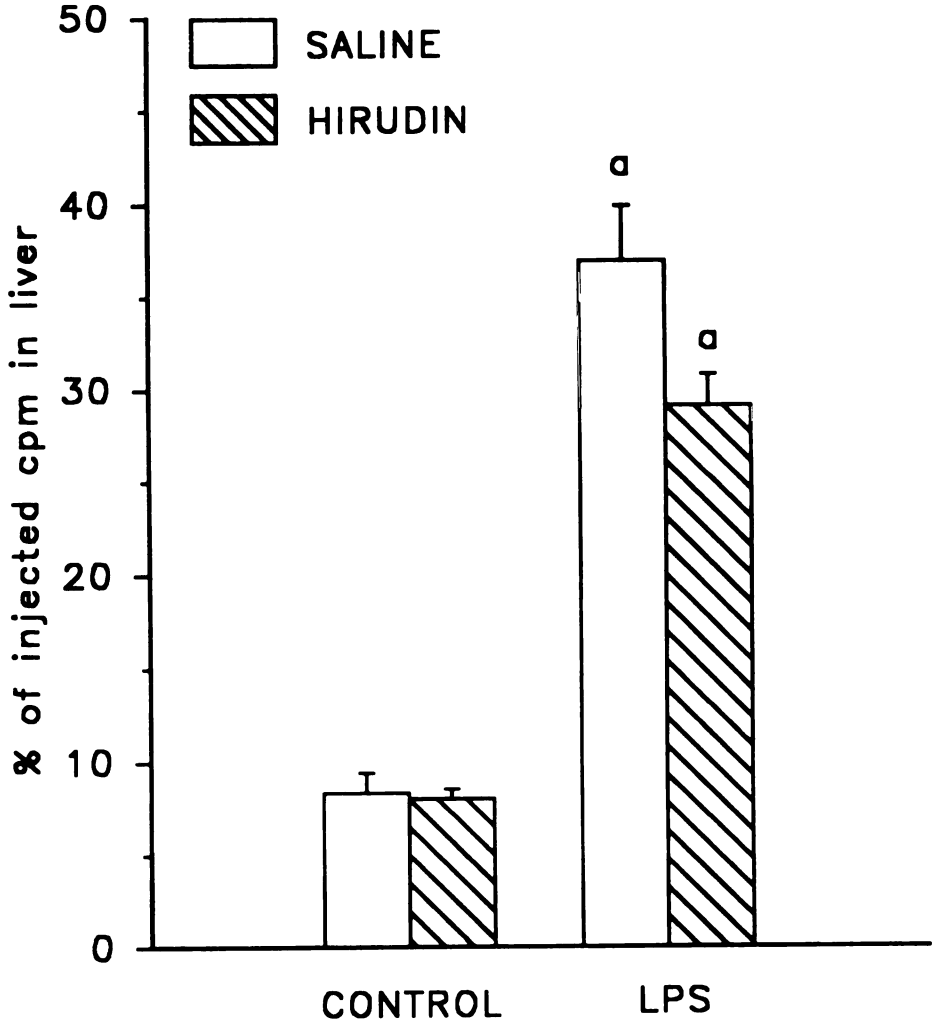

50
40
30
20
10
0
% of injected cpm in liver
SALINE
HIRUDIN
a
a
CONTROL
LPS

Figure 4.12

Figure 4.13. The effects of heparin administered before and after LPS on liver injury. Animals were treated with heparin (HEP, 2000 U/kg, iv) or its saline vehicle (SAL) 1 hr prior to (-1hr) or 1.5, 2.5 or 3.5 hrs after the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after the administration of LPS, blood was collected for assessment of liver injury as measured by plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 8-14 per group.

a, significantly different from respective SAL/LPS group

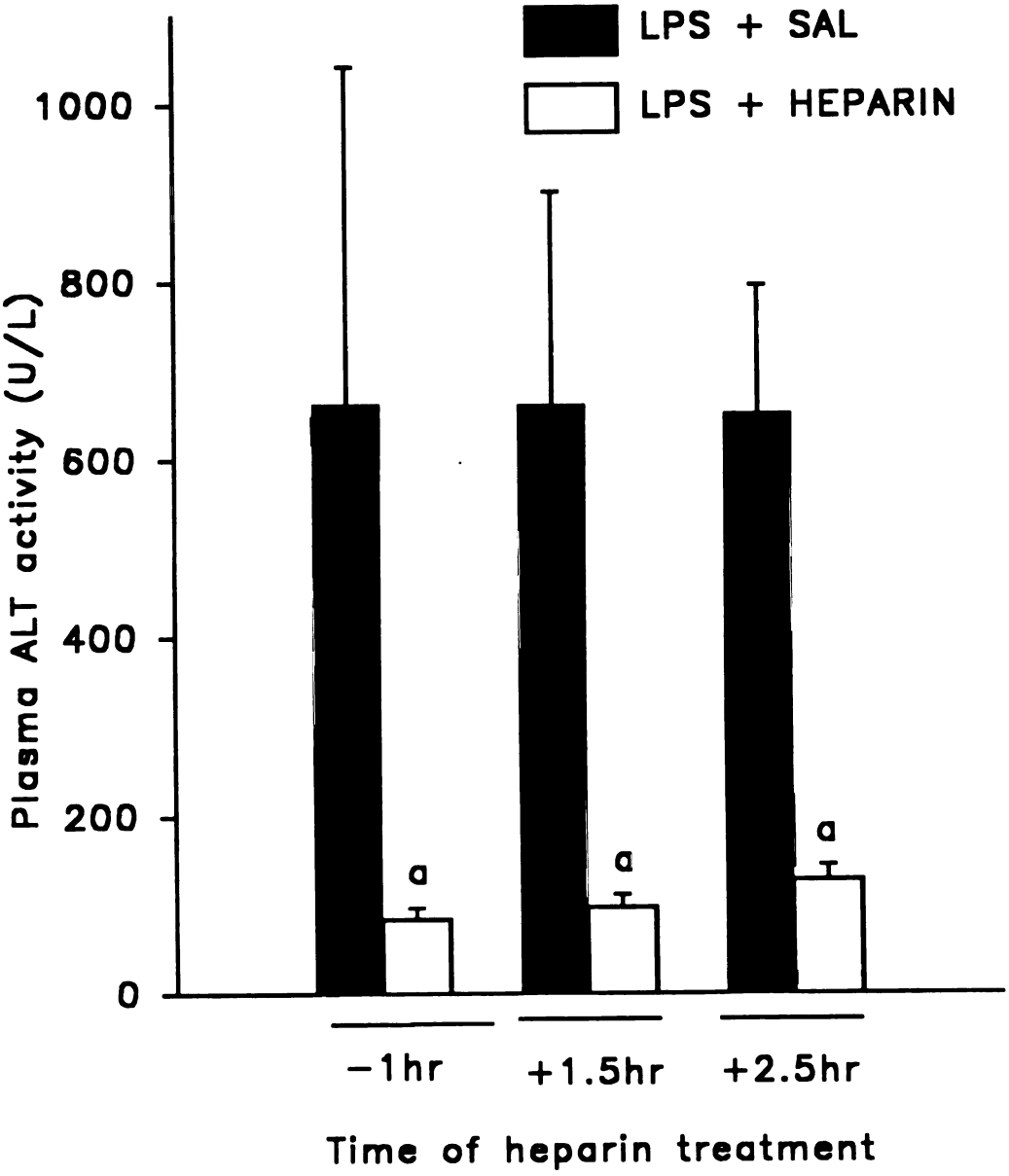

LPS + SAL
LPS + HEPARIN
1000
800
600
400
200
0
Plasma ALT activity (U/L)
a
a
a
−1hr
+1.5hr
+2.5hr
Time of heparin treatment

Figure 4.13

Figure 4.14. The effects of hirudin administered after LPS on liver injury. Animals were treated with LPS (4 mg/kg, iv) or its saline vehicle. Hirudin (36,000 U/kg, sc) or its saline vehicle was administered 2.5 and 4.5 hrs after LPS. Six hours after the administration of LPS, blood was collected for assessment of liver injury as measured by plasma ALT activity. Results are expressed as mean ± SEM, N = 6-9 per group.

a, significantly different from respective SAL/LPS group

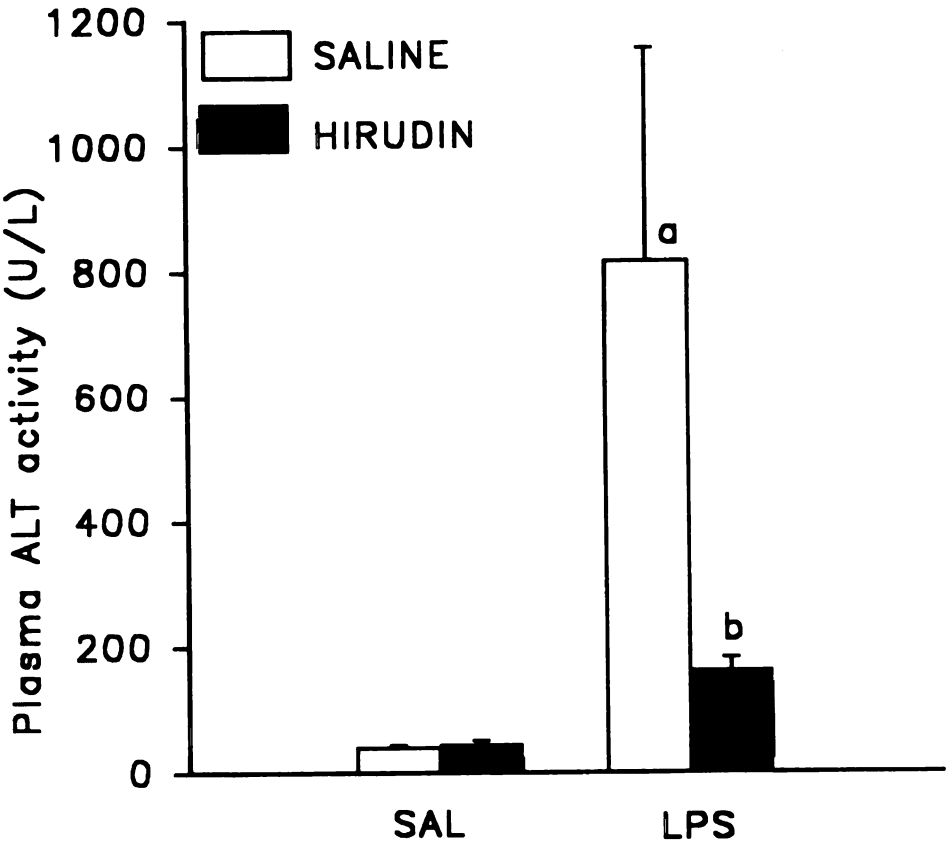

1200
1000
800
600
400
200
0
Plasma ALT activity (U/L)
SALINE
HIRUDIN
a
b
SAL
LPS

Figure 4.14

4. E. Discussion

The pathogenesis of LPS-induced liver injury entails actions of several cellular and soluble inflammatory mediators, including but not limited to neutrophils, Kupffer cells, TNF-α, platelets and the coagulation system (Hewett *et al.*,1992; Iimuro *et al.*,1994; Hewett *et al.*,1993; Pearson *et al.*,1995; Hewett *et al.*,1995). Understanding the mechanism by which exposure to LPS culminates in hepatic injury necessitates knowing when and how these inflammatory mediators interact. In this model, neutrophils and platelets begin to accumulate in the liver within 1 hr after LPS administration, and peak concentrations of cytokines such as TNF-α occur 90 minutes after LPS administration (Pearson *et al.*,1995). These events precede the onset of liver injury, which is not detectable as increased plasma ALT activity until nearly 4 hrs after LPS administration (Pearson *et al.*,1995).

Results from this and other studies indicate that the coagulation system is critical to the development of LPS-induced hepatotoxicity (Hewett *et al.*,1995; Margaretten *et al.*,1967). Our results suggest that the coagulation system does not mediate LPS-induced thrombocytopenia or the platelet and neutrophil accumulation observed in livers of rats exposed to LPS. In addition, the protection from injury by heparin or hirudin administered after LPS at times when neutrophils and platelets have accumulated within the liver suggests that thrombin is a "distal" mediator of liver injury; that is, its action occurs after the accumulation of inflammatory cells in the liver and after exposure to cytokines

like TNF-α (Pearson *et al.*, 1995; Hewett *et al.*, 1993).

These findings raise a question as to how the coagulation system contributes to the development of hepatotoxicity. It has been hypothesized that the coagulation system contributes to tissue injury through the formation of occlusive fibrin thrombi that results in decreased tissue perfusion and ischemia. However, treatment of rats with ancrod, a snake venom enzyme that anticoagulates blood by depleting plasma of fibrinogen, failed to prevent liver lesions after LPS exposure. This result suggests that the role of the coagulation system in the development of LPS-induced liver injury is independent of fibrinogen and of the formation of insoluble fibrin thrombi (Hewett *et al.*, 1995). The location of the lesion further argues against ischemia as the basis for injury, inasmuch as studies of hepatic ischemia/reperfusion in the rat have shown that resulting damage is characterized by centrilobular tissue injury, whereas LPS exposure results primarily in midzonal hepatocellular necrosis (Jaeschke *et al.*, 1990; Kehrer *et al.*, 1990). Furthermore, in this model the liver injury begins within 4 hrs after LPS exposure, and results with heparin and hirudin suggest that the coagulation system becomes important between 2.5 and 3.5 hrs after LPS administration, i.e. just prior to the onset of overt liver injury (Pearson *et al.*, 1995). Thus, there is a relatively short period of time between activation of the coagulation system and the onset of liver injury, a finding that does not support an ischemia-based mechanism, which requires a longer duration of reduced blood flow to cause injury to the liver (Jaeschke *et al.*, 1990; Jaeschke *et al.*, 1991a; Hughes *et al.*, 1992).

The exact role of the coagulation system in this model of hepatotoxicity remains unclear. Anticoagulants such as warfarin or heparin that interfere with the actions of thrombin are hepatoprotective, but ancrod, an anticoagulant which does not inhibit thrombin or prevent its formation, failed to prevent liver injury (Hewett *et al.*, 1995). These findings point to thrombin as a mediator of LPS-induced liver injury. Thrombin, a serine protease, has long been known for its role in the coagulation system, and activation of either the intrinsic or extrinsic pathways of coagulation by LPS culminates in the formation of thrombin from prothrombin. To evaluate further the role of the coagulation system and thrombin in particular, we utilized the anticoagulant, heparin, and the selective thrombin inhibitor, hirudin.

Hirudin, a peptide initially isolated from the saliva of leeches (*Hirudo medicinalis*), is an extremely potent and selective inhibitor of thrombin. It forms a highly stable complex with thrombin and blocks its catalytic site (Fenton *et al.*, 1991; Markwardt, 1989; Talbot, 1989; Fenton, 1989). The anticoagulant, heparin, can also inhibit thrombin but lacks the specificity of hirudin. Heparin increases the affinity of antithrombin III (ATIII) for thrombin, but ATIII also has affinity for other coagulation factors, namely Factors IXa, Xa, XIa and XIIa and other proteoloytic enzymes such as kallikrein, trypsin and plasmin (Olson *et al.*, 1994). Heparin's effect on blood cells is complex. In regard to platelets, heparin can affect platelet aggregation and can cause thrombocytopenia in human beings. In addition, activated platelets can release platelet factor 4, a protein that can neutralize heparin. Heparin inhibits certain neutrophil functions

in vitro, including degranulation and neutrophil-induced platelet aggregation via inhibition of cathepsin G, a protease released from activated neutrophils that effects hepatocellular killing *in vitro* (Ferrer-Lopez *et al.*, 1992; Webb *et al.*, 1993; Ho *et al.*, 1995). Morever, the administration of heparin can influence inflammatory events by increasing extracellular superoxide dismutase (Becker *et al.*, 1994), activating the complement system, binding to endothelium (Olson *et al.*, 1994) and inhibiting soluble phospholipase A_2 (Dua *et al.*, 1994).

