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THE ROLE OF THE INNATE IMMUNE SYSTEM IN THE LPS-INDUCED POTENTIATION OF ALLYL ALCOHOL HEPATOTOXICITY

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THE ROLE OF THE INNATE IMMUNE SYSTEM IN THE LPS-INDUCED POTENTIATION OF ALLYL ALCOHOL HEPATOTOXICITY

By

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ABSTRACT

THE ROLE OF THE INNATE IMMUNE SYSTEM IN THE LPS-INDUCED POTENTIATION OF ALLYL ALCOHOL HEPATOTOXICITY

By

Rosie Antionette Sneed

Lipopolysaccharide (LPS) causes liver damage at relatively large doses in rats (>2 mg/kg). Smaller doses, however may modify the response to other hepatotoxicants. Pretreatment of rats with a small dose of LPS (100 μg/kg) significantly increased the hepatotoxicity of a subsequent, nonlethal dose of allyl alcohol (30 mg/kg). The presence of exogenous LPS accelerated the development of liver injury compared to controls and the lesions produced resembled those caused by a higher dose of allyl alcohol. Doses of LPS up to 4 orders of magnitude lower than 100 μg/kg were able to potentiate the hepatotoxicity of allyl alcohol significantly, and the presence of LPS caused a leftward shift in the dose-response curve of allyl alcohol. Allyl alcohol had to be metabolized to the aldehyde, acrolein, for the potentiation to be seen; however, LPS did not exert its effect by altering the activity of alcohol dehydrogenase. Likewise, LPS did not affect hepatocellular levels of reduced glutathione. Inhibition of either Kupffer cell function or depletion of circulating neutrophils afforded animals

significant protection from LPS-induced enhancement of allyl alcohol hepatotoxicity. These data indicated that these cell populations played a significant role in this model of liver injury. Kupffer cells are key regulators of the host response to infection. They exert their effects on adjacent cells via a number of powerful biochemical mediators such as cytokines and prostaglandins. Inhibiting the synthesis of one of these mediators, tumor necrosis factor-alpha (TNF), has been shown to be protective in models of liver injury using high doses of LPS. Administration of PTX, an inhibitor of TNF synthesis, protected animals from LPS-induced potentiation of allyl alcohol hepatotoxicity, but the administration of a more specific anti-TNF serum did not. These results suggest that PTX protected animals through one of its other pharmacological properties such as increasing the intracellular levels of cyclic adenine monophosphate (cAMP) in Kupffer cells or attenuation of free radical formation. Direct exposure of cultured hepatocytes to LPS did not increase their sensitivity to allyl alcohol, and neither did direct exposure to TNF. These data suggest that neither LPS nor TNF alone was sufficient to sensitize hepatocytes to allyl alcohol. The data may also suggest that a more complex series of events occurs *in vivo* than simple exposure to TNF. In conclusion, LPS potentiates the hepatotoxicity of allyl alcohol, and cells of the innate immune system play a critical role in the ability of LPS to potentiate the hepatotoxicity of allyl alcohol.

To Mother and Father, the people who always encouraged me to follow my dreams.

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LIST OF ABBREVIATIONS

AA	allyl alcohol
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ANIT	alpha-naphthylisothiocyanate
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
BCA	bicinchoninic acid
BPI	bactericidal/ permeability increasing protein
C	Celsius
cAMP	cyclic adenine monophosphate
CAP	ceramide activated kinase
CD	cluster of designation
CETP	cholesterol ester transport protein
CO_2	carbon dioxide
D	dexter
E. coli	*Escherichia coli*
g	gravity
gm	gram
$GdCl_3$	gadolinium chloride
GSH	reduced glutathione
GSSH	oxidized glutathione
Ig	immunoglobulin
HPLC	high performance liquid chromatography
IκB	inhibitory subunit of N kappa B
IL-1α	interleukin 1 alpha
IL-6	interleukin 6
ip	intraperitoneal
iv	intravenous
KC	Kupffer cell
kD	kilodalton
KDO	2-keto-3-deoxy-D-manno-octulosonic acid
kg	kilogram
KOH	potassium hydroxide
$KHCO_3$	potassium bicarbonate
L	liter
LBP	LPS binding protein
LPS	lipopolysaccharide
M	molar

min	minute
MAP	mitogen activated protein
μg	microgram
mCD14	membrane-bound CD14
mg	milligram
ml	milliliter
mmol	millimole
mM	millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
4-MP	4-methylpyrazole chloride
N	number
NAD^+	oxidized nicotinamide dinucleotide
NADH	reduced nicotinamide dinucleotide
ND	none detected
NADPH	reduced nicotinamide dinucleotide phosphate
ng	nanogram
nm	nanometer
$NO^{\cdot}$	nitric oxide
NOS	nitric oxide synthase
NF-κB	nuclear factor kappa B
O_2	molecular oxygen
p	protein
PAF	platelet activating factor
pH	power of hydrogen
PMN	polymorphonuclear leukocyte
PGD_2	prostaglandin D2
PGE_1	prostaglandin E1
PGE_2	prostaglandin E2
$PGF_{1\alpha}$	prostaglandin F1 alpha
PGI_2	prostaglandin I 2
6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin F1 alpha
po	per os
PTX	pentoxifylline
Sal	saline
sCD14	soluble CD14
SEM	standard error of the mean
TNF-α	tumor necrosis factor alpha
TNF-β	tumor necrosis factor beta
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
U	unit

UV	ultraviolet

INTRODUCTION

The innate immune system serves as the first line of defense against microbial invaders for mammalian organisms. Due to its ability to respond to biological insult without prior exposure, the innate immune system is an invaluable asset in the prevention of a bacterial infection. Individuals who have defects in this branch of the immune system are more susceptible to bacterial disease than other individuals (Anderson *et al.,* 1985; Giger *et al.,* 1987; Patarroyo and Makgoba, 1989). Although the activation of the innate immune system is most often beneficial to its host, it can also be detrimental. An excessive or inappropriate response of the innate immune system can result in injury to organs, derangement of blood circulation or death.

Lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria is an ancient signal of bacterial invasion. Cells of the innate immune system respond to this substance and activate the various host defense mechanisms. LPS is also one of the things which can trigger an overreaction from the innate immune system.