Since heparin can have significant interactions with both neutrophils and platelets, cells which are critical to LPS-induced liver injury (Hewett *et al.*, 1992; Pearson *et al.*, 1995), we chose to evaluate the role of thrombin in this model by using the more selective thrombin inhibitor, hirudin. Results from this study indicate that thrombin is needed for the manifestation of LPS-induced hepatotoxicity, since treatment with hirudin significantly attenuated liver injury as measured by plasma ALT activity and histologic evidence of necrosis.

In these studies, hirudin treatment attenuated LPS-induced thrombocytopenia, as measured by both platelet concentration and ^{111}In-radiolabeled platelets in blood. This suggests that thrombin may contribute, somewhat, to the thrombocytopenia observed after LPS administration by a mechanism likely due to the direct platelet aggregating effect of thrombin. Although a similar trend was observed in studies conducted with heparin, it was not statistically significant. The discrepancies between these findings may result from heparin's inhibitory effect on platelet function and further supports the hirudin's specificity and value in *in vivo* studies.

Thrombin is necessary for fibrin clot formation, but the actions of thrombin are not limited to the coagulation system. Thrombin can influence many cells including endothelium, neutrophils and platelets through receptor-mediated actions. For example, thrombin can influence cell adherence by stimulating the expression of adhesion molecules and the release of cytokines (Garcia *et al.*, 1986; DeMichele *et al.*, 1992). Such an event could contribute to the inflammatory cell accumulation in the livers after LPS exposure. Furthermore, thrombin is an activator of platelets and a chemoattractant and activator of neutrophils (Morin *et al.*, 1990; Bizios *et al.*, 1986; Cohen *et al.*, 1991).

In this model, thrombin does not appear to promote the accumulation of neutrophils or platelets in the liver since accumulation was unaffected by heparin administration. Other studies have shown that heparin does not alter neutrophil sticking to mesenteric venules in response to LPS as measured by intravital fluorescence microscopy and luminol-dependent chemiluminescence techniques (Suzuki *et al.*, 1988). Also, the administration of heparin or hirudin attenuated liver injury in this model after these inflammatory cells had accumulated within the liver. It is possible that thrombin may activate these cells after their hepatic accumulation. A mechanism by which thrombin stimulates the inflammatory cells already present within the sinusoids would be consistent with the findings that LPS-induced liver injury is dependent upon neutrophils and platelets as well as thrombin. Of potential interest in this regard is the recent observation that thrombin-activated platelets can increase the release of cytotoxic factors from neutrophils *in vitro* (Gomez *et al.*, 1989).

Whether thrombin acts via such a mechanism *in vivo* remains to be determined.

Kupffer cells, the resident macrophages of the liver, play a critical role in LPS-induced liver injury (24; Shibayama *et al.*, 1991). Little is known about the effect of thrombin on these cells, however thrombin alters the production of cytokines and arachidonic acid metabolites in other types of macrophages *in vitro* (Bar-Shavit *et al.*, 1983a; Bar-Shavit *et al.*, 1983b; Jones *et al.*, 1990; Podjarny *et al.*, 1989). Accordingly, thrombin might contribute to the pathogenesis of liver injury by stimulating the release of inflammatory mediators from Kupffer cells. Thrombin can activate endothelium to release coagulation factors and cytokines and to express adhesion molecules (Bizios *et al.*, 1986; DeMichele *et al.*, 1992). These events could contribute to the activation of inflammatory cells. Thus, thrombin's critical role in LPS-induced hepatocellular injury may derive from one or more of its effects on cells in the hepatic sinusoid.

It is possible that thrombin may contribute to parenchymal cell necrosis by a direct effect on these cells. High and low affinity binding sites for thrombin on hepatocytes have been reported, although the function of these "receptors" is not known (Weyer *et al.*, 1988). Thrombin stimulates glycogenolysis in isolated, perfused livers by a cyclooxygenase-dependent mechanism (Yamanaka *et al.*, 1992). However, the direct effects of thrombin on liver parenchymal cells and the possibility that thrombin may directly contribute to hepatocellular injury remain to be determined.

The finding that heparin or hirudin provides protection when given after

the accumulation of neutrophils and platelets within the liver provides insight into the mechanism(s) by which LPS exposure culminates in liver injury. For example, this finding suggests that the mere presence of neutrophils and platelets within the liver is not sufficient to promote hepatocellular injury. Thus, it raises the possibility that thrombin may act directly on these inflammatory cells to stimulate the release of mediators that causes tissue injury. It is also possible that neutrophils and platelets alter hepatic parenchymal cells in a way that makes them susceptible to injury by an activated coagulation system.

That thrombin is a distal link in the chain of events that produce liver injury may prove useful in the clinical setting. In experimental studies, drugs that interfere with cellular and soluble mediators have usually been administered to animals prior to the administration of endotoxin. In this study, we have found an agent that affords protection when administered after LPS exposure and in close temporal apposition to the onset of liver injury. In endotoxemic patients, for whom prophylactic treatment is not practical, this quality has considerable importance.

In summary, results from this study increase current understanding of the mechanism(s) by which LPS exposure culminates in hepatocellular damage. Results from studies in animals were pretreated with heparin or hirudin suggest that the coagulation system, although critical for the genesis of liver damage, is not needed for the recruitment of platelets and neutrophils into the liver. Furthermore, the novel finding that thrombin is a critical yet distal mediator of liver injury raises an interesting question as to how thrombin mediates

hepatocellular injury during inflammation.

CHAPTER 5

THE ROLES OF PAF AND LEUKOTRIENES IN LPS-INDUCED LIVER INJURY

5. A. Abstract

The intravenous administration of lipopolysaccharide (LPS) to rats results in liver lesions characterized by the infiltration of both platelets and neutrophils into the liver and by midzonal hepatocellular necrosis. The liver injury is dependent on neutrophils, platelets and the coagulation system, as removal or inhibition of any of these components inhibits the development of hepatocellular necrosis. Platelet activating factor (PAF) and the cysteinyl leukotrienes (LTs) are potent inflammatory lipids that are critical for the development of some LPS-mediated alterations. To test the hypothesis that PAF, alone or in combination with LTs, contributes to the development of liver injury during LPS exposure, we conducted studies with the PAF receptor antagonist, WEB 2086, and the 5-lipoxygenase inhibitor, Zileuton. Female, Sprague-Dawley rats were pretreated with WEB 2086 (10 mg/kg, ip) alone or with Zileuton (40 mg/kg, po) 1 hour before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Treatment with WEB 2086, alone or in combination with Zileuton, did not inhibit LPS-mediated hepatic neutrophil infiltration or liver injury, as assessed by histologic evaluation and increases in plasma alanine aminotransferase activity. Pretreatment with these agents also had no effect on the activation of the coagulation system or on the thrombocytopenia induced by LPS. These results suggest that PAF, alone or in combination with 5-lipoxygenase products, is not a critical mediator of LPS-induced hepatocellular injury in this model.

5. B. Introduction

Platelet activating factor (PAF, 1-0-alkyl-2(R)-acetyl-glycero-3-phosphocholine) is a lipid mediator that is critical for the development of numerous LPS-mediated alterations, including hypotension (Casals-Stenzel *et al.*,1988), lung injury (Olson *et al.*,1990; Yue *et al.*, 1981) and death (Casals-Stenzel *et al.*,1988; Salari *et al.*,1990; Rabinovici *et al.*,1990). PAF is produced by leukocytes, platelets and endothelial cells and has proinflammatory properties. For example, it can mediate neutrophil chemotaxis and activation (Worthen *et al.*,1983; Montrucchio *et al.*,1993; Takahashi *et al.*,1991), increase vascular permeability and alter vascular tone (Hosford *et al.*, 1993; Buxton *et al.*,1986). PAF also stimulates the production of other soluble mediators such as eicosanoids, cytokines and superoxide anion (Hosford *et al.*, 1993; Snyder,1990; Takahashi *et al.*,1991).

A recent study indicated that antagonism of PAF receptors alone is insufficient to prevent liver injury in the rat after the intraperitoneal administration of LPS (Yoshikawa *et al.*,1992). Similarly, administration of a 5-lipoxygenase inhibitor afforded no protection. However, the combination of a PAF receptor antagonist and a 5-lipoxygenase inhibitor prevented hepatic injury and lethality (Yoshikawa *et al.*,1992). This study supported the hypothesis that PAF in combination with LTs is critical for the development of hepatic injury and suggested that PAF and LTs may have redundant actions in this model.

Like PAF, the LTs can mediate inflammatory responses, edema and

changes in vascular tone. With regard to other alterations following LPS exposure, the LTs are involved in the development of leukopenia (Cook *et al.*, 1985). In addition, LTC_4 and LTD_4 have been detected in the bile of LPS-treated rats (Hagmann *et al.*, 1985), and the intravenous administration of LPS results in LT formation *in vivo* through a mechanism that depends upon complement (Jaeschke *et al.*, 1992c). The LTs are critical for LPS-induced liver injury in another experimental model of endotoxemia, namely in mice sensitized with galactosamine (Tiegs *et al.*, 1988). However, in the rat the liver injury observed after the intraperitoneal administration of LPS liver injury is not mediated by 5-lipoxygenase products, alone, but rather by the combination of LTs and PAF (Yoshikawa *et al.*, 1992). Since studies evaluating the role of the cysteinyl LTs appear to be model-dependent, we thought it important to evaluate the role of the LTs in a model of liver injury resulting from the intravenous administration of LPS in the rat.

The pathogenesis of hepatocellular injury that follows the intravenous administration of LPS in the rat has been well characterized and is dependent upon neutrophils, platelets, Kupffer cells and tumor necrosis factor-*alpha* and requires activation of the coagulation system (Hewett *et al.*, 1992; Pearson *et al.*, 1995; 24; Iimuro *et al.*, 1994; Hewett *et al.*, 1993; Hewett *et al.*, 1995). Although several studies have implicated PAF and LTs in the development of liver injury in other models, the roles of PAF and LTs appears to be dependent upon species and route of administration of LPS. Therefore, studies were conducted to evaluate the roles of these mediators in the development of liver

injury in the rat after the intravenous administration of LPS. To evaluate the roles of PAF and LTs, rats were pretreated with either the PAF receptor antagonist, WEB 2086, alone or in combination with the 5-lipoxygenase inhibitor, Zileuton. We evaluated the effects of the coadministration of WEB 2086 and Zileuton on LPS-induced liver injury, thrombocytopenia and activation of the coagulation system.

5. C. Materials and methods

5. C. 1. Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12) and Kit 59 for determination of plasma alanine aminotransferase activity (ALT) were purchased from Sigma Chemical Company (St. Louis, MO). Dilution of blood and lysis of erythrocytes for platelet enumeration were performed in platelet Unopettes (Baxter Scientific Products, McGaw Park, IL). Plasma fibrinogen concentration was measured in a BBL Fibrometer (Becton, Dickinson and Company, Hunt Valley, MD) using Data-Fi fibrinogen determination kit (Baxter Scientific Products, McGaw Park, IL). PAF was purchased from Calbiochem (San Diego, CA). WEB 2086 was a generous gift from Boehringer Ingelheim Corporation (Ridgefield, CT). Zileuton was a generous gift from Abbott Laboratories (Abbott Park, IL).

5. C. 2. Animals

See Chapter 2, section C. 2.

5. C. 3. Verification of drug efficacy

Preliminary studies were conducted to establish an effective dosing regimen for the PAF receptor antagonist, WEB 2086, and the 5-lipoxygenase inhibitor, Zileuton. The administration of WEB 2086 (10 mg/kg, ip) inhibited PAF-induced hypotension by 86%. PAF receptor antagonism occurred by 30 minutes after the administration of WEB 2086 and was maintained up to 8 hours thereafter. These results have been published in detail elsewhere (Baile *et al.*,1995b). The efficacy of 5-lipoxygenase inhibition with Zileuton was verified by evaluating the *ex vivo* production of LTB_4 after stimulation of whole blood with the calcium ionophore A23187. Six hours after the administration of Zileuton (40 mg/kg, po), LTB_4 production in whole blood was inhibited by 85% (Bailie *et al.*1995a).

5. C. 4. Experimental protocols

To address the role of PAF in liver injury, animals were pretreated with WEB 2086 (10 mg/kg, ip) or saline vehicle 1 hour before the administration of LPS (4 mg/kg, iv) or saline vehicle. Six hours after the administration of LPS, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was collected into sodium citrate (0.38% final concentration) from the descending aorta, and liver sections were

fixed in 10% neutral buffered formalin for histologic evaluation. Plasma was assayed for ALT activity, a marker of hepatocellular injury.

To address the possible redundancy in the roles of PAF and LTs in liver injury, a combination of WEB 2086 and Zileuton was employed. Rats were treated with either WEB 2086 (10 mg/kg, ip) or saline vehicle and with either Zileuton (40 mg/kg, po) or 0.2% methylcellulose vehicle 1 hour before the administration of LPS (4 mg/kg, iv). Six hours after LPS administration, animals were anesthetized as described above and blood and liver sections were collected.

Blood platelets were enumerated in a hemacytometer after erythrocyte lysis and dilution in platelet Unopettes. Plasma ALT activity was used as a marker of hepatic parenchymal cell injury and was determined spectrophotometrically using Sigma Kit 59 (Bergmeyer *et al.*, 1978). Plasma fibrinogen concentration was determined from the thrombin clotting times of diluted plasma in a fibrometer (Hewett *et al.*, 1995).

5. C. 5. Statistical analysis

Data were analyzed with a completely random, two-way analysis of variance (ANOVA). Homogeneity of variance was tested with the F-max test prior to analysis (Steel and Torrie, 1980). Nonhomogeneous data were log-transformed prior to analysis. Comparisons between treatment means were performed using Tukey's omega test. The criterion for significance was $p \leq 0.05$. Results are expressed as mean ± SEM.

5. D. Results

The administration of LPS to rats resulted in hepatocellular necrosis within 6 hrs. This liver injury was assessed by elevations in plasma alanine aminotransferase (ALT) activity, an indication of a loss in parenchymal cell integrity. Pretreatment with the PAF receptor antagonist, WEB 2086, did not prevent LPS-induced hepatocellular damage (Figure 5.1).

To address the possibility that 5-lipoxygenase metabolites and PAF may have critical but redundant actions, animals were cotreated with WEB 2086 and Zileuton. As before, the administration of LPS produced a significant elevation in plasma ALT activity, indicative of liver injury (Figure 5.2). Cotreatment with WEB 2086 and Zileuton did not prevent this liver injury. The findings with ALT activity were consistent with results of histologic evaluation of liver sections. LPS administration resulted in a pronounced infiltration of neutrophils into the liver tissue and multifocal, irregularly shaped areas of midzonal hepatocellular necrosis (Figure 5.3). These lesions were characterized by hypereosinophilic parenchymal cells with small pyknotic nuclei and indistinct cytoplasmic borders. Neither LPS-induced sinusoidal neutrophilia nor hepatocellular necrosis were altered by pretreatment with WEB 2086 and Zileuton.

As reported previously (Hewett *et al.*,1995; Pearson *et al.*,1995), the intravenous administration of LPS resulted in a decrease in blood platelet concentration (Figure 5.4) and plasma fibrinogen concentration (Figure 5.5), a marker of activation of the coagulation system. Pretreatment with WEB 2086

and Zileuton did not significantly alter LPS-induced thrombocytopenia (Figure 5.4) or the decrease in plasma fibrinogen concentration (Figure 5.5).