Recently, activation of the innate immune system has been associated with the enhancement of adverse tissue responses to certain xenobiotic agents. Laboratory animals treated with activators of the innate immune system are more susceptible to agents such as carbon tetrachloride

(Chamulitrat *et al.*, 1994), D-galactosamine (Galanos *et al.*, 1979), and halothane (Lind *et al.*, 1994). It is interesting to speculate whether this also occurs in humans. If so, the state of activation of the innate immune system may explain some of the idiosyncratic responses to toxic xenobiotics observed in humans. When the innate immune system has been stimulated, an otherwise nontoxic dose of a xenobiotic agent becomes toxic.

In this dissertation, the following hypothesis will be tested: Activation of the inflammatory cells of the innate immune system by lipopolysaccharide and the consequent production of mediators by these cells is important in the mechanism by which lipopolysaccharide enhances allyl alcohol-induced hepatotoxicity. In this introduction, the innate immune system, biology of lipopolysaccharide and its effects on the liver, the interaction of the innate immune system with lipopolysaccharide, the ability of lipopolysaccharide to potentiate xenobiotic hepatotoxicity, and the toxicology of allyl alcohol will be discussed. In subsequent chapters, experimental evidence supporting the above hypothesis will be presented and discussed.

Chapter 1

GENERAL INTRODUCTION

1. A. The Innate Immune System

The mammalian immune system is composed of two main divisions: the acquired immune system and the innate immune system. The acquired immune system must have prior exposure to pathogens before a full response is mounted. Thus it serves as a secondary, long-term line of host defense. The various populations of lymphocytes make up this branch of the immune system. In contrast, the innate immune system is designed for a rapid response to invasive agents and is nonspecific in its actions. It's activity does not require prior exposure. The cells comprising this system are myeloid in origin, and their precursors are located in the bone marrow. Members of this branch of the immune system include macrophages, neutrophils, eosinophils, and basophils.

1. A. 1. Macrophages

Macrophages are terminally differentiated mononuclear phagocytes that reside in most tissues. The cells are believed to originate mainly in the bone marrow and travel to their tissue of destination in the form of blood monocytes. Precursor populations of macrophages have also been found in other organs, and these most likely contribute to the fixed macrophage populations of the body.

Macrophages exhibit both heterogeneity among tissues as well as within a given tissue. For example, in mice, the populations of macrophages within various tissues express different cell surface proteins and have different levels of biochemical activity and phagocytic ability (Unanue, 1990). In the murine spleen, two populations of macrophages can be distinguished based upon phagocytic activity and expression of MHC class II molecules (Unanue, 1990).

These cells have numerous functions that protect the host from invading pathogens. They entrap and kill microorganisms and clear body fluids of soluble immune complexes. They take up and process antigen for presentation to lymphocytes. Macrophages are also highly secretory. Their products include eicosanoids, growth factors, complement proteins, nitric oxide, cytokines, reactive oxygen species, chemokines and enzymes (Decker, 1990). These products represent a mechanism by which macrophages communicate with other cells and regulate the immune response.

1. A. 2. Kupffer Cells

Kupffer cells are named after their discoverer, Baron von Kupffer, and are the largest single population of macrophages in the mammalian body.

These are the resident macrophages of the liver and are situated in the hepatic sinusoids. The strategic position of Kupffer cells in the hepatic sinusoids allows them to play a major role in clearing the portal blood of particulate matter, especially bacteria and bacterial products. Kupffer cells form one part of the two-part system used by the liver to detoxify and metabolize LPS (Fox *et al.,* 1989). They perform the first part of the detoxification process by removing some of the polysaccharide moieties; the modified LPS is then passed onto hepatocytes for further metabolism.

In intact animals, Kupffer cells play a major role in the liver injury caused by large doses of LPS (2 mg/kg and above). Blockade of Kupffer cell function protects the host from many of the damaging effects of endotoxemia (Marshall *et al.,* 1987; Iimuro *et al.,* 1994). The ability of LPS-stimulated Kupffer cells to affect and modify the functions of adjacent parenchymal cell populations is possibly one reason for the injurious effects produced by the resident liver macrophages. Another may be the direct toxic effects of some of the biochemical mediators produced by these cells.

1. A. 2. 1. Hepatic Architecture

The liver is the largest gland in the body, with both exocrine and endocrine functions. This organ is situated between the mesenteric venous blood flow and the rest of the body, and the liver serves as the gate-keeper between the intestine and the systemic blood. The mesenteric veins combine to form the hepatic portal vein, the main blood supply to the liver. A second, much smaller blood supply comes from the hepatic artery.

Histologically, the liver is composed predominantly of cuboidal epithelial cells arranged in radiating cords that are in turn arranged in laminae. The spaces between the radiating cords are called sinusoids and they converge at the central vein. The distal portion of the sinusoids originates at the portal triad, a structure composed of a branch of the portal vein, a branch of the hepatic artery, one or more bile ducts, and a lymph channel. The sinusoids can be thought of as specialized capillary beds. They are lined with fenestrated endothelial cells, Kupffer cells, Ito cells, and pit cells. Blood flows from the portal triads towards the central veins. Eventually the central veins converge to form the hepatic veins that drain into the inferior vena cava.

1. A. 2. 2. Location and Function of Kupffer Cells

Kupffer cells are one of the four types of hepatic sinusoidal cells. They have two origins: 1) from proliferation of existing hepatic populations and, 2) from precursors in the bone marrow (Fawcett, 1986). They are variable in shape and seem to be able to change their position within the sinusoidal space. While they are mainly found in contact with the endothelium, they may also be found within the space of Disse (Bouwens and Wisse, 1992; Wisse *et al.*, 1996).

The surface of Kupffer cells is irregular, characterized by surface folds, villous projections and vermiform bodies. A thin glycocalyx is present; most of the cell surface of Kupffer cells is in contact with the blood flowing from the portal triad. Their cytoplasm is richer in organelles than the neighboring endothelial cells. Peroxidase is present in the lumens of the endoplasmic reticulum, and peroxidase activity is a means to distinguish Kupffer cells from endothelial cells.

Different populations of Kupffer cells have been identified based on size, phagocytic activity and surface antigens. Periportal Kupffer cells are larger, have more lysosomal and cathepsin G activity, and are able to phagocytose more latex particles than Kupffer cells isolated from the centrilobular or midzonal regions of the liver lobule (Sleyster and Knook, 1982).

Macrophage subpopulations can be distinguished using the ED family of cell surface antigens. ED1 has been found on monocytes and all macrophage populations while ED2 has been associated with resident macrophages (Sato *et al.,* 1998; Yamate *et al.,* 1999). Using monoclonal antibodies to the ED1 and ED2 surface antigens, Armbrust and Ramadori (1996) distinguished between two populations in the liver. Large ED1+/ ED2+ Kupffer cells are located mainly in the periportal and centrilobular regions. Smaller ED1+/ED2- cells are found along the midzonal region of the sinusoids.

The position of Kupffer cells in the hepatic sinusoids allows them extensive access to the portal blood flow. Kupffer cells are highly phagocytic cells, and in addition to removing bacterial LPS as mentioned above, they actively remove particulate matter from blood passing over them. When laboratory animals are treated with colloidal carbon, the carbon particles are rapidly phagocytosed by the host's macrophages, including Kupffer cells. The accumulated carbon can be seen under light microscopy (Fawcett, 1986).

1. A. 2. 3. Interaction of Kupffer Cells and Hepatocytes

Kupffer cells are in contact with the other cell types of the liver and play a role in liver homeostasis in both normal and disease states. These macrophages can affect the level of protein synthesis, protein phosphorylation, and glycogenolysis in neighboring hepatocytes. For example, in a series of *in vitro* studies involving hepatocyte:Kupffer cell cocultures, West and colleagues (1985, 1986, 1988) found that unstimulated Kupffer cells stimulate protein synthesis in hepatocytes as measured by ^{3}H-leucine incorporation.

In contrast to results with unstimulated Kupffer cells, when hepatocytes were cultured with Kupffer cells stimulated with LPS, protein synthesis was inhibited. The factors involved in this process were soluble because these results could be reproduced by culturing hepatocytes in medium from unstimulated Kupffer cells and LPS- or *E. coli*-stimulated Kupffer cells. The level of inhibition of protein synthesis was directly related to the production of nitrates and nitrites, suggesting a role for nitric oxide ($NO^{\cdot}$) (Billiar *et al.,* 1989a, Billiar *et al.,* 1989b). Furthermore, the level of inhibition of protein synthesis was also related to the amount of L-arginine in the culture medium, and the inhibitor of nitric oxide synthesis, N^{G}monomethyl-L-arginine, prevented most of the observed inhibition of protein synthesis.

Conditioned medium from LPS-treated Kupffer cells enhanced the phosphorylation of some proteins and inhibited the phosphorylation of others in cultured hepatocytes (Castelijn *et al.,* 1988a). These changes in phosphorylation could be reproduced with the prostaglandins PGD_2, PGE_1, and PGE_2, indicating that Kupffer cells use eicosanoids as a means of intercellular communication in the liver.

Another example of this communication is the role of PGD_2 in the increase in glucose levels seen during endotoxemia. In the isolated, perfused liver, LPS exposure produces a spike in PGD_2 levels before an increase in glucose output (Castelijn *et al.,* 1988b). This increase in glucose can be blocked with aspirin, an irreversible inhibitor of cyclooxygenase. In addition, PGD_2 alone can stimulate glucose production in cultured hepatocytes. LPS alone could not, indicating that a cell type other than hepatocytes is producing the PGD_2. Since the major eicosanoid produced by LPS-stimulated Kupffer cells is PGD_2, Kupffer cells are the most likely candidate (Kuiper *et al.,* 1988).

1. A. 3. Neutrophils

Neutrophils are another cell population important in the innate immune system. They are derived from the same pluripotent stem cell as blood

monocytes. Mature neutrophils are released into the general circulation and have a half-life in the circulation of approximately six hours. They soon move out of the blood stream and enter the extravascular pool of neutrophils. These cells are "front line" phagocytes responsible for early defense against bacterial infection. They are the first cells to arrive at sites of inflammation and are responsible for the early killing of invading microorganisms. Neutrophil granules are storage sites for the various enzymes and mediators used by these cells as part of their role in tissue inflammation (Gallin, 1989).

Even though neutrophils are largely beneficial in their activities, they can cause massive hepatocellular injury and necrosis. Large numbers of neutrophils accumulate in the sinusoids of rats treated with high doses of LPS (2 mg/kg and above) (Hewett *et al.,* 1992). These neutrophils release proteases and reactive oxygen species into the intercellular environment for the purpose of killing invading bacteria; however, the mediators also have toxic effects on the hepatic parenchymal cells, causing cell death and injury. Reduction of circulating neutrophil populations alone or disrupting their ability to migrate into tissues can protect rats (Jaeschke *et al.,* 1991; Hewett *et al.,* 1992; Hewett *et al.,* 1993) and mice (Xu *et al.,* 1994) from the liver injury associated with exposure to high doses of LPS over 2 mg/kg, demonstrating that

neutrophils, like Kupffer cells, play a vital role in the liver injury associated with high blood levels of LPS.

1. A. 4. Eosinophils and Basophils

Eosinophils and basophils form the balance of the innate immune system. These cells do not interact directly with LPS but are responsible for protection against parasitic infections (eosinophils) and mediating the allergic response (eosinophils and basophils) (Fawcett, 1986).

1. A. 5. Interaction of LPS and the Innate Immune System

Cells of the innate immune system are very sensitive to the presence of LPS. In fact, many of the effects of LPS do not arise from direct action of LPS on the various systems of the host, but are mediated through the actions on the innate immune system.

The focus of this dissertation is the role of the innate immune system in LPS effects on allyl alcohol hepatotoxicity. The previous sections provided background on the innate immune system. The next section will begin with

an introduction to the topic of LPS as a prelude to a discussion of the interaction of LPS with the innate immune system.

1. B. Lipopolysaccharide

1. B. 1. Structure

LPS is a component of the outer membrane of Gram-negative bacteria (Anderson *et al.,* 1985; Raetz *et al.,* 1988; Rietschel *et al.,* 1994). The exact structure of the molecule varies among species and strains of bacteria, but all LPS molecules share three specific characteristics. These are the O-antigen, the core, and lipid A (Figure 1.1). The most variable region of LPS is the O-antigen. The O-antigen is the outermost portion of a molecule of LPS and is in contact with the external environment. Composed of twenty to forty repeating sugar units, the O-antigen is responsible for the antigen specificity of a given LPS molecule. The core, the central region of LPS, is embedded in the outer surface of the membrane. It serves as the bridge between the O-antigen and the innermost portion, lipid A. The core contains two unusual sugars: a seven-carbon sugar (heptose), and a 2-keto-3-deoxy-D-manno-octulosonic acid

(KDO). The final portion, lipid A, is the most conserved region of LPS. It is also responsible for most of the biological activity associated with LPS. The lipid A portion of LPS is fully embedded in the membrane, and its fatty acid tails oppose the fatty acid tails of the phospholipids that make up the internal side of the outer membrane lipid bilayer.