Figure 5.1. The effects of LPS and WEB 2086 on plasma ALT activity. Animals were treated with WEB 2086 (10 mg/kg, ip) or its saline vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 5-8 per group.

a, significantly different from respective value in the absence of LPS

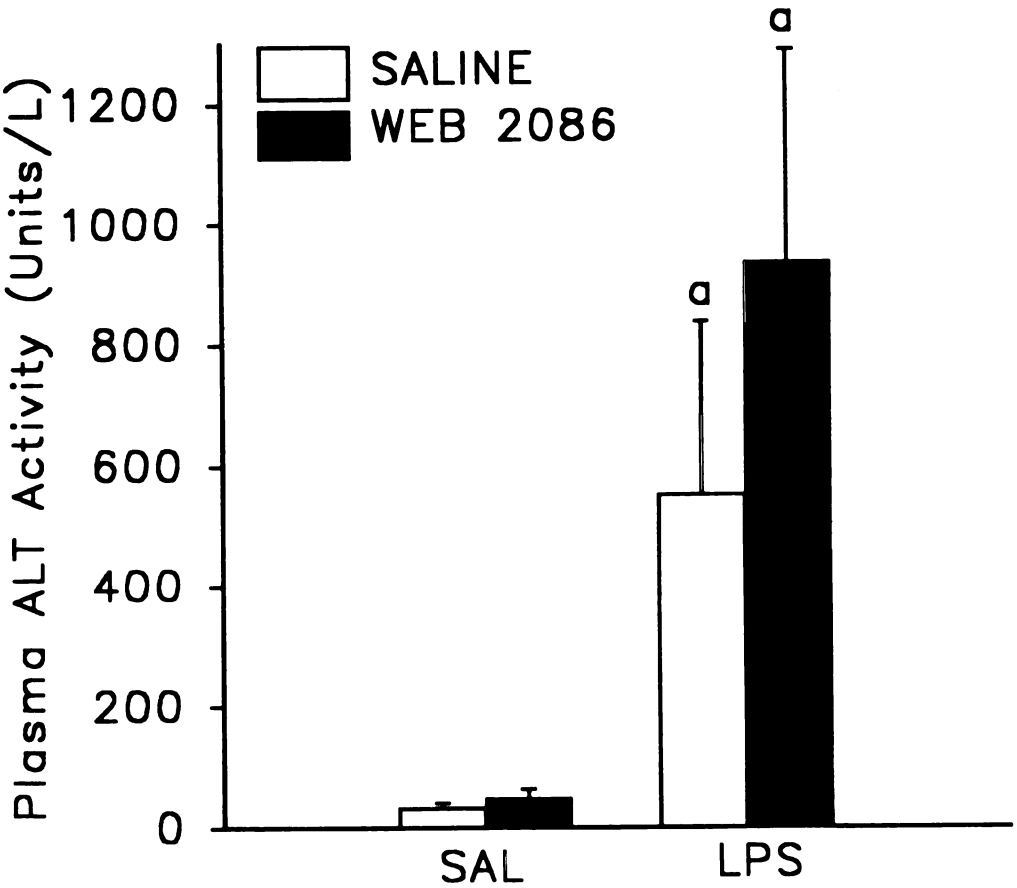

SALINE
WEB 2086
Plasma ALT Activity (Units/L)
1200
1000
800
600
400
200
0
a
a
SAL
LPS

FIGURE 5.1

Figure 5.2. The effects of LPS and WEB 2086/Zileuton on plasma ALT activity. Animals were treated with WEB 2086 (10 mg/kg, ip) plus Zileuton (40 mg/kg, po) or their respective vehicles (saline and methylcellulose (MC), respectively) 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 5-8 per group.

a, significantly different from respective value in the absence of LPS

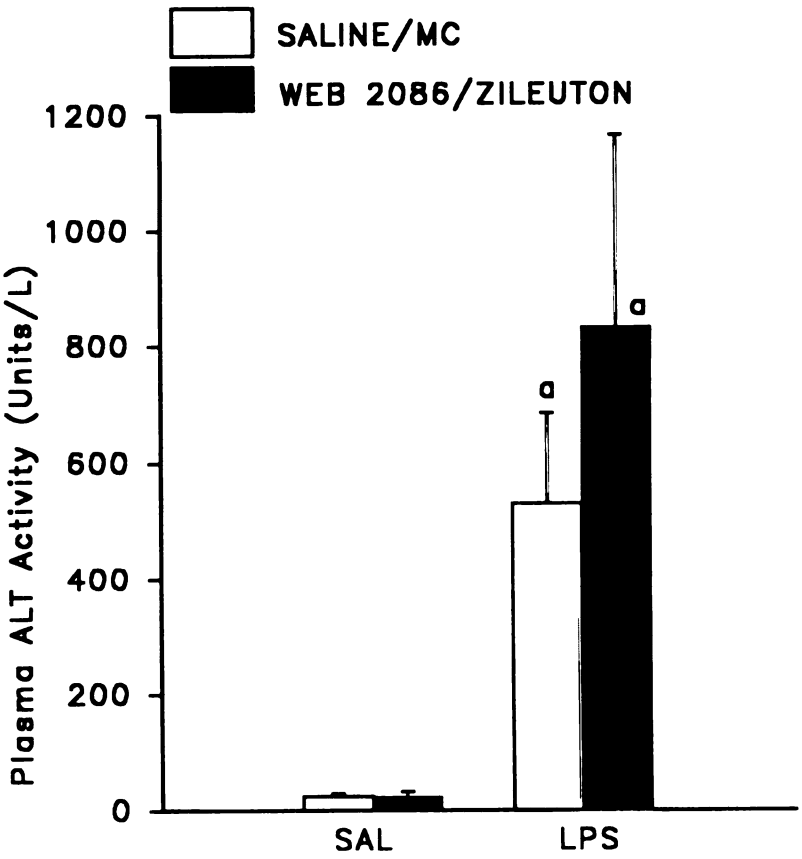

SALINE/MC
WEB 2086/ZILEUTON
Plasma ALT Activity (Units/L)
1200
1000
800
600
400
200
0
a
a
SAL
LPS

Figure 5.2

Figure 5.3. The effects of LPS and WEB 2086/Zileuton on liver injury. Animals were treated with WEB 2086 (10 mg/kg, ip) plus Zileuton (40 mg/kg, po) or their respective vehicles (saline and methylcellulose (MC), respectively) 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, liver sections were collected, fixed and processed for histologic evaluation. LPS administration resulted in a marked sinusoidal neutrophilia and midzonal hepatocellular necrosis. Pretreatment with WEB 2086 and Zileuton did not alter either the neutrophilia or the hepatocellular necrosis observed after LPS administration.

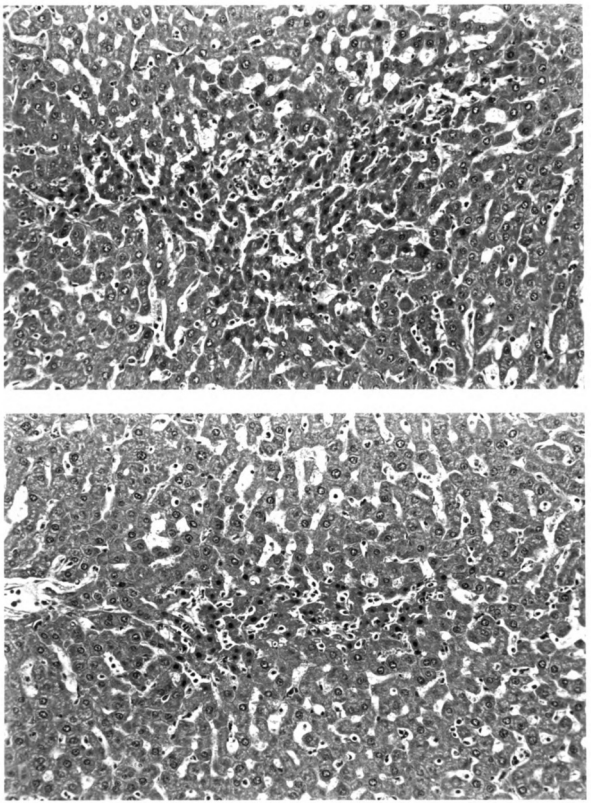


Figure 5.4. The effects of LPS and WEB 2086/ Zileuton on blood platelet concentration. Animals were treated with WEB 2086 (10 mg/kg, ip) plus Zileuton (40 mg/kg, po) or their respective vehicles (saline and methylcellulose (MC), respectively) 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for enumeration of blood platelets as described in Methods. Results are expressed as mean ± SEM, N = 5-8 per group.

a, significantly different from respective value in the absence of LPS

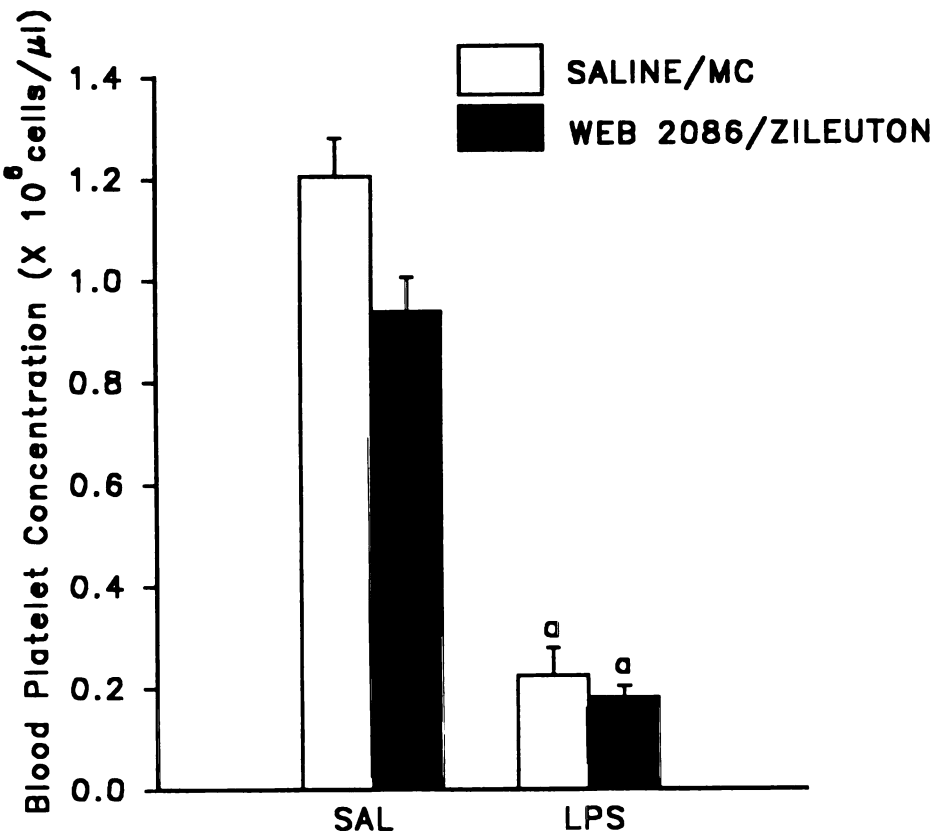
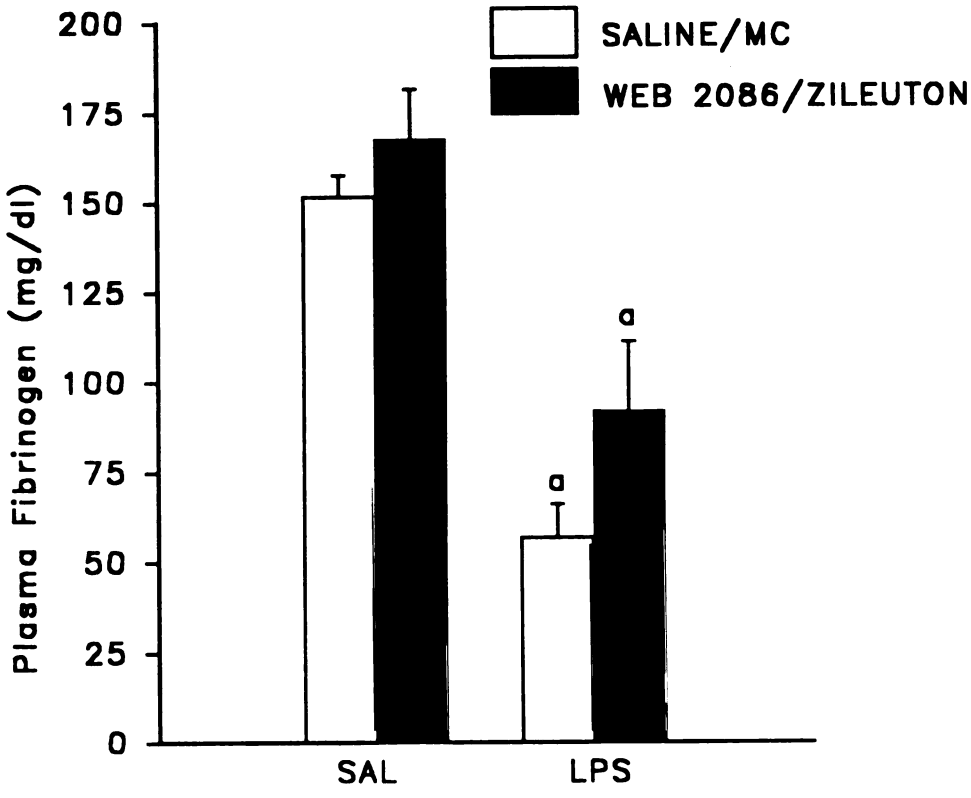

Blood Platelet Concentration (X 10^6 cells/μl)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SALINE/MC
WEB 2086/ZILEUTON
a
a
SAL
LPS

Figure 5.5. The effects of LPS and WEB 2086/ Zileuton on plasma fibrinogen concentration. Animals were treated with WEB 2086 (10 mg/kg, ip) plus Zileuton (40 mg/kg, po) or their respective vehicles (saline and methylcellulose (MC), respectively) 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after the administration of LPS, blood was collected for measurement of plasma fibrinogen concentration as described in Methods. Results are expressed as mean ± SEM, N = 5-8 per group.

a, significantly different from respective value in the absence of LPS


SALINE/MC
WEB 2086/ZILEUTON
Plasma Fibrinogen (mg/dl)
200
175
150
125
100
75
50
25
0
a
a
SAL
LPS

5. E. Discussion

Results from this study suggest that PAF, alone or in combination with LTs, is not required for the thrombocytopenia, activation of the coagulation system or liver injury that follows the intravenous administration of *E. coli* LPS to rats. In preliminary studies, the effectiveness of the treatment regimens for WEB 2086 and Zileuton were verified (Bailie *et al.*, 1995a; Bailie *et al.*, 1995b). The results indicated that these drugs effectively antagonized the actions of PAF and LT biosynthesis *in vivo*. Similar doses of WEB 2086 (1-10 mg/kg) have been used in another rat model in which it protected against the lethal effects of intravenous administration of 15 mg/kg *E. coli* LPS (Casals-Stenzel *et al.*, 1988), supporting the contention that the lack of effect of WEB 2086 on LPS-induced liver injury was not due to insufficient antagonism of PAF.

Our finding that blockade of PAF and LTs did not prevent liver injury in this model is in contrast to results using other models of endotoxemia. For example, pretreatment with the PAF receptor antagonist, CV 3988, in combination with the 5-lipoxygenase inhibitor, ONO-1078, prevented liver injury in the rat after LPS exposure (Yoshikawa *et al.*, 1992). The contrasting results may derive from a difference in experimental models. In the study of Yoshikawa *et al.* (Yoshikawa *et al.*, 1992), animals were exposed to a larger dose of LPS (7 mg/kg) by intraperitoneal rather than intravenous injection and liver injury was assessed 3 hours, rather than 6 hours later. In addition, the liver injury they observed was not as pronounced as that observed in this study.

The contrasting nature of the results may be a consequence of differences in the route of administration of LPS and/or the degree of hepatocellular damage between the two studies. In addition, the differences in routes of administration of LPS are associated with temporal differences in the development of injury, since the onset of liver damage after the intravenous administration of 4 mg/kg LPS does not begin until 4 hours after exposure (Pearson *et al.*, 1995). Thus, in the study that revealed a role for PAF and LTs in liver injury after the intraperitoneal administration of LPS, hepatocellular injury was evaluated at a time before it appears in response to intravenous administration. This temporal difference suggests that liver injury in the two models arises by different mechanisms, and such a difference may underlie the divergent findings regarding the roles PAF and LTs.

Another model of LPS-induced liver injury entails mice that are sensitized with galactosamine, a protein synthesis inhibitor. In the galactosamine/LPS model, inhibition of LT biosynthesis prevented liver injury, whereas PAF antagonism with WEB 2086 did not (Tiegs *et al.*, 1988). The contrast between this finding and ours may result from species differences. In addition, the administration of galactosamine may increase the sensitivity of hepatocytes to damage induced by LTs and may also invoke different inflammatory processes. In any case, these differences in results likely arise from biologically significant differences among animal models. If so, these findings emphasize that a specific inflammatory mediator that is critical in one model of endotoxemia may be inconsequential in another.