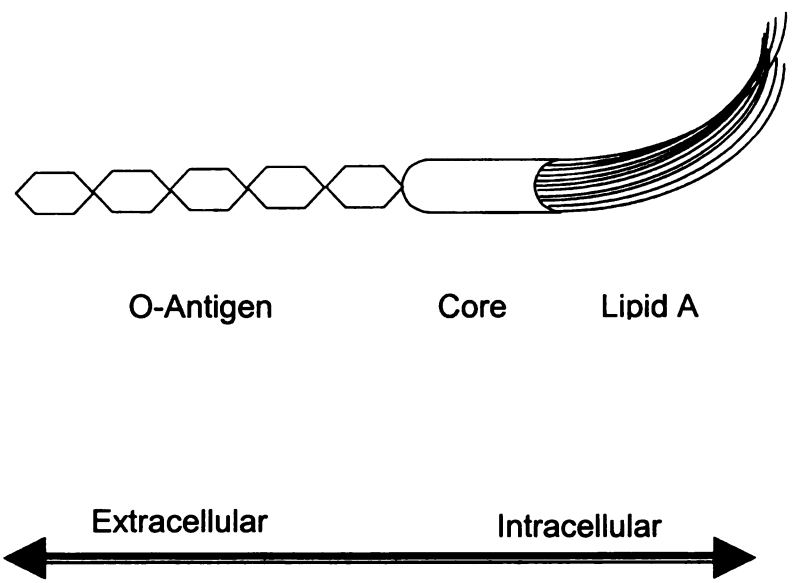
LPS has been classified into three categories based on the structure of the O-antigen: smooth, semi-rough, and rough. In the smooth strains of LPS, a full complement of twenty to forty sugar residues is present in the O-antigen. The O-antigen is greatly reduced in the semi-rough strains, whereas the rough strains lack both the O-antigen and part of the core (Morrison and Ulevitch, 1978).

1. B. 2. Sources of LPS in Host Systems

LPS may enter the body of the host animal by two major routes: from organisms outside of the host (exogenous) and from organisms inhabiting the intestinal tract (endogenous). Most of the toxic effects of LPS are associated with infection by Gram-negative bacteria, an exogenous source of LPS. The proliferation of bacteria and the release of their products into the host's system lead to the clinical conditions known as septic shock, adult respiratory distress syndrome, and multiple system organ failure.

Figure 1.1. Structure of LPS. The typical LPS molecule has three distinct divisions. The O-antigen is the outermost portion of the molecule and faces into the environment. The middle portion is the Core and contains a unique seven-carbon sugar, KDO. Lipid A is the innermost portion of the molecule.

Lipopolysaccharide



The diseases associated with bacterial infections arise from a wide variety of causes including tissue trauma, contagion, and nosocomial infections. The elderly, very young, and immunocompromised are the most vulnerable, but any individual can be affected (Durham *et al.,* 1990). Even with intervention with antibiotics and supportive therapy, many patients diagnosed with one of the above conditions still die. This is thought to be due the enhanced release of LPS from the bacteria killed by the antibiotic and the amount of systemic damage that has occurred before the patient is treated.

Endogenous LPS arises from the Gram-negative bacteria mammals normally carry in their large bowel. These microorganisms ferment ingesta, further processing it and releasing valuable nutrients. A small amount of the LPS released by the turnover of gut bacteria reaches the portal circulation and is cleared by the Kupffer cells (Jones and Summerfield, 1988; Fox *et al.,* 1989; Nakao *et al.,* 1994). If the gut wall is compromised by intoxication or disease, an increased amount of LPS may enter the portal circulation and overwhelm the normal hepatic clearance mechanisms.

1. B. 3. Effects of LPS on Mammalian Physiology

LPS is a biologically active compound in mammalian systems (Raetz *et al.,* 1988; Vogel, *et. al.*, 1990; Raetz, *et. al.*, 1991; Wright and Kolesnick, 1995) and may have either positive or negative responses in a given host. Several beneficial effects have been found from exposure to LPS. The oldest benefit known is the chemotherapeutic effect of bacterial products in cancer treatment. This was first noted in the late 1800's. Researchers discovered that LPS could induce hemorrhage and necrosis in tumors (Vogel and Hogan, 1990). In time, they found the causative agent to be the cytokine, tumor necrosis factor, which is elicited by exposure to LPS (Carswell *et al.,* 1975). Other beneficial effects of LPS include stimulation of the innate immune system to resist infection. The C3H/HeJ mouse, which is hyporesponsive to LPS, has less resistance to pathogens than the C3H strains that exhibit a normal response to LPS (O'Brien *et al.,* 1980). Sublethal doses of LPS are also radioprotective when given 16 to 24 hours prior to exposure (Neta *et al.,* 1986a). The protective effect of LPS was found again to be due to the induction of cytokines such as tumor necrosis factor- alpha, interleukin-1 and colony stimulating factor (Fujita *et al.,* 1983; Neta *et al.,* 1986a; Neta *et al.,* 1986b).

LPS has a number of detrimental effects on the host as described above. On the physiological level, LPS acts as a pyrogen. It raises the

normal temperature set point in the hypothalamus (Dinarello, 1983). The body raises the core temperature in response to the new set point by vasoconstriction, reduced sweating, piloerection, and shivering. LPS exposure causes hypoglycemia by inhibiting the glucocorticoid induction of phosphoenolpyruvate carboxykinase (Rippe and Berry, 1972). LPS toxicity also causes disorders of the circulatory system, notably disseminated intravascular coagulation, hypotension, and infarcts. It is the circulatory disorders that are associated with the lethal effects of LPS, and organs that have extensive vascular beds such as liver, lung, and kidney are very susceptible. The organ of interest for this dissertation is the liver.

1. B. 3. 1. Effects of LPS on the Liver

Gram-negative bacteria and LPS have been shown to be hepatotoxic by several investigators (Levy, *et. al.*, 1967; Balis, *et. al.*, 1979; Utili, *et. al.*, 1977; Hirata, *et. al.*). Microscopic changes in the liver are seen 30 minutes after the injection of a sublethal dose of LPS in mice (100-300 μg) (Levy *et al.,* 1967). At this time, Kupffer cells become more rounded in appearance and bulge into the hepatic sinusoids, and neutrophils begin to accumulate in the sinusoids. Neutrophils are present in the sinusoids by 60 minutes. This is accompanied by mononuclear cells and eosinophils.