The administration of PAF results in neutrophil accumulation in lungs of rabbits (Worthen *et al.*, 1983) and rats (Chang, 1994). In addition, PAF receptor antagonism affords protection against pulmonary leukocyte accumulation observed after the intrathoracic administration of *E. coli* LPS in guinea pigs (Bozza *et al.*, 1994). However, in rats treated with *S. enteritidis* LPS, PAF blockade does not prevent pulmonary neutrophil sequestration (Chang, 1994). Thus, the role of PAF in LPS-mediated neutrophil accumulation in the lungs is model-dependent.

Compared to the lungs, little is known about possible contributions of PAF and LTs in the sequestration of neutrophils within the liver after LPS exposure. Results from this study suggest that PAF and LTs are not required for sinusoidal neutrophilia during LPS exposure in rats, since histopathologic analysis revealed larger numbers of neutrophils in livers from LPS-treated animals irrespective of pretreatment with WEB 2086 and Zileuton (Figure 5.3). In this model, neutrophils appear to accumulate in the liver by a different mechanism, such as the expression of P selectin on sinusoidal endothelium (Coughlan *et al.*, 1994).

Neutropenia is often used as an indicator of neutrophil accumulation in tissues. The role of PAF in neutropenia is controversial, inasmuch as some findings have supported a role for PAF in LPS-induced neutropenia (Coughlan *et al.*, 1994) whereas others suggested that PAF is not involved (Okamoto *et al.*, 1986; Rabinovici *et al.*, 1990). Similarly, the role of LTs in LPS-induced neutropenia is unclear since inhibition of 5-lipoxygenase attenuated neutropenia in the rat after the intravenous administration of 15 mg/kg *S. enteritidis* LPS

(Cook *et al.*,1985), but did not alter neutropenia in sheep after a 20 minute infusion of *E. coli* LPS (Kuratomi *et al.*,1993). Our results indicate that, in the rat given LPS intravenously at a relatively small dose, neither LTs nor PAF are responsible for neutropenia. Thus, as for liver injury, the roles of PAF and LTs in LPS-induced neutropenia are highly model-dependent.

The hepatocellular damage after LPS exposure in the model we used is dependent on both platelets and an activated coagulation system (Pearson *et al.*,1995; Hewett *et al.*,1995). Prior to the onset of liver injury, blood platelet concentration decreases and plasma fibrinogen concentration is reduced, marking activation of the coagulation system (Pearson *et al.*,1995). Pretreatment with WEB 2086 and Zileuton did not alter LPS-induced thrombocytopenia or the decrease in plasma fibrinogen concentration. This suggests that PAF and LTs are not critical mediators of either of these phenomena in this model. Results of studies evaluating the roles PAF and LTs in LPS-induced thrombocytopenia and activation of the coagulation system have been inconsistent. In a rat model in which LPS was infused over a 4 hour period, PAF was critical for the development of thrombocytopenia and activation of the coagulation system (Imanishi *et al.*,1991; Imura *et al.*,1986). However, PAF receptor antagonism did not prevent the thrombocytopenia observed after intravenous administration of a larger dose (14.4 mg/kg) of LPS (Rabinovici *et al.*,1990). Similarly, contrasting results have been reported in models of endotoxemia in rabbits (Ou *et al.*,1994; Okamoto *et al.*,1986). As for liver injury, differences in species, LPS source or dose, route of

administration or the time of evaluation may underlie the disparities in results.

In conclusion, our results suggest that PAF and the cysteinyl LTs are not critical mediators of the thrombocytopenia, activation of the coagulation system, hepatic neutrophil accumulation or hepatotoxicity that occurs in this model employing intravenous administration of LPS to rats. In light of other published studies of the roles of PAF and LTs, it appears that these lipid mediators contribute to pathophysiologic alterations in some, but not all animal models of endotoxemia. This emphasizes that the role and importance of a specific inflammatory mediator in the pathogenesis of tissue injury may vary with species, nature of LPS exposure or other factors that differ from one animal model to another.

CHAPTER 6

THE ROLE OF CYCLOOXYGENASE PRODUCTS IN LPS-INDUCED HEPATOTOXICITY

6. A. Abstract

The intravenous administration of gram-negative bacterial lipopolysaccharide (LPS) to rats results in activation of the coagulation system, thromobocytopenia and liver damage. Hepatic lesions are characterized by the infiltration of both platelets and neutrophils and by midzonal parenchymal cell necrosis. The genesis of liver injury is dependent on numerous soluble and cellular inflammatory mediators, including platelets, neutrophils, Kupffer cells and components of the coagulation system. Cyclooxygenase products, including thromboxane, are critical to the development of liver damage in other models of endotoxemia. After intravenous LPS administration (4 mg/kg), plasma thromboxane B_2 concentrations steadily increased over a 6 hr period, indicating that LPS exposure increases biosynthesis of cyclooxygenase products. To test the hypothesis that cyclooxygenase metabolites contribute to the development of hepatocellular damage, animals were pretreated with aspirin (450 mg/kg, po). Pretreatment with aspirin reduced but did not prevent liver injury, as marked by a 50% reduction in the LPS-induced elevation in plasma alanine aminotransferase activity and by histologic evaluation. Pretreatment with aspirin had no effect on LPS-induced thrombocytopenia, activation of the coagulation system or hepatic platelet accumulation. These results suggested that cyclooxygenase products contribute to the genesis of hepatotoxicity. To evaluate whether thromboxane A_2 is involved in liver injury, animals were pretreated with a thromboxane synthase inhibitor, dazmegrel (50

mg/kg, po). Pretreatment with dazmegrel prevented the LPS-induced elevation in plasma thromboxane concentration. However, it did not alter LPS-induced liver injury, activation of the coagulation system, thrombocytopenia or hepatic platelet accumulation. Taken together, these findings suggest that cyclooxygenase products, but not thromboxane, contribute to the development of hepatotoxicity in rats treated intravenously with LPS.

6. B. Introduction

Numerous studies have implicated cyclooxygenase products in the pathogenesis of LPS-induced lethality in several animal models. In rats, LPS administration stimulates the production of PGI_2 and TxA_2, as measured by the stable metabolites 6-keto-$PGF_{1\alpha}$ and TxB_2, respectively (Cook *et al.*,1980; Olanoff *et al.*,1985; Wise *et al.*,1980; Halushka *et al.*,1981; Ishiguro *et al.*,1994). Inhibition of the formation of cyclooxygenase metabolites with aspirin and ibuprofen attenuates the lethal effects of LPS (Cook *et al.*,1982; Wise *et al.*,1980; Halushka *et al.*,1981; Ishiguro *et al.*,1994). In addition, pretreatment with aspirin attenuates liver injury after the intravenous administration of LPS in rats (Ishiguro *et al.*,1994). However, the role of cyclooxygenase metabolites in the pathogenesis of LPS-induced liver injury is model-dependent, since aspirin and ibuprofen do not afford protection from the hepatotoxic effects of LPS in mice that have been sensitized with galactosamine

(Tiegs *et al.*, 1988; Wendel *et al.*, 1986).

With the development of newer pharmacologic interventions, considerable attention has focused on the role of TxA_2 in endotoxemia, and in particular, its role in LPS-induced hepatotoxicity. Studies with thromboxane synthase inhibitors and thromboxane receptor antagonists have supported a role for TxA_2 in the development of liver injury in rats treated with intravenous *Salmonella enteritidis* LPS (Cook *et al.*, 1982; Wise *et al.*, 1980) and in mice treated with LPS one week after priming with *C. parvum* (Nagai *et al.*, 1989). However, TxA_2 may not be critical for the development of LPS-induced hepatotoxicity in all models, since thromboxane synthase inhibition does not afford protection in a rat model of sepsis in which a fecal suspension is instilled into the peritoneal cavity (Cook *et al.*, 1982; Butler *et al.*, 1983) or in a model of *E. coli* LPS infusion in the rat (Furman *et al.*, 1984). Thus, the role and importance of TxA_2 as a determinant of liver injury during endotoxemia may vary with biological and/or experimental differences among animals models.

One model of endotoxemia involves a single intravenous administration of *E. coli* LPS to rats. To determine the time course of TxA_2 production in this model, plasma TxB_2 concentration was measured at various times after LPS administration. To evaluate the role of cyclooxygenase metabolites, especially thromboxane, the effect of pretreatment either with aspirin or with the thromboxane synthase inhibitor, dazmegrel, on LPS-induced liver injury was determined. Finally, to gain insight in the possible mechanisms by which prostanoids contribute to hepaototoxicity *in vivo*, we evaluated the effects of

aspirin and dazmegrel on activation of the coagulation system, thrombocytopenia and hepatic platelet accumulation during LPS exposure.

6. C. Materials and methods

6. C. 1. Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12), heparin (Type II, disodium salt), aspirin, indomethacin and Kit 59 for determination of alanine aminotransferase (ALT) activity were purchased from Sigma Chemical Company (St. Louis, MO). Determination of plasma fibrinogen concentrations was performed with Data-Fi fibrinogen reagents (Baxter Scientific Products). Blood platelet concentrations were determined in a Baker System 2000 Blood Cell Analyzer. ^{111}Indium-oxine was purchased from Medi-Physics division of Amersham Life Science (Arlington Heights, IL). Prostaglandin E_1 was purchased from Calbiochem (San Diego, CA). Enzyme immunoassay (EIA) kits for measurement of TxB_2 were purchased from Cayman Chemical (Ann Arbor, MI). C-18 columns for the extraction of lipids were purchased from Waters Chromatography (Millipore, Milford, MA). Dazmegrel was a generous gift from Pfizer Phamaceuticals (England).

6. C. 2. Animals

See Chapter 2, section C.2.

6. C. 3. Quantification of tissue platelet distribution

See Chapter 2, section C.6.

6. C. 4. Time course of TxB_2 production

Rats were treated with LPS (4 mg/kg, iv) or saline vehicle. Two, four and 6 hours after the administration of LPS, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn from the descending aorta into a syringe containing sodium citrate (0.38% final concentration) and indomethacin (0.1 mM final concentration). Plasma (0.5 ml) was stored at -20^0 C until it was assayed for TxB_2 immunoreactive substance. Prostanoids were extracted from plasma with Sep-Pak C-18 cartridges. Measurement of TxB_2 was performed by the University of Michigan Ligand Core laboratory using a TxB_2 EIA kit (Cayman Chemical Company, Ann Arbor, MI).

6. C. 5. Aspirin study

Aspirin (200 mg/ml) was dissolved in sterile water (pH 12) containing 0.95M sodium carbonate. Once aspirin was in solution, the pH was adjusted to 8.0 with 1 M HCl. Animals were treated with ^{111}In-labeled platelets 2 hours before the administration of LPS. Aspirin (450 mg/kg) or vehicle (water, pH 8.0) was administered by gavage 1 hour before the administration of LPS (4 mg/kg, iv) or its saline vehicle. This treatment regimen has been shown to

inhibit platelet aggregation *ex vivo* within 1 hour after aspirin administration (Bailie et al., 1995). Six hours after the administration of LPS, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn from the descending aorta into sodium citrate (0.38% final concentration) and indomethacin (0.1 mM final concentration). The liver, lungs, spleen and kidneys were excised for determination of radiolabeled platelet distribution. Liver sections were fixed in 10% neutral buffered formalin and processed for histologic evaluation. Blood platelet concentrations were determined, and plasma samples were assayed for plasma TxB_2 and fibrinogen concentration and for ALT activity.

6. C. 6. Dazmegrel study

Dazmegrel (25 mg/ml) was dissolved in sterile water (pH 12). Once in solution, the pH was adjusted to 8.5 with 1 M HCl. Animals were treated with ^{111}In-labeled platelets 2 hours before the administration of LPS. Dazmegrel (50 mg/kg) or its vehicle (water, pH 8.5) was administered by gavage 1 hour before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after the administration of LPS, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and a midline laparotomy was performed. Blood was drawn from the descending aorta into sodium citrate (0.38% final concentration) and indomethacin (0.1 mM final concentration). The liver, lungs, spleen and kidneys were excised for determination of radiolabeled platelet distribution. Liver sections were fixed in 10% neutral buffered formalin and processed for

histologic evaluation. Blood platelet concentrations were determined and plasma samples were assayed for plasma TxB_2 and fibrinogen concentration and for ALT activity.

6. C. 7. Data analysis

A one-way analysis of variance (ANOVA) on ranks was used to evaluate results of the time course of plasma TxB_2 production. Comparisons between treatment and control were performed with Dunn's method (Steel and Torrie, 1980). For aspirin and dazmegrel studies, all data were analyzed with a 2 X 2 multifactorial, completely random ANOVA. Data with nonhomogeneous variances were square root- or log-transformed prior to analysis. Comparisons between groups were performed with Tukey's omega test (Steel and Torrie, 1980). The criterion for significance for all studies was $p \leq 0.05$. Results are expressed as mean ± standard error of the mean (SEM). For all results presented, N represents the number of individual animals used in the study.

6. D. Results

6. D. 1. Time course of TxB_2 production

LPS administration resulted in an elevation in plasma TxB_2 concentration. Plasma TxB_2 was elevated 2 hours after LPS administration and remained elevated thereafter (Figure 6.1).

6. D. 2. Aspirin study

Pretreatment with aspirin prevented the LPS-induced elevation in plasma TxB_2 concentration measured 6 hours after the administration of LPS, confirming inhibition of cyclooxygenase activity (Figure 6.2). Plasma alanine aminotransferase (ALT) occurs in hepatic parenchymal cells and its release into the plasma is commonly used as an indicator of hepatocellular damage. As shown in previous studies, LPS administration resulted in liver injury, as marked by elevations in plasma ALT activity (Figure 6.3). Pretreatment with aspirin reduced the increase in ALT activity by 50%, suggesting a reduction in liver injury. This finding was consistent with histolgic evaluation of liver sections (data not shown).

The administration of LPS resulted in activation of the coagulation system, as marked by a decrease in plasma fibrinogen concentration (Figure 6.4). Aspirin had no effect on LPS-induced activation of coagulation. Consistent with previous studies, LPS also resulted in thrombocytopenia (Figure 6.5) and a pronounced accumulation of radiolabeled platelets in the liver (Figure 6.6). Pretreatment with aspirin altered neither the thrombocytopenia nor the hepatic platelet accumulation. Table 6.1 shows the distribution of radiolabeled platelets in blood, spleen, kidneys and lungs for each treatment group. LPS exposure resulted in a significant decrease in radiolabeled platelets in blood, and this decrease was not altered by pretreatment with aspirin. LPS exposure decreased splenic radioactivity, and this was inhibited with aspirin. The distribution of radiolabeled platelets in the lungs and kidneys was not altered by the

administration of LPS irrespective of aspirin treatment.

6. D. 3. Dazmegrel study

As with aspirin, pretreatment with dazmegrel inhibited the LPS-induced elevation in plasma TxB_2 concentration (Figure 6.7), verifying the inhibition of thromboxane synthase activity. However, pretreatment with dazmegrel did not inhibit the hepatotoxicity observed after LPS exposure, as measured by plasma ALT activity (Figure 6.8). This finding was consistent with histologic evaluation of liver sections (data not shown). Similar to the findings with aspirin, dazmegrel had no effect on LPS-induced thrombocytopenia (Figure 6.9), activation of the coagulation system (Figure 6.10) or hepatic platelet accumulation (Figure 6.11). The distribution of radiolabeled platelets in blood and other tissues in the dazmegrel study was similar to that reported for aspirin (data not shown).