Thrombi are also present at this time. At two hours after LPS treatment, Kupffer cells and endothelial cells are swollen. This, along with the thrombi and infiltrating leukocytes, slows and alters the pattern of blood flow through the hepatic sinusoids (McCuskey *et al.,* 1982). The number of neutrophils in the liver continues to increase.

LPS has some direct effects on hepatocytes; however, these effects (including alterations in bile and fatty acid synthesis) are not considered to be toxic (Utili *et al.,* 1977; Feingold *et al.,* 1992). Most of the liver injury from treatment with LPS is due indirectly to effects of LPS on the innate immune system. These various indirect effects of LPS on the liver are mediated by cytokines, free radicals, eicosanoids, and enzymes produced by cells of the innate immune system (Figure 1.2).

1. B. 3. 2. Biochemical mediators of LPS-induced hepatotoxicity

Cytokines

Cytokines are proteins or glycoproteins produced by cells of the immune system. They serve as a means of communication between the producing cell and the target cell, and their effects may be local or systemic.

Elevated levels of cytokines are found in the blood of both human patients suffering from bacterial infections and experimental animals administered LPS or Gram-negative bacteria. The plasma levels of tumor necrosis factor-alpha and interleukin-6 in patients admitted to hospitals with serious bacterial infections are elevated, and this elevation correlates with disease severity and death (Brantzaeg *et al.,* 1991; Dofferhoff *et al.,* 1991).

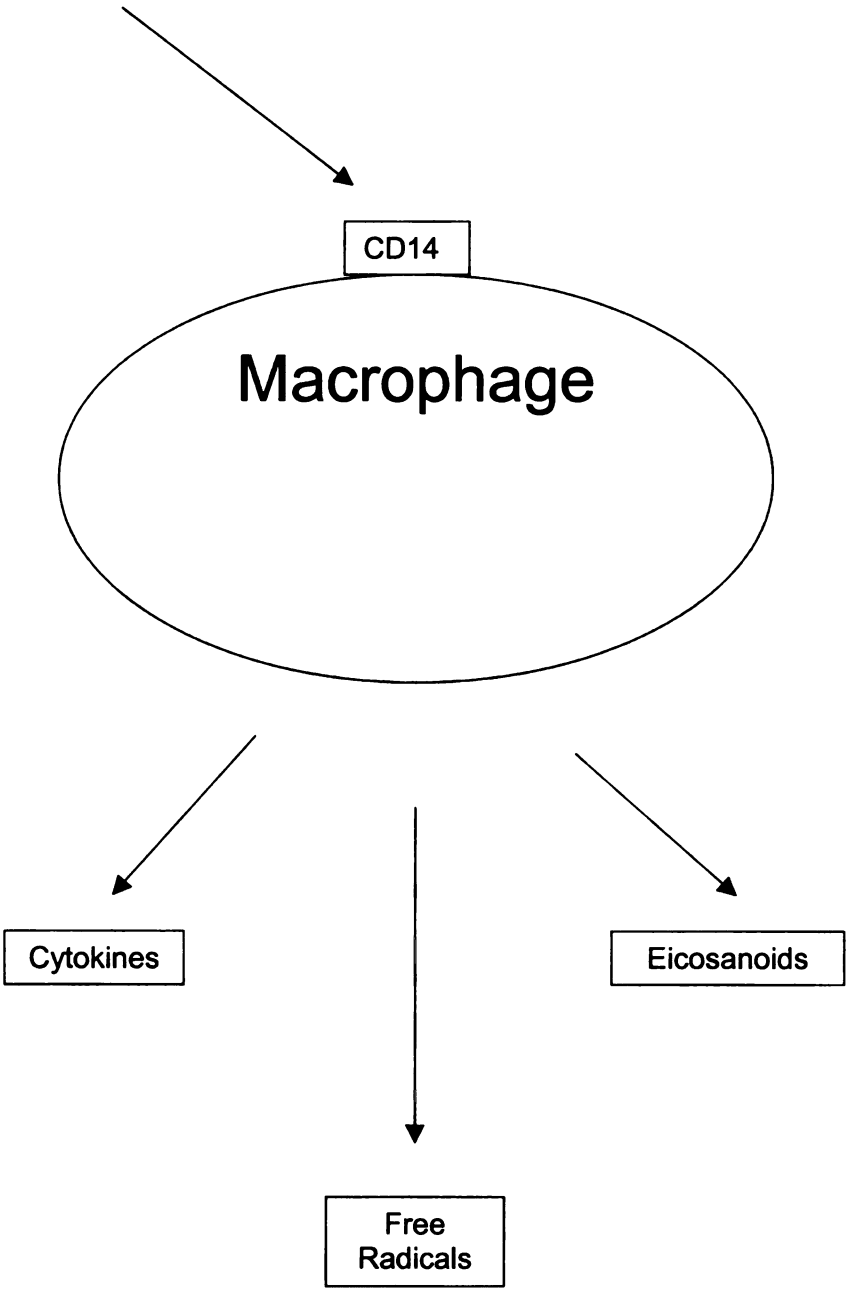
Tumor necrosis factor-alpha

The term "tumor necrosis factor" is used for two cytokines produced in response to inflammatory stimuli. The alpha form is released by cells of the monocyte/macrophage series and is also known as cachectin (Beutler *et al.,* 1985; Beutler and Cerami, 1987; Bemelmans *et al.*, 1996). The beta form is produced by lymphoid cells and is called lymphotoxin (Paul and Ruddle, 1988). Both have similar biological effects.

Tumor necrosis factor-alpha (TNF) is considered to be the pivotal proinflammatory cytokine of the host response to LPS (Tracey and Cerami, 1993). It is the first cytokine detected after exposure to bacteria or endotoxin: blood concentrations peak between 90 to 120 minutes in humans (Hesse *et al.,* 1988; Michie *et al.,* 1988) and at 120 minutes in baboons (Creasey *et al.,* 1991). TNF can stimulate the production of the other major cytokines of inflammation, interleukin-1 (IL-1) and interleukin-6 (IL-6) (Dinarello *et al.,* 1986; Fong *et al.,* 1989; Helle *et al.,* 1991).

Figure 1.2. Products of LPS-stimulated macrophages. The binding of the LPS binding protein (LBP):LPS complex to the CD14 receptor activates a number of intracellular pathways, resulting in the release of a variety of biochemical mediators of inflammation.