Figure 6.1. Plasma TxB_2 concentration during LPS exposure. Animals were treated with LPS (4 mg/kg, iv) or its saline vehicle. Two, four and six hours later, blood samples were collected for measurement of plasma TxB_2 concentration as described in Methods. Since the values from saline-treated rats did not change with time, these values were grouped and represented as control (i.e., 0 hr). Results are expressed as mean ± SEM, N = 3-5 per group. a, significantly different from control (i.e., 0 hr)

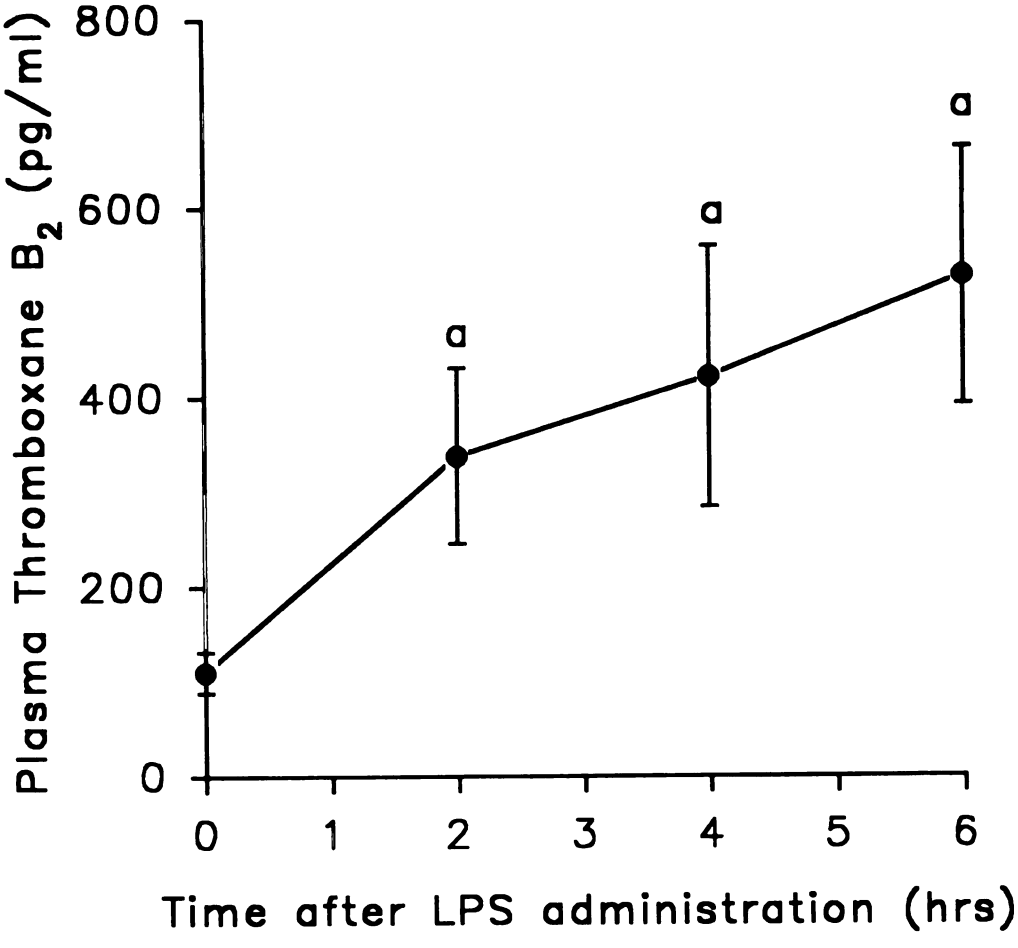

Plasma Thromboxane B_2 (pg/ml)
800
600
400
200
0
a
a
a
0
1
2
3
4
5
6
Time after LPS administration (hrs)

FIGURE 6.1

Figure 6.2. The effects of LPS and aspirin on plasma TxB_2 concentration. Animals were treated with aspirin (450 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma TxB_2 concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of aspirin

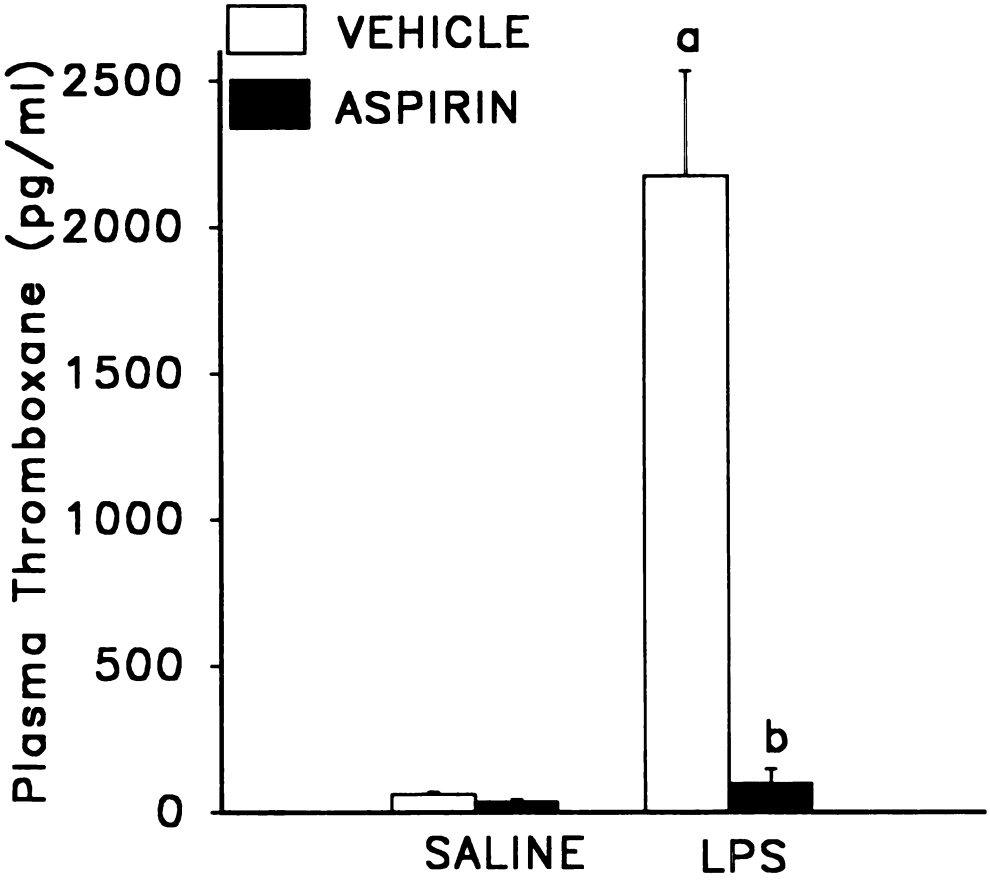

VEHICLE
ASPIRIN
a
b
Plasma Thromboxane (pg/ml)
2500
2000
1500
1000
500
0
SALINE
LPS

FIGURE 6.2

Figure 6.3. The effects of LPS and aspirin on plasma ALT activity. Animals were treated with aspirin (450 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of aspirin

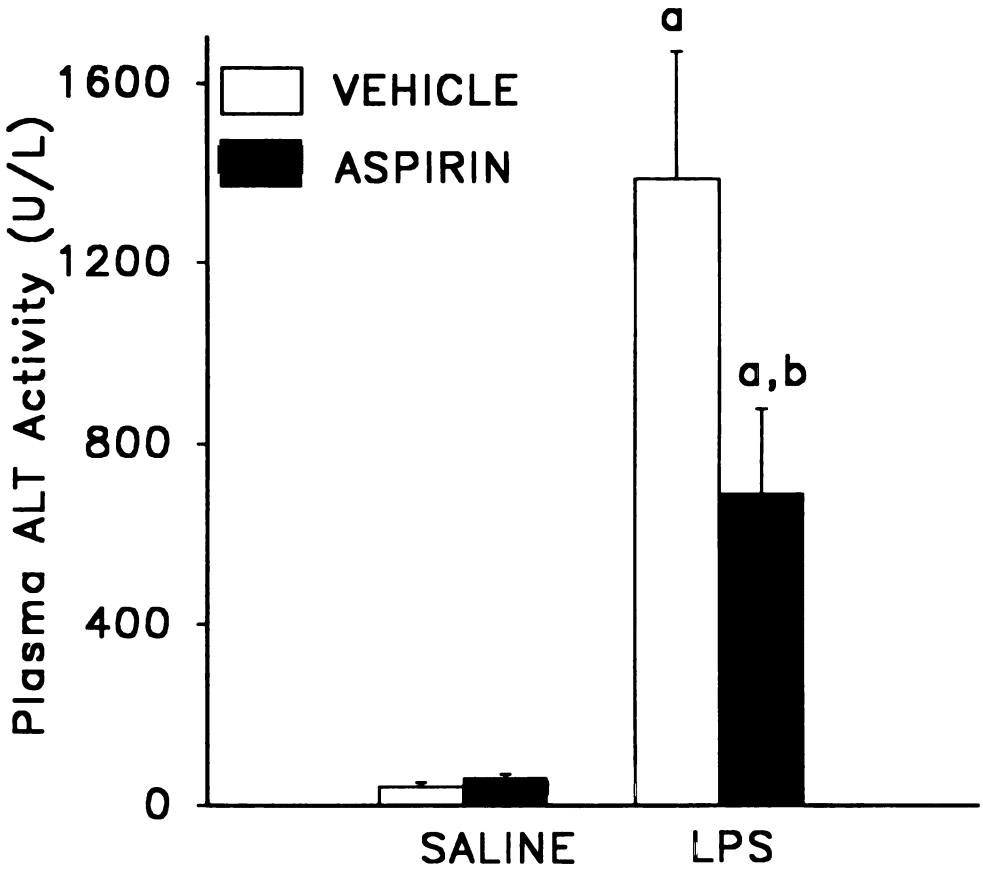

a
1600
VEHICLE
ASPIRIN
1200
Plasma ALT Activity (U/L)
a,b
800
400
0
SALINE
LPS

Figure 6.3

Figure 6.4. The effects of LPS and aspirin on plasma fibrinogen concentration. Animals were treated with aspirin (450 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma fibrinogen concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

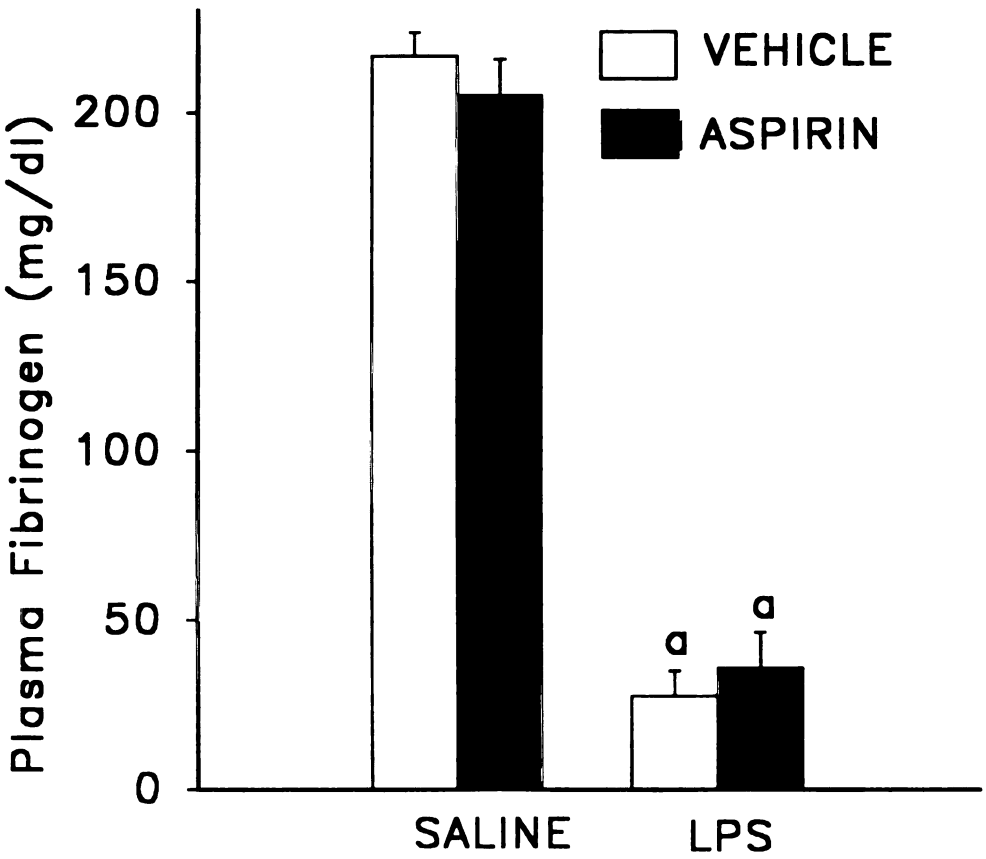

VEHICLE
ASPIRIN
Plasma Fibrinogen (mg/dl)
200
150
100
50
0
a
a
SALINE
LPS

FIGURE 6.4

Figure 6.5. The effects of LPS and aspirin on blood platelet concentration. Animals were treated with aspirin (450 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of platelet concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

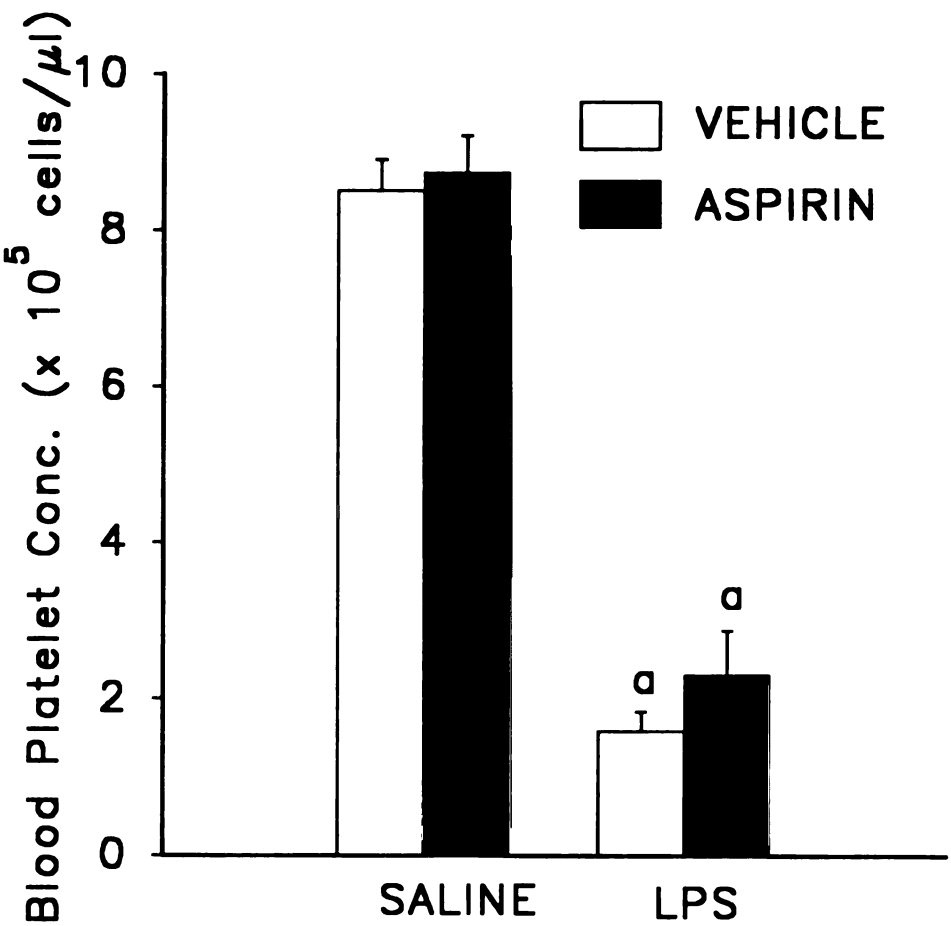

Blood Platelet Conc. (x 10^5 cells/μl)
10
8
6
4
2
0
VEHICLE
ASPIRIN
a
a
SALINE
LPS

FIGURE 6.5

Figure 6.6. The effects of LPS and aspirin on hepatic platelet accumulation. All animals were treated with ^{111}In-labeled platelets (iv) 2 hrs before the administration of LPS. Animals were treated with aspirin (450 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, liver sections were collected for measurement of radioactivity in a gamma counter as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

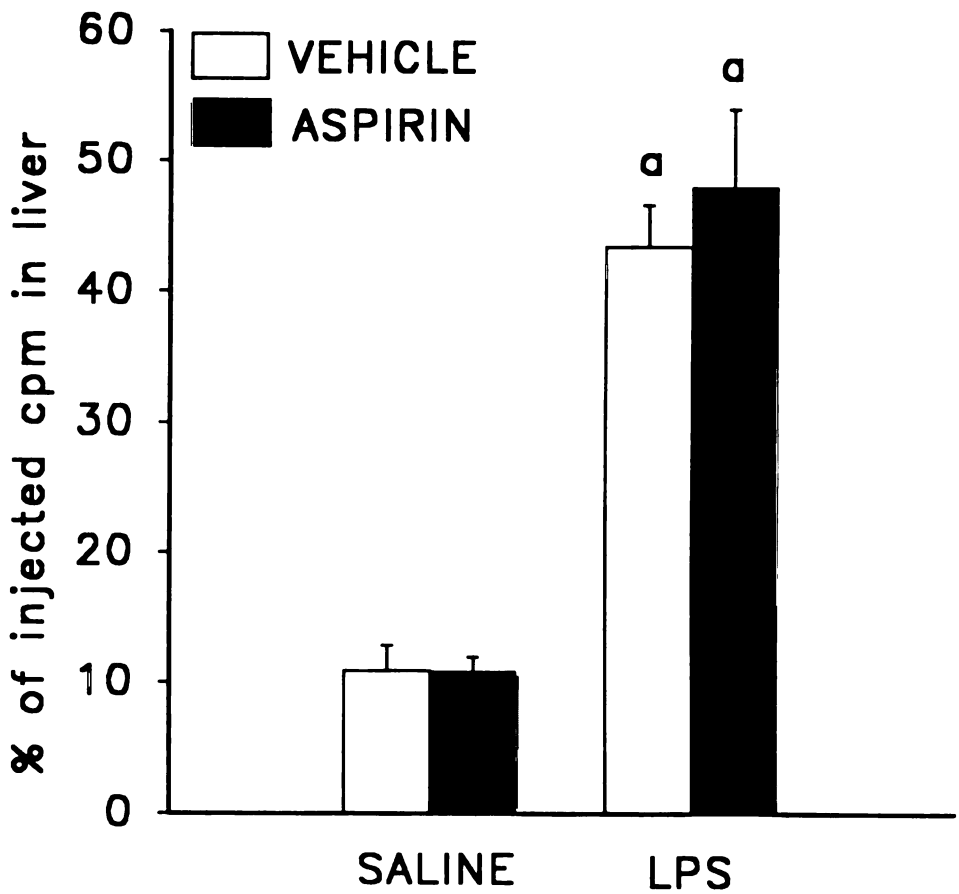

60
50
40
30
20
10
0
% of injected cpm in liver
VEHICLE
ASPIRIN
a
a
SALINE
LPS

Figure 6.6

TABLE 6.1

Effects of Aspirin and LPS on Platelet Distribution in the Blood, Spleen, Kidneys and Lungs

	TREATMENT GROUPS			
TISSUE	SALINE + VEH	SALINE + ASPIRIN	LPS + VEH	LPS + ASPIRIN
BLOOD	4.6 ± 0.6	3.7 ± 0.4	0.7 ± 0.2[a]	1.2 ± 0.5[a]
SPLEEN	15.8 ± 0.6	14.0 ± 0.9	9.5 ± 0.8[a]	14.6 ± 2.5
KIDNEYS	1.5 ± 0.5	1.6 ± 0.4	2.2 ± 0.8	2.4 ± 0.5
LUNGS	3.3 ± 0.8	2.7 ± 0.3	2.2 ± 0.2	2.5 ± 0.2

Rats were given ^{111}In-labeled platelets 2 hrs before the administration of LPS. Rats were pretreated with either aspirin (450 mg/kg, po) or its vehicle (VEH) 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after the administration of LPS, blood and organs were collected for measurement of radioactivity as described in Methods. Results are expressed as percent of injected radioactivity in the entire organ (mean ± SEM), N = 6-11 per group.

[a]significantly different from respective value in the absence of LPS

[b]significantly different from respective value in the absence of aspirin

Figure 6.7. The effects of LPS and dazmegrel on plasma TxB_2 concentration. Animals were treated with dazmegrel (50 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma TxB_2 concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

b, significantly different from respective value in the absence of dazmegrel

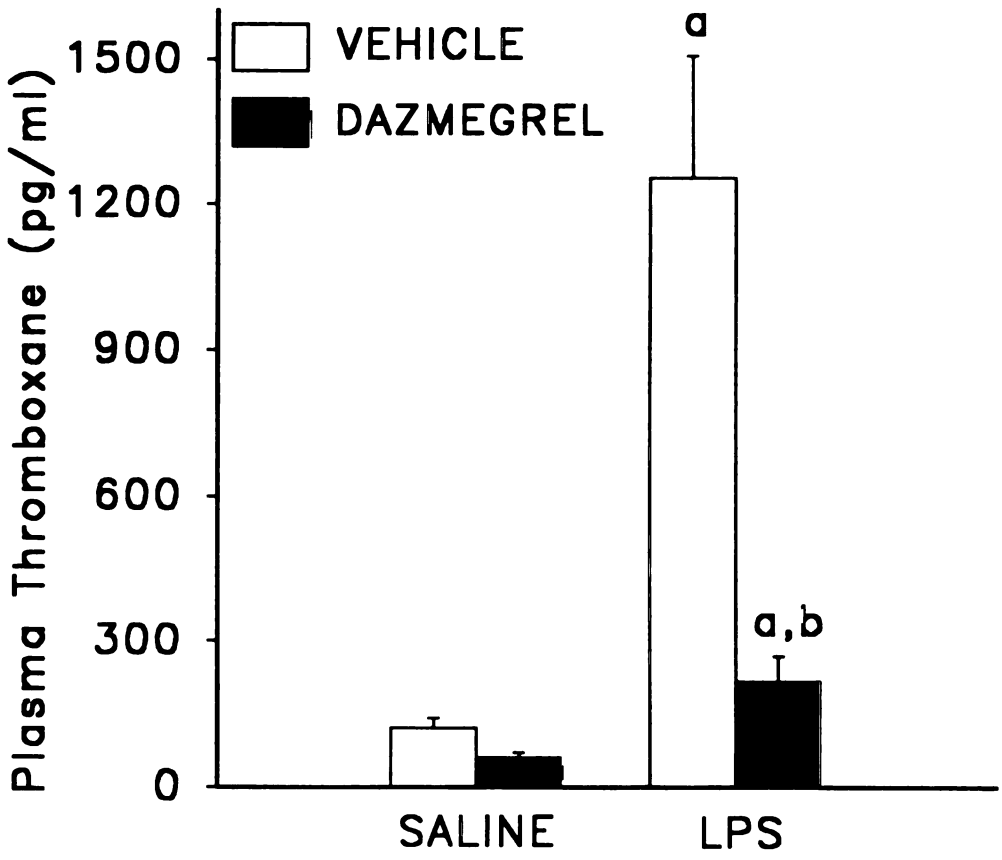

VEHICLE
DAZMEGREL
Plasma Thromboxane (pg/ml)
1500
1200
900
600
300
0
a
a,b
SALINE
LPS

FIGURE 6.7

Figure 6.8. The effects of LPS and dazmegrel on plasma ALT activity. Animals were treated with dazmegrel (50 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma ALT activity as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

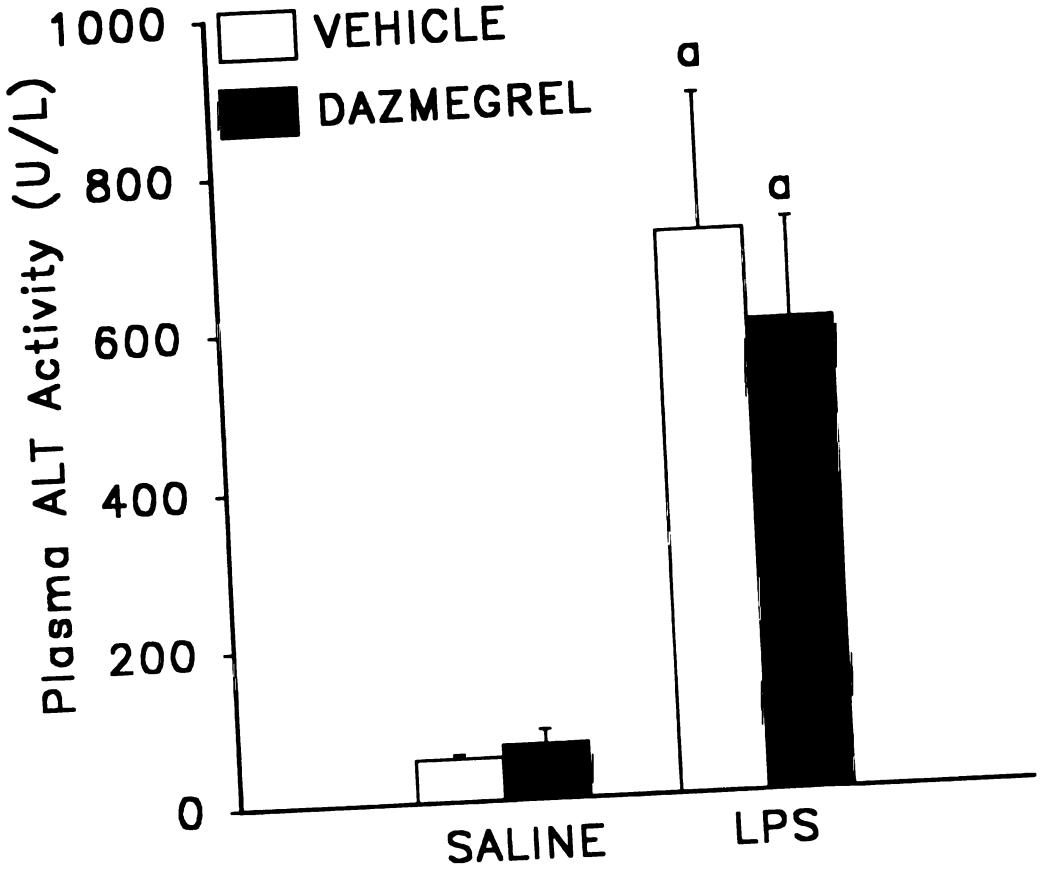

1000
800
600
400
200
0
Plasma ALT Activity (U/L)
VEHICLE
DAZMEGREL
a
a
SALINE
LPS

Figure 6.8

Figure 6.9. The effects of LPS and dazmegrel on blood platelet concentration. Animals were treated with dazmegrel (50 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of platelet concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

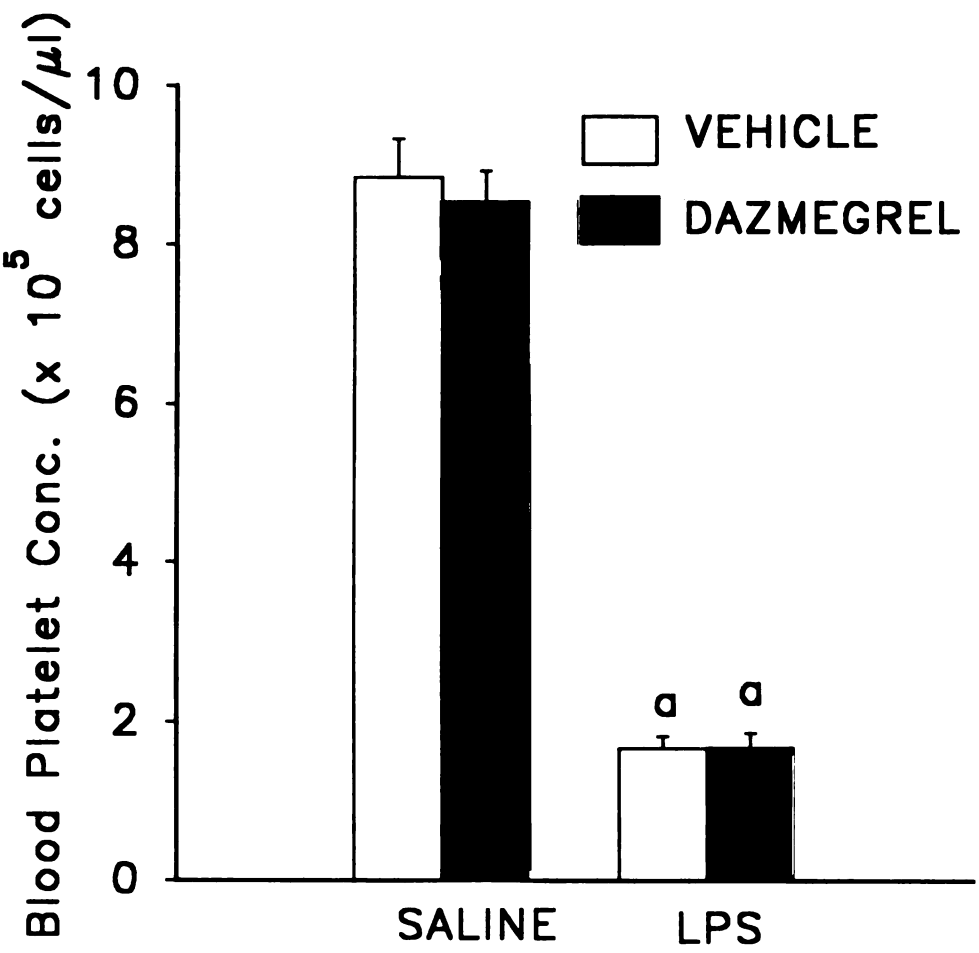

Blood Platelet Conc. (x 10^5 cells/μl)
10
8
6
4
2
0
VEHICLE
DAZMEGREL
a
a
SALINE
LPS

FIGURE 6.9

Figure 6.10. The effects of LPS and dazmegrel on plasma fibrinogen concentration. Animals were treated with dazmegrel (50 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma fibrinogen concentration as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.

a, significantly different from respective value in the absence of LPS

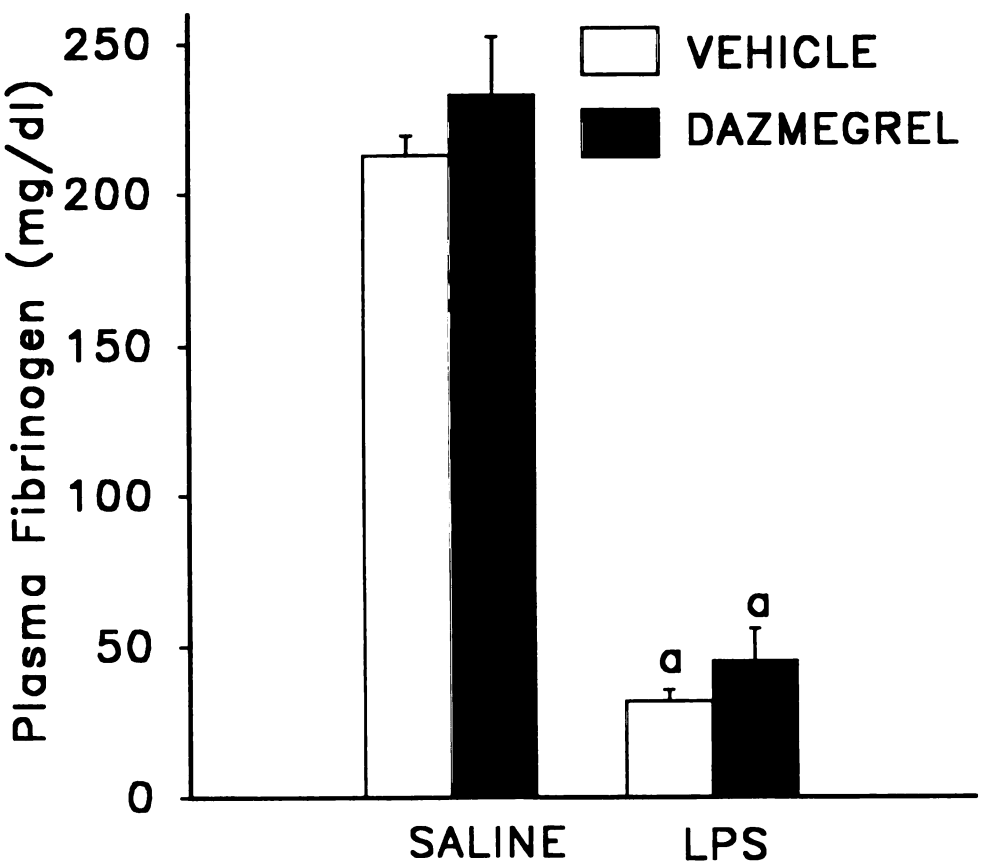

Plasma Fibrinogen (mg/dl)
250
200
150
100
50
0
VEHICLE
DAZMEGREL
a
a
SALINE
LPS

Figure 6.10

Figure 6.11. The effects of LPS and dazmegrel on hepatic platelet accumulation. All animals were treated with ^{111}In-labeled platelets (iv) 2 hrs before the administration of LPS. Animals were treated with dazmegrel (50 mg/kg, po) or its vehicle 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after LPS administration, liver sections were collected for measurement of radioactivity in a gamma counter as described in Methods. Results are expressed as mean ± SEM, N = 6-11 per group.
a, significantly different from respective value in the absence of LPS