LBP:LPS



Inhibition of tumor necrosis factor-alpha activity, either by passive immunization with anti-tumor necrosis factor-antibodies (Beutler *et al.,* 1985; Tracey *et al.,* 1987) or pharmacologically with pentoxifylline (Hewett *et al.,* 1993), protects laboratory animals from the damaging effects of both endotoxemia and bacteremia. The signs and symptoms associated with inflammation and cachexia can be mimicked by administration of exogenous TNF (Tracey *et al.,* 1988).

Interleukin-1

Interleukin-1 is a cytokine produced by a variety of cell types and tissues, including macrophages, epidermal, epithelial, lymphoid, and vascular tissues (Moore *et al.,* 1980; Dinarello, 1988). It is the second of the proinflammatory cytokines to reach a peak in plasma after exposure to endotoxin (240-300 min in baboons; 120 minutes in humans) (Creasey *et al.,* 1991; Zabel *et al.,* 1989) and is found in the blood of human patients and experimental animals suffering from endotoxemia and bacteremia (van der Meer and Vogels, 1991; Brandtzaeg *et al.,* 1991; Creasey *et al.,* 1991).

Interleukin-1 is a potent mediator of the inflammatory response. It enhances the growth of T lymphocytes, induces the expression of adhesion molecules on endothelium, elicits the release of other biochemical mediators such as prostaglandins, IL-6, TNF, and granulocyte-macrophage colony stimulating factor (Kunkel *et al.,* 1986;

Durum and Oppenheim, 1989), and induces neutrophil emigration into tissues (Cybulsky *et al.,* 1986; Movat *et al.,* 1987). In mice, blockade of the IL-1 receptor significantly alters the inflammatory response to LPS (McIntyre *et al.,* 1991). Neutrophil migration from the bone marrow is significantly attenuated. In addition, serum levels of hepatic acute phase proteins and IL-6 are significantly lowered in the murine model (McIntyre *et al.,* 1991).

Interleukin-6

Following administration of LPS, blood concentrations of IL-6 peak at 120 minutes in humans (Zabel *et al.,* 1989) and at 180 minutes in rats (Kispert 1992). IL-6 is produced by a number of cell types such as activated macrophages and T lymphocytes, endothelial cells, and fibroblasts (Durum and Oppenheim, 1989; Jirik *et al.,* 1989; Helle *et al.,* 1991; Callery *et al.,* 1992). The production of IL-6 is greatly influenced by the other two proinflammatory cytokines, TNF and IL-1. Both TNF and IL-1 have a stimulatory effect on IL-6 production (Durum and Oppenheim, 1989; Evans *et al.,* 1992; Brouckaert *et al.,* 1993).

Interleukin-6 plays a key role in the acute phase response characteristic of endotoxemia. This cytokine is a major initiator and regulator of the hepatic synthesis of acute phase proteins such as fibrinogen, alpha-2-macroglobulin, and C-reactive protein (Zentella *et al.,* 1991; Kispert 1992).

Eicosanoids

The eicosanoids are a family of unsaturated, twenty-carbon fatty acid derivatives with powerful regulatory actions on host physiology. They include prostaglandins, leukotrienes, and thromboxanes. These lipids mediate a wide variety of bodily functions including the production of pain and fever, induction of blood clotting, regulation of blood pressure, induction of labor, regulation of gastric acid production and inflammation. The eicosanoids have some hormone-like properties, but unlike hormones, they generally exert their actions locally.

Eicosanoids are involved in the inflammatory response. They are produced by cells of the immune system and regulate many of the activities of neighboring cells such as cytokine production (Callery *et al.,* 1990) and glycogenolysis (Casteleijn *et al.,*1988c). The pattern of eicosanoid production varies among cell types. Neutrophils have a greater production of leukotrienes compared to macrophages, which produce more prostaglandins.

Eicosanoids are produced by cells of the innate immune system in response to both normal physiologic stimuli and LPS. These compounds or their metabolites have been detected in the plasma and bile of septic patients and of experimental animals given endotoxin or otherwise exposed to products of Gram-negative bacteria. Inhibition of one or more of the eicosanoids has been shown to increase survival in some animal

models. For example, the use of non-steroidal anti-inflammatory drugs such as aspirin, naproxen, and ibruprofen increases the survival of endotoxemic or septic animals (Ball *et al.,* 1986; Albrecht *et al.,* 1997). Inhibitors of the 5-lipoxygenase pathway or leukotriene receptor antagonists have also proven helpful in ameliorating the effects of endotoxic/septic shock (Keppler *et al.,* 1987; Tiegs and Wendel, 1987; Nagai *et al.,* 1989; Matuschak *et al.,* 1990).

The thromboxanes

The thomboxanes are vasoactive eicosanoids that mediate many of the vascular events associated with endotoxic and septic shock. Thromboxane A_2 (TxA_2) is a potent vasoconstrictor and causes platelet aggregation. It has a very short plasma half-life (~30 seconds), and thus the stable derivative, thromboxane B_2 (TxB_2), is usually used an indicator of TxA_2 in plasma. Thromboxanes reach peak plasma concentrations rapidly after bolus iv injections of endotoxin (Ball *et al.,* 1986). Inhibition of thromboxane synthesis or pharmacological antagonism of TxA_2 increases survival in animal models in which endotoxin is given as a bolus iv injection.

The prostaglandins

The prostaglandins mediate a wide range of physiological functions. They play important roles in the regulation of acid production by gastric chief cells, the absorptive activities of the cells of the proximal renal

tubules, induction of labor, and many other physiological processes. With respect to this dissertation, prostaglandins produced by Kupffer cells in response to exposure to endotoxin will be the focus.

Prostaglandins are produced by macrophages, including Kupffer cells, after treatment with endotoxin. The metabolites that have been identified are PGE_1, PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2), (Bowers *et al.,* 1985; Brouwer *et al.,* 1988; Casteleijn *et al.,*1988c; Okumura *et al.,*. 1987.)

Like prostaglandins in other organs of the body, the prostaglandins produced by Kupffer cells have regulatory functions after exposure to endotoxin. They regulate the production of the proinflammatory cytokines (TNF, IL-1, IL-6) (Karck *et al.,* 1988; Callery *et al.,* 1990; Peters *et al.,* 1990; Grewe *et al.,* 1994; Roland *et al.,* 1994) and inducible nitric oxide synthase (NOS) (Gaillard *et al.,*1991; Harbrecht *et al.,* 1995), and cause the release of glucose from hepatocytes (Casteleijn *et al.,* 1988b).

The leukotrienes

The presence of LPS triggers the production of leukotrienes *in vivo,* and the cysteinyl forms are eliminated in the bile (Hagmann *et al.,* 1985). Leukotrienes are highly chemotactic for neutrophils and are produced by hepatic macrophages during sepsis (Doi *et al.,* 1993). This presence of leukotrienes causes the migration of neutrophils into the liver and the associated liver injury. Rodriguez de Turco and Spitzer (1990) showed that

the dominant arachidonic acid metabolites produced by these infiltrating cells are leukotrienes. Additionally, there is an increase in 5-lipoxygenase activity in the liver (Kawada *et al.,* 1992).

The exact role of leukotrienes in endotoxin-induced liver injury is somewhat controversial. Inhibitors of leukotriene synthesis or leukotriene antagonists are protective in models of endotoxin-induced liver injury *in vivo* involving presensitization with galactosamine (Tiegs and Wendel, 1988; Keppler *et al.,* 1987; Matuschak *et al.,* 1990) or with *Corynebacterium parvum* (Nagai *et al.,* 1989). However, in one *in vivo* model in which only endotoxin was administered, inhibition of 5-lipoxygenase with zileutin was not protective (Pearson *et al.,* 1997). These different results may indicate that the fundamental role of leukotrienes is different in the respective models.

Free radicals

Reactive oxygen species

Cells of the myeloid lineage can generate reactive intermediates of oxygen as a means of killing invading bacteria. Molecular oxygen is converted to the superoxide anion radical. The enzyme, superoxide dismutase, then converts the superoxide anion to hydrogen peroxide, and subsequently the hydroxyl radical is produced.

The production of superoxide anion and related free radicals has been linked to liver injury in a number of models. Macrophages and neutrophils

primed by previous exposure to endotoxin or other stimulants, such as phorbol myristate acetate, produce much higher levels of oxygen-derived free radicals than controls (Arthur *et al.,* 1986; Arthur *et al.,* 1988; Shiratori *et al.,* 1988; Aida and Pabst, 1990; Bautista *et al.,* 1990; Mayer and Spitzer, 1991). When the subsequent release of oxygen-derived free radicals is blocked by allopurinol (Arthur *et al.,* 1985) or superoxide dismutase (Arthur *et al.,* 1985; Shiratori *et al.,* 1988), liver injury is prevented.

Nitric Oxide

Nitric oxide ($NO^{\cdot}$) is a free radical that is produced by most cells of the mammalian body. The physiological effects mediated by $NO^{\cdot}$ are quite varied and are dependent on the cell producing the radical and the local environment. The enzyme that produces $NO^{\cdot}$, nitric oxide synthase (NOS), has two forms, constitutive and inducible. The constitutive form of the enzyme is found in numerous cell types such as endothelial cells and neurons. Macrophages have an inducible form of NOS which becomes upregulated after exposure to LPS (Gaillard et al., 1991; Laskin et al., 1994). In both instances, the $NO^{\cdot}$ is derived from L-arginine in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and nitirc oxide synthase.

Macrophages use $NO^{\cdot}$ as a cytotoxic molecule. It has been detected during macrophage-induced killing of a variety of targets, including tumor

cells, *Entamoeba histolytica*, *Leishmania major*, and bacteria (Liew *et al.*, 1990a; Liew *et al.*, 1990b; Lin and Chadee, 1992; Aono *et al.*, 1994; Fukumura *et al.*, 1996; Saito *et al.*, 1996; Sveinbjornsson *et al.*, 1996; Lavnialkova *et al.*, 1997; MacMicking *et al.*, 1997; Vouldoukis *et al.*, 1997; Albina and Reichner, 1998). Inhibition of $NO^{\cdot}$ synthesis prevented cytotoxicity in the above models.

1. B. 4. Cellular Effects of LPS

1. B. 4. 1. LPS Binding Protein

The mammalian body has evolved a system by which LPS is transferred from the general circulation to cells of the innate immune system. Several proteins that can bind LPS are present in blood and greatly enhance the movement of small amounts of circulating LPS to monocytes, macrophages, and neutrophils. One of the most studied of these proteins is LPS-binding protein.

LPS binding protein (LBP) is an acute phase protein synthesized by hepatocytes (Ramadori *et al.*,1990). Trace amounts are normally found in the bloodstream of healthy individuals (0.5μg/ml), and the concentration

rises as high as 50 μg/ml 24 hours after the induction of an acute phase response (Tobias *et al.,* 1986, 1988). Structurally, LBP is a 60-kD glycoprotein. It has sequence homology with bactericidal/permeability increasing protein (BPI; 69% amino acid identity with human BPI) and cholesterol ester transport protein (CETP; 23% amino acid identity) (Schumann *et al.,* 1990). This molecule has a high affinity site for binding lipid A (Schumann *et al.,* 1990) and can bind both rough and smooth forms of LPS (Tobias *et al.,* 1989). LBP serves as an opsonizing protein, mediating the attachment of LPS-coated particles and intact Gram-negative bacteria to macrophages (Schumann *et al.,* 1990).

The combined presence of LBP and LPS significantly enhances the production of TNF by rabbit peritoneal macrophages as compared to LPS alone. Addition of LBP to peritoneal macrophages exposed to LPS accelerates the expression of TNF (4.5 hours versus 8 hours) and increases the stability of TNF mRNA (>5 hour versus 1 hour) (Heumann *et al.,* 1992; Mathison *et al.,* 1992). Immunodepletion of LBP inhibits production of TNF in whole blood and in preparations of monocytes, indicating that this protein is a key element in the innate immune system response to LPS (Schumann *et al.,* 1990; Heumann *et al.,* 1992).

1. B. 4. 2. CD14

CD14 was first recognized as a marker for monocytes and macrophages. Now investigators realize that this cell surface molecule is the cellular receptor for complexes of LPS and LBP (Kirkland *et al.,* 1989; Wright *et al.,* 1990). CD14 is a 55 kD glycoprotein attached to the cell membrane by a phosphatidylinositol glycan anchor and is mobile in the plane of the membrane (Haziot *et al.,* 1988; Simmons *et al.,* 1989). CD14 allows macrophages, monocytes and neutrophils to respond to very low circulating concentrations of LPS:LBP complexes (Wright *et al.,* 1990; Haziot *et al.*, 1993b). LPS alone is able to bind to the CD14 molecule; however, this occurs at much higher concentrations of LPS than in the presence of LBP (Hailman *et al.,* 1994). Additionally, a soluble form of CD14 (sCD14) is present in blood and acts as the equivalent of the membrane-bound CD14 (mCD14) for cells which do not have mCD14 (e.g. endothelial cells and astrocytes) (Bazil *et al.,* 1989; Pugin *et al.,* 1993; Haziot *et al.,* 1993a; Goldblum *et al.,* 1994; Tobias *et al.,*1995; Jack *et al.,* 1995; Goldenbock *et al.,* 1995). The soluble CD14 appears to originate from CD14 molecules shed from the surface of circulating monocytes (Bufler *et al.,* 1995).