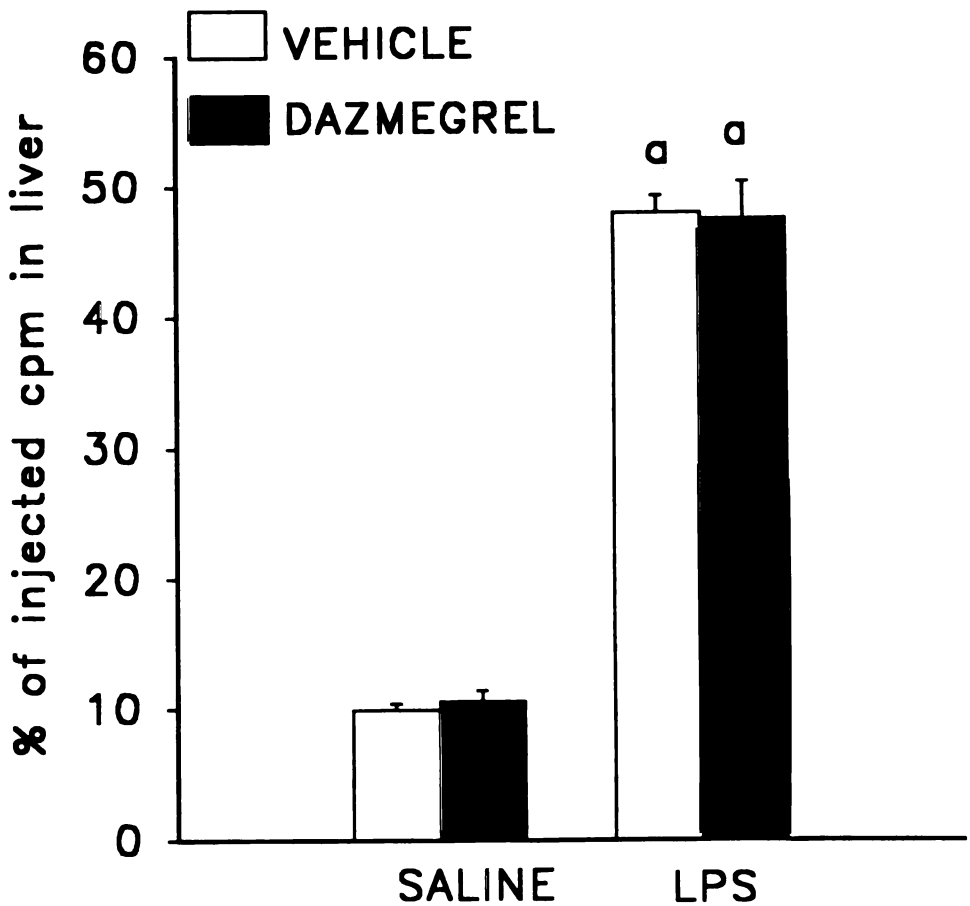

VEHICLE
DAZMEGREL
% of injected cpm in liver
60
50
40
30
20
10
0
a
a
SALINE
LPS

Figure 6.11

6. E. Discussion

The intravenous administration of LPS to rats stimulates TxA_2 production, as measured by increased plasma TxB_2 concentration. Concentrations of TxB_2 are elevated 2 hours after the administration of LPS and tend to increase thereafter. Results from this study suggest that cyclooxygenase products, but not thromboxane, contribute to the pathogenesis of LPS-induced hepatotoxicity. In addition, cyclooxygenase products apparently do not contribute to the activation of the coagulation system, thrombocytopenia or hepatic platelet accumulation observed after LPS administration.

TxA_2, a potent vasoconstrictor and activator of platelets, is released from numerous cells, including activated platelets and LPS-treated Kupffer cells (Kawada *et al.*,1992; Moscat *et al.*,1987; Ishiguro *et al.*,1994). Once activated, platelets release coagulation factors, express a procoagulant surface that stimulates the intrinsic pathway of coagulation and protect coagulation factors from proteolytic degradation (Walsh,1981a; Walsh *et al.*,1981b). Since LPS-induced hepatotoxicity is dependent upon Kupffer cells, platelets and the coagulation system (Iimuro *et al.*,1994; Pearson *et al.*,1995; Hewett *et al.*,1995; Margaretten *et al.*,1967) and TxA_2 could mediate interactions between them, this prompted an evaluation of the role of TxA_2 in LPS-induced thrombocytopenia, hepatic platelet accumulation and activation of the coagulation system. However, results with aspirin and dazmegrel suggest that thromboxane does not contribute to these events. This finding contrasts results

from another model of endotoxemia in which thromboxane synthase inhibitors attenuated thrombocytopenia (Olanoff *et al.*, 1985) and the elevation in fibrin degradation products (Wise *et al.*, 1980) in rats treated intravenously with 20 mg/kg *Salmonella enteritidis* LPS. The contrasting nature of the results may be a consequence of differences in the bacterial source and dose of LPS.

In these studies, pretreatment with aspirin attenuated LPS-induced liver injury but the thromboxane synthase inhibitor, dazmegrel, had no effect. Taken together, these findings suggest that prostanoids other than thromboxane might be critical to the development of hepatotoxicity. PGD_2 is the main prostanoid produced by unstimulated Kupffer cells, and its production is increased upon Kupffer cell activation (Ujihara *et al.*, 1988; Grewe *et al.*, 1992; Dieter *et al.*, 1989). In certain tumor cell lines, PGD_2 and its serum metabolite, delta^{12}-PGJ_2, are cytotoxic (Ikai *et al.*, 1995; Ujihara *et al.*, 1988). This cytotoxicity is characterized by changes in cell membrane integrity and damage to actin and keratin filaments (Ikai *et al.*, 1995). It is not known whether PGD_2 has similar cytotoxic effects on hepatic parenchymal cells. However, PGD_2 specifically binds to hepatocytes (Kuiper *et al.*, 1988; Casteleijn *et al.*, 1988a; Casteleijn *et al.*, 1988b; Kuiper *et al.*, 1989) and can alter hepatocellular function. In the isolated perfused liver, PGD_2 increases calcium efflux, glycogenolysis and portal pressure (Altin *et al.*, 1988). The relationship of these alterations to hepatocellular necrosis is unknown, but, the possibility that PGD_2, by altering intracellular calcium, might increase the susceptibility of hepatocytes to injury from other inflammatory mediators is intriguing.

These studies suggest that thromboxane is not critical to the pathogenesis of liver injury during LPS exposure and are in contrast to findings in another model of endotoxemia in which rats were treated with a larger dose of LPS. The bolus, intravenous injection of 20 mg/kg *S. enteritidis* LPS results in a peak of plasma TxB_2 concentration within 30 minutes that decreases thereafter (Cook *et al.*, 1980). Significant hepatocellular damage occurs within 4 hours after the administration of LPS. In this model of endotoxemia, pretreatment with cyclooxygenase inhibitors, thromboxane synthase inhibitors or thromboxane receptor antagonists affords protection from lethality, liver injury, thrombocytopenia and activation of the coagulation system (Olanoff *et al.*, 1985; Wise *et al.*, 1980; Cook *et al.*, 1982; Wise *et al.*, 1980; Cook *et al.*, 1980).

This model using *S. enteritidis* LPS is different from the one we used in which the intravenous administration of a smaller dose of *E. coli* LPS to rats resulted in a more gradual increase in plasma TxB_2 over a 6 hour period. In addition, in the model we have employed, the onset of liver injury does not occur until nearly 4 hours after the administration of LPS and is not pronounced until about 6 hours after LPS exposure (Pearson *et al.*, 1995). Thus, the time course of thromboxane production and the onset of hepatocellular injury appear to differ significantly in these two models. The dissimilarities may be a consequence of both the bacterial source and dose of LPS. These contrasting findings underscore that different inflammatory mediators may have prominance in various models of endotoxemia.

Based on the time course of plasma TxB_2 production, results in the model employing bolus, intravenous administration of a modest dose of *E. coli* LPS appear to resemble more closely models in which endotoxemia is induced by the intraperitoneal administration of a fecal suspension (Cook *et al.*, 1982; Butler *et al.*, 1983) or the infusion of *E. coli* LPS over a 4 hour period (Furman *et al.*, 1984). In these models, the elevation of plasma TxB_2 is sustained over a 4-8 hour period after the initiation of endotoxemia. In addition, thromboxane synthase inhibitors do not prevent lethality or liver injury (Cook *et al.*, 1982; Butler *et al.*, 1983; Furman *et al.*, 1984). Thus, in models in which the elevation of plasma TxB_2 is sustained for several hours, thromboxane does not appear to be critical to the development of lethal or hepatotoxic effects. By contrast, in models of endotoxemia in which the elevation in plasma TxB_2 is more pronounced and transient, thromboxane is critical for the development of liver injury (Wise *et al.*, 1980; Cook *et al.*, 1982). Together, these results suggest that the role of importance of thromboxane in various models of endotoxemia may be defined by the duration and magnitude of TxB_2 production.

In summary, results from this study suggest that thromboxane is not a critical mediator of liver injury, thrombocytopenia, hepatic platelet accumulation and activation of the coagulation system during LPS exposure. These findings also suggest that prostanoids other than thromboxane partially contribute to liver injury in this model. In light of other published results on the role of thromboxane, these findings emphasize differences among animal models of endotoxemia. Furthermore, these findings suggest that the nature of

thromboxane generation in response to LPS may vary among models and this may define its critical role in the pathogenesis of tissue injury.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The overall aim of this dissertation was to examine the mechanisms by which exposure to LPS culminates in liver injury. In particular, the roles of host-derived, cellular and soluble mediators in the pathogenesis were evaluated. Major emphasis was placed on the role of platelets in hepatotoxicity. Studies were conducted to evaluate how platelets might contribute to tissue injury and also how platelets interact with other inflammatory mediators. Results from studies presented in this dissertation suggest that several inflammatory mediators, including platelets, neutrophils, Kupffer cells, thrombin and cyclooxygenase products, contribute to the manifestation of hepatocellular damage. In addition, results from these studies suggest that the full pathogenesis of liver injury depends upon complex interactions between these inflammatory mediators.

7. A. Temporal relationships between alterations in the blood and liver during LPS exposure

The administration of a single, bolus injection of LPS resulted in the accumulation of both platelets and neutrophils within 1 hour, followed by continued increases over the next several hours. Concurrent with the hepatic neutrophil infiltration was a pronounced decrease in blood neutrophils, followed by a release of immature, band neutrophils into the blood. The hepatic infiltration of inflammatory cells preceded the increase in plasma TNF-α

concentration that was maximal 1.5 hours after LPS exposure. Plasma TxB_2 also increased continuously after the administration of LPS throughout the 6 hour duration of these studies. A consumptive coagulopathy began between 2-3 hours after LPS administration. This was characterized by decreased plasma fibrinogen concentration and blood platelet numbers. All of these events preceded the onset of hepatocellular injury, as marked by elevations in the plasma of the liver specific enzyme, ALT. Liver damage progressed rapidly between 4 and 6 hours after LPS exposure. Six hours after LPS administration, liver injury was characterized by primarily, midzonal hepatocellular necrosis with a pronounced sinusoidal neutrophilia.

7. B. The role of platelets in LPS-induced hepatotoxicity

Results from studies presented in Chapter 2 demonstrated that animals became thrombocytopenic prior to the onset of liver injury. In addition, studies with ^{111}In-labeled platelets, suggested that platelets accumulate in the liver shortly after the administration of LPS. Prior depletion of platelets with a polyclonal antiserum afforded protection from liver injury. These results support the main hypothesis of this thesis that platelets contribute to LPS-induced liver injury.

Taking all of the values from LPS-treated animals evaluated in this dissertation, there appears to be a weak negative correlation between the

numbers of platelets in the blood and plasma ALT activity. This correlation suggests that the degree of thrombocytopenia is associated with the development of liver injury. However, there was no correlation between the numbers of radiolabeled platelets in the liver and the degree of liver injury. Thus, blood platelet numbers appear to more closely relate to liver injury than hepatic platelet accumulation. It is possible that only a few platelets need to accumulate in the liver to contribute to the development of liver injury. The relationship between blood platelets and liver injury may be related to the coagulation system, as there is a very strong negative correlation between activation of the coagulation system and liver injury. In general, these correlations appear to support the contention that platelets contribute to the development of liver injury.

Since platelets accumulated in the liver prior to the onset of liver injury and they were critical for the development of this liver injury, these results suggest that hepatic platelet accumulation is a critical event in the pathogenesis. Thus, studies were conducted to address the possible mechanisms by which platelets accumulate in the liver after LPS exposure. In particular, studies were conducted to evaluate the contributions of other inflammatory mediators, such as neutrophils, Kupffer cells, thrombin and TxA_2, in the manifestation of hepatic platelet accumulation.

Results from these studies suggest that LPS exposure stimulates the accumulation of platelets in the liver by mechanisms that are independent of neutrophils, the coagulation system, thrombin and TxA_2. However, studies

with $GdCl_3$ suggest that Kupffer cells partially contribute to the accumulation of platelets. Since Kupffer cell inactivation did not abolish the platelet infiltration, these studies suggest that another mechanism contributes to their accumulation.

Since platelets are critical in the pathogenesis of liver injury, this result raised the question of how platelets mediate this injury. Results from platelet depletion studies suggest that platelets are necessary for the activation of the coagulation system after LPS. Since studies with heparin and hirudin suggest that an activated coagulation system and subsequent thrombin formation is required for the development of liver injury, these findings, taken together, imply that platelets might contribute in hepatotoxicity simply by promoting the activation of the coagulation system.

Platelets could also promote liver injury by releasing soluble inflammatory mediators such as cyclooxygenase and lipoxygenase products and PAF. However, studies with a PAF receptor antagonist, and 5-lipoxygenase and thromboxane synthase inhibitors, suggest that these mediators are not critical for the development of liver injury after LPS exposure. Therefore, it is not likely that platelets contribute to tissue injury by mechanisms involving these mediators.

7. C. The roles of other inflammatory mediators in LPS-induced hepatotoxicity

7. C. 1. The coagulation system

Both platelets and the coagulation system interact during thrombosis. To elucidate some of these interactions *in vivo*, studies were conducted with antibodies to platelets and anticoagulants. Results from platelet depletion studies suggest that platelets contribute to the activation of the coagulation system after LPS exposure. Conversely, studies in animals pretreated with heparin or hirudin, suggest that the coagulation system does not contribute to LPS-induced hepatic platelet accumulation or thrombocytopenia. Taken together, these studies suggest that during LPS exposure, platelets mediate the activation of the coagulation system whereas the coagulation system does not influence platelets.

The results of these studies suggested that the coagulation system does not inhibit LPS-induced thrombocytopenia and hepatic platelet and neutrophil accumulation, yet contributes to the development of liver injury. These findings, taken together with the time course of the decrease in plasma fibrinogen concentration, inferred that the coagulation system is not activated until several hours after LPS exposure. To test this hypothesis, heparin or the selective thrombin inhibitor, hirudin, was administered at various times after the injection of LPS. These anticoagulants can be administered 2.5 hours after LPS exposure and still inhibit the development of liver injury. Thus, the coagulation system is a distal mediator of liver injury, in that it is not activated until several

hours after LPS exposure and appears to act shortly before the onset of hepatocellular injury.

This finding provides novel insight in the mechanisms by which LPS exposure results in liver injury. First, it suggests that the coagulation system, in particular, thrombin, appears to be critical shortly be the appearance of hepatocellular damage. More interesting, this result suggests that all the prior events induced by LPS exposure, such as hepatic platelet and neutrophil infiltration and cytokine release, are not sufficient to produce liver injury. Thus, liver injury involves interactions between the coagulation system and other inflammatory mediators. This raises the possibility that Kupffer cells, neutrophils and platelets exposed to LPS and subsequent mediator release may increase the susceptibility of hepatic parenchymal cells to damage by the coagulation system. It is also possible that thrombin may act on these other inflammatory cells and stimulate their release of a mediator that leads to hepatocellular damage.

7. C. 2. Lipid mediators

Results with WEB 2086 and Zileuton suggest that PAF, alone or in combination with 5-lipoxygenase products, is not a critical mediator of LPS-induced thrombocytopenia, activation of the coagulation system and liver injury. In addition, results from studies with Dazmegrel suggest that TxA_2 is not a critical mediator of these alterations as well. Since these mediators have been

shown to contribute to liver injury in other models of endotoxemia, these results emphasize differences between experimental models. Some of these differences may be attributed to differences among bacterial sources and doses of LPS, route of administration and animal species. It is interesting to note that, among animal models, there is no differences between the critical cells involved in liver injury (i.e. neutrophils and Kupffer cells are critical in every model). However, the role of soluble mediators appears to differ greatly among models. This suggests that the roles cytokines and soluble mediators in tissue injury can vary and may be a consequence of species variability and the nature of the endotoxin insult.

Pretreatment with aspirin attenuated liver injury, but did not alter hepatic platelet accumulation, thrombocytopenia or activation of the coagulation system. Thus, cyclooxygenase products other than thromboxane, perhaps PGD_2, contribute partially to the development of hepatocellular damage. However, the exact cyclooxygenase metabolites involved in liver injury are not known.

Table 7.1 summarizes many of the effects of LPS and various agents on liver injury that have been presented or discussed in this dissertation. It is by no means comprehensive but provides a summary of some critical results as well as some interactions between inflammatory mediators.

Table 7.1 Endotoxin-induced liver injury and alterations by cotreatment with various agents. This table summarizes the effects of various agents on liver injury as well as other parameters known to be involved in LPS-induced liver injury. Included are all the agents presented in this dissertation as well as agents applied to the intravenous LPS model in the rat. Key: P = protection; W = worse; N = no protection; NA = not applicable; U = unknown; PP = partial protection.

Co-Rx	Action	ALT/ AST	Bili-rubin	Histo	PMNs in blood	PMNs in liver	Plts in blood	Plts in liver	ECs	TNFα	F'gen
None		↑	↑	+++	↓→↑	↑	↓	↑	↓	↑	↓
anti-PMN Ig	↓ blood PMNs	P	N	P	NA	P	N	N	N	↑↑	PP
cyclo-phosph	↓ white cells	P	N	P	NA		N				N
anti-platelet serum	↓ blood pts	P	P	P	N	N	NA	U	U	N	P
$GdCl_3$	inhib KCs	P	P	P		N	PP	PP		N	N
anti-TNF or PTX	↓ TNFα	P	P	P	U	N	U	U	U	NA	P
heparin or warfarin	inhib throm-bin	P	P	P	U	N	N	U	U	U	NA
ancrod	↓f'gen	N	N	N	N	N	U	U	U	U	NA
WEB 2086 Zileuton	↓PAF+ LTs	N	N	N		N	N				N
aspirin	↓TxB2	PP	U	PP		N	N	N			N
dazmegrel	↓TxB2	N	U	N		N	N	N			

7. D. Interactions between inflammatory mediators in the pathogenesis of LPS-induced liver injury

Studies conducted in this dissertation suggest that inflammatory mediators interact in the manifestation of liver injury during LPS exposure. Results from these studies and others show that prior removal or inhibition of neutrophils, platelets, Kupffer cells, TNF-α or thrombin inhibits, almost completely, the development of liver injury. Thus, each of these mediators contributes to liver injury in some way.

However, liver injury involves interactions between these mediators. For instance, platelet depletion attenuates liver injury and activation of the coagulation system, but does not inhibit hepatic neutrophil accumulation or the production of TNF-α. Thus, the presence of neutrophils and TNF-α is not sufficient to produce liver injury. This suggests that liver injury arises from the interaction of platelets and the coagulation system with neutrophils and TNF-α.

These theme is supported by nearly all the studies in this dissertation. For example, pretreatment with heparin or hirudin, attenuates liver injury but does not alter hepatic neutrophil and platelet accumulation, further supporting the hypothesis that liver injury is a result of interactions between these mediators. Similar findings were found with Kupffer cells. Liver injury and hepatic platelet accumulation is reduced in animals treated with $GdCl_3$, despite hepatic neutrophil infiltration, activation of the coagulation system and elevated TNF-α concentrations. Thus, neutrophils, an activated coagulation system and

TNF-α are not sufficient to result in liver injury. This suggests that Kupffer cells are critical for the development of liver injury by mechanisms involving interactions with these other mediators. Future studies using in vitro methods such as cocultures and isolated perfused livers could help elucidate some of these interactions.

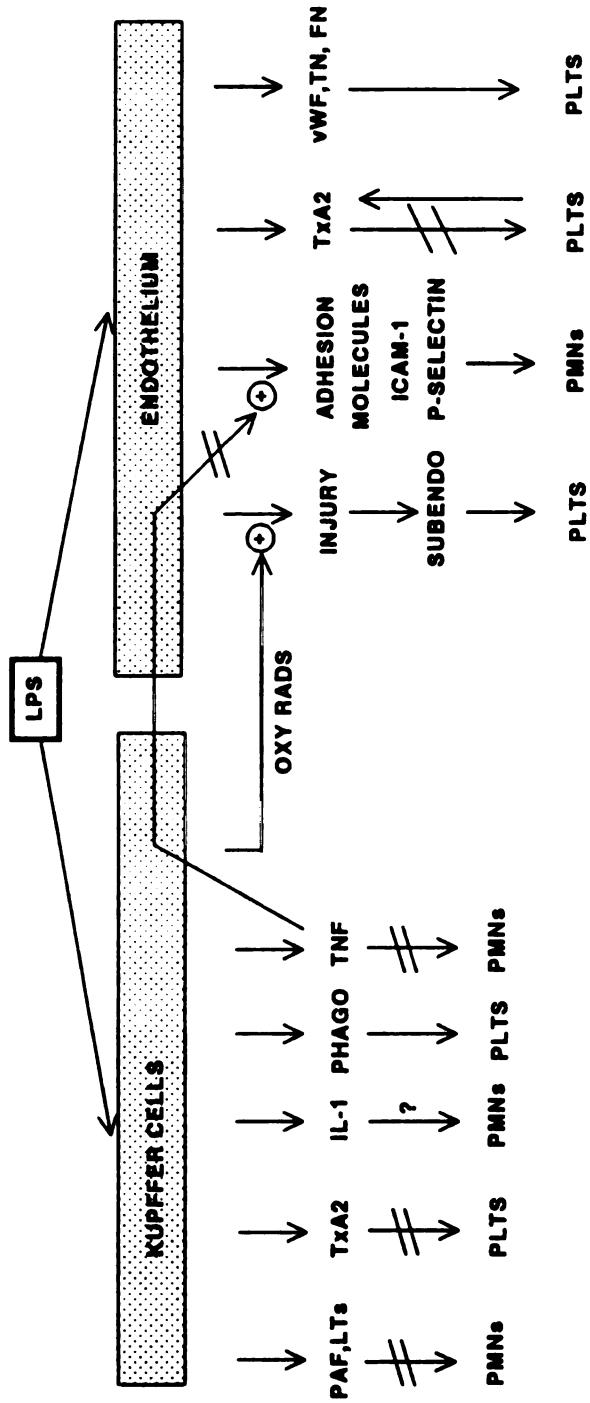
Figure 7.1 summarizes many of the findings presented in this dissertation. Exposure to LPS likely alters the functions of Kupffer cells and stimulates the accumulation of both platelets and neutrophils in the liver. The mechanism by which LPS stimulates the infiltration of inflammatory cells is possibly due to a direct effect on the endothelium and the subsequent upregulation of adhesion molecules and/or overt endothelial cell damage. The Kupffer cells are involved in the accumulation of platelets in the liver, by a mechanism that is independent of thromboxane. These inflammatory cells are possibly involved in the pathogenesis of liver injury via the release of soluble mediators. However, thromboxane, PAF and LTs do not appear to be critical mediators of liver injury. Platelets are critical for the activation of the coagulation system and subsequent thrombin formation. Since thrombin is a distal mediator of liver injury and can influence all of these cells present within the liver, the mechanism by which thrombin promotes hepatocellular damage is not clear. For instance, thrombin could directly effect susceptible hepatocytes. In addition, thrombin can stimulate the release of potent inflammatory mediators from Kupffer cells, neutrophils, platelets and the endothelium. It is possible that thrombin contributes to hepatocellular damage

by evoking the release of cytotoxic mediators from these inflammatory cells.

Figure 7.1 The mechanisms by which inflammatory mediators interact in the development of liver injury. LPS acts on several cells, including the endothelium (EC) and Kupffer cells (KC). LPS exposure also stimulates the production of TNF-α, although its cellular source and biologic effects are not known *in vivo*. Kupffer cells contribute to LPS-induced hepatic platelet accumulation. Platelets are critical for the activation of the coagulation system and subsequent thrombin formation. The precise mechanisms by which thrombin contributes to liver injury are not known, however thrombin can influence other hepatic cells (dashed lines) such as platelets, Kupffer cells, neutrophils, endothelium and parenchymal cells.

FIGURE 7.1

MECHANISMS OF NEUTROPHIL AND PLATELET ACCUMULATION IN LIVER



7.E. Hypothetical mechanism of LPS-induced liver injury

Figure 7.2 highlights some of the potential mechanisms by which LPS exposure results in liver injury. Numerous *in vivo* and *in vitro* studies have demonstrated that the exposure to LPS alters Kupffer cell and endothelial cell function. Since these cells can bind LPS and are already present within the liver, it is likely that LPS first interacts with these cells. Such an interaction could promote the expression of adhesion molecules or the release of inflammatory mediators which contribute to the accumulation of both platelets and neutrophils within the liver. Kupffer cells partially contribute to the accumulation of platelets, but not neutrophils, by an unknown mechanism. Plasma TNF-α activity is elevated shortly after the onset of hepatic platelet and neutrophil accumulation. The cellular source of this TNF-α is not known, but evidence from $GdCl_3$ treated animals suggest it is not solely produced by Kupffer cells. TNF-α is critical for the pathogenesis of liver injury, although its cellular targets *in vivo* are not clearly defined. Evidence from neutrophil depletion studies suggest that TNF-α may stimulate neutrophils and it is the neutrophil-derived mediators that contribute to hepatocellular damage. Platelets are critical for the activation of the coagulation system and subsequent thrombin, a "distal" event since plasma fibrinogen concentrations are not decreased until nearly 2-3 hours after LPS exposure. Thrombin is critical mediator of liver injury that acts shortly before the onset of hepatocellular damage. The mechanisms by which thrombin contributes to liver injury are not known. However, *in vitro* studies suggest that thrombin can interact with all

the cells present in LPS-treated livers, including platelets, endothelium, neutrophils, Kupffer cells and hepatic parenchymal cells. Thus, future studies in isolated organ and cell systems could help elucidate the mechanisms by which thrombin formation culminates in parenchymal cell injury.

Figure 7.2 Proposed mechanism of LPS-induced liver injury. (1) LPS directly alters Kupffer cells and the endothelium. The stimulation of adhesion molecules or overt endothelial cell injury contributes to the accumulation of inflammatory cells within the liver. (2) In addition, Kupffer cells contribute to the hepatic platelet accumulation. (3) LPS exposure also results in the release of TNF-α into plasma after platelets and neutrophils have already begun to accumulate within the liver tissues. The sources and targets of TNF are not clearly defined. (4) Platelets contribute to LPS-induced activation of the coagulation system and subsequent thrombin formation. (5) Thrombin appears to be a critical yet distal mediator of liver injury. The cellular targets of thrombin (dashed lines) and the mechanism by which its formation contributes to parenchymal cell injury remain unclear.

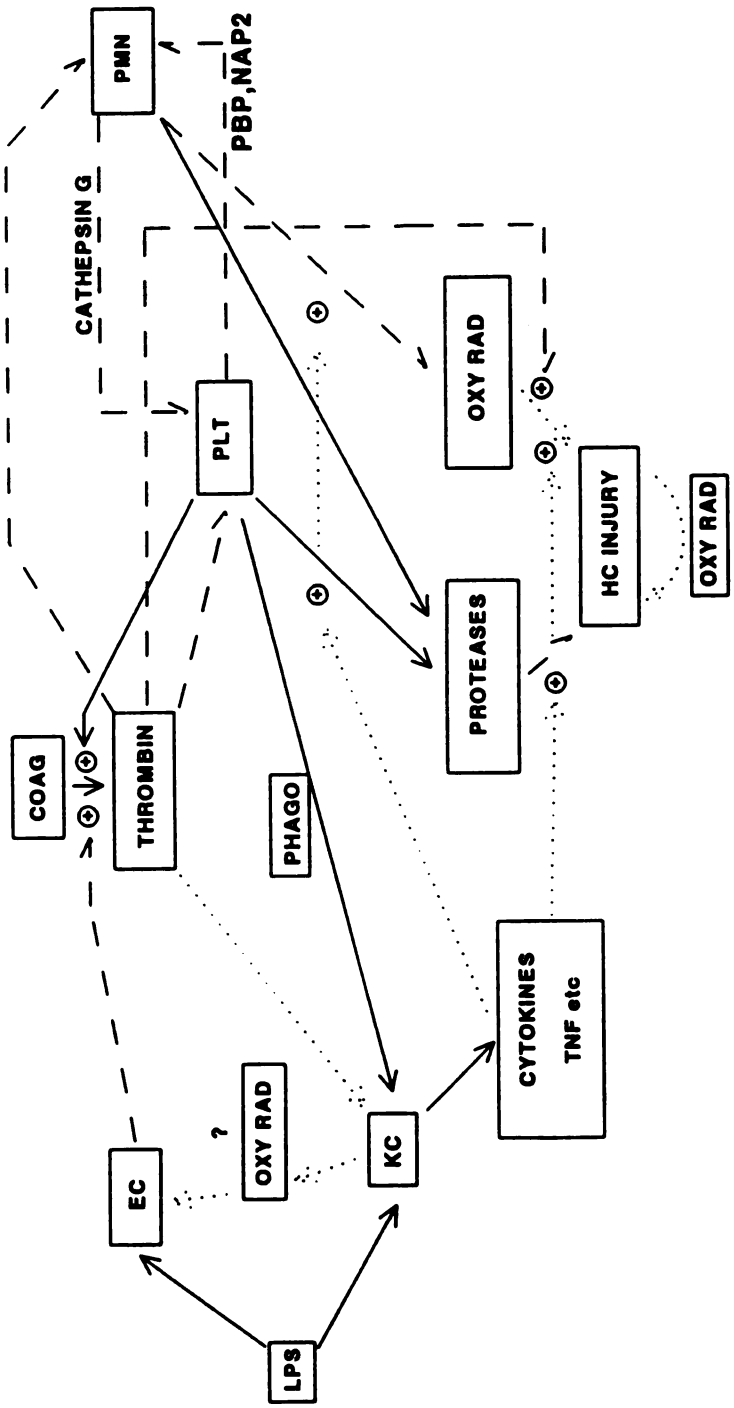


FIGURE 7.2

MECHANISMS AND CONSEQUENCES OF INFLAMMATORY CELL ACTIVATION

LIST OF REFERENCES

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