1. B. 4. 3. Intracellular Mechanisms of Action

Many of the biological effects of LPS have been described above. Here, the cellular basis of some of these effects will be discussed.

LPS induces rapid protein tyrosine phosphorylation (4-5 minutes) in both murine peritoneal macrophages and RAW 264.7 cells, and this phosphorylation results in the release of arachidonic acid metabolites (Weinstein *et al.,* 1991). Inhibition of protein tyrosine phosphorylation by herbimycin A causes a dose-dependent decrease in the concentrations of arachidonic acid metabolites produced by RAW 264.7 cells (Weinstein *et al.,* 1991). LPS also induces tyrosine phosphorylation of at least two mitogen-activated protein (MAP) kinases (Weinstein *et al.,* 1992). These data correlate with another set of experiments in which mice are protected from lethal endotoxemia by administration of tyrphostins, compounds that inhibit protein tyrosine phosphorylation (Novogrodsky *et al.,* 1994).

In recent years, experimental evidence indicates that LPS can activate monocytes and macrophages via the sphingomyelin pathway. The lipid sphingomyelin is concentrated in the outer leaflet of the plasma membrane of mammalian cells (Kolesnick and Golde, 1994). Ceramide is the fatty acid in an amide linkage at position two, and hydrolysis of the phosphodiester bond by sphingomyelinase yields free ceramide and phosphocholine (Kolesnick and Hermer, 1991). It is possible that free

ceramide interacts with ceramide activated protein (CAP) kinase, a proline-directed protein kinase which may phosphorylate p42 MAP kinase (Raines *et al.,* 1993; Joseph *et al.,* 1993; Liu *et al.,* 1994). In addition to activating a portion of the MAP kinase system, ceramide may also induce the degradation of inhibitory κB (IκB), the cellular inhibitor of nuclear factor-κB (NF-κB), resulting in translocation of NF-κB to the nucleus (Yang *et al.,* 1993).

The overall structure of ceramide, the putative second messenger molecule of this signal transduction pathway, and lipid A are similar. A specific region of each molecule, the glycerol backbone, is essentially identical and is required to evoke a response from cells (Joseph *et al.,* 1994). In HL-60 cells, lipid A is able to induce phosphorylation of CAP kinase within 30 seconds of exposure. This result is similar to those obtained with ceramide (Joseph *et al.,* 1994). LPS and lipid A are not believed to stimulate the production of ceramide from sphingomyelin but are thought to mimic ceramide in the cell and thus activate the CAP kinase-dependent pathway.

Ceramide is believed to participate in the cellular actions of the proinflammatory cytokines, TNF and IL-1β. Both of these cytokines may use the sphingomyelin pathway to exert their effects (Mathias *et al.,* 1991; Mathias *et al.,* 1993). Investigators have shown that TNF can increase

intracellular levels of ceramide and lead to translocation of NF-κB into the nucleus (Yang *et al.,* 1993).

1. B. 5. Summary

LPS is a powerful elicitor of host responses. This molecule can initiate many biochemical pathways in mammalian hosts and some of these pathways can lead to organ damage or death. Most of the hepatotoxic effects associated with LPS are mediated indirectly through the innate immune system, of which the Kupffer cells and neutrophils are critical components. Due to the important role of macrophages in regulating the host response to LPS and due to their status as the largest, single population of macrophages in the mammalian body, Kupffer cells have a major role in host responses to LPS exposure. A number of the biochemical mediators of LPS-activated Kupffer cells have systemic effects as well as local ones. The presence of these mediators in abnormal amounts may increase the susceptibility of host organs to injury when an exposure to a second toxicant occurs.

1. C. LPS Potentiation of Xenobiotic Hepatoxicity

In recent years, numerous researchers have shown that LPS has biological effects beyond those mentioned above. This bacterial component is also able to modify the hepatotoxicity of other xenobiotic agents *in vivo*. A wide variety of chemicals that work by different mechanisms and affect different portions of the liver lobule have synergistic effects with LPS (Table 1.1). Thus far, no common mechanism has been discovered.

The source of the LPS which potentiates the liver injury may be exogenous or endogenous in nature. The exact source is dependent on the hepatotoxicant. The inhibition of endogenous LPS by polymyxin B protects animals from the hepatotoxicity of alpha-naphthylisothiocyanate (Calcamuggi *et al.,* 1992) whereas exogenous LPS potentiates liver injury in the case of halothane (Lind *et al.,* 1994).

1. C. 1. Role of the Innate Immune System

The innate immune system appears to play a role in some models of xenobiotic-induced hepatotoxicity. For example, researchers have found that inactivation of Kupffer cells with $GdCl_3$ protects animals from the toxic

effects of carbon tetrachloride (Edwards *et al.,* 1993), ethanol (Adachi *et al.*, 1994), cadmium (Sauer *et al.,* 1997), and allyl alcohol (Przybocki *et al.,* 1992).

Work by other researchers shows that Kupffer cells are protective in xenobiotic-induced hepatotoxicity and that liver injury occurs after the hepatic sinusoidal structure has been compromised. Both ethanol and allyl alcohol cause enlargement of the fenestrae of the endothelial cells and reduce the phagocytic capacity of the Kupffer cells (Nolan, 1975; te Koppele *et al.,* 1991). The compromised Kupffer cells are unable to clear the endotoxin properly from the portal blood. Treatment of rats with D-galactosamine reduces the clearance of endotoxin from the blood (Nakao *et al.,* 1994).

The state of activation of Kupffer cells and macrophages has a role in how the host responds to many xenobiotics. Laboratory rodents treated with stimulators of macrophage activity, such as retinol, are much more sensitive to hepatotoxicants than controls. Retinoids are activators of macrophages as measured by increased levels of chemiluminescence (Guzman *et al.,* 1991) and tumoricidal activity (Tachibana *et al.,* 1984; Moriguchi *et al.,* 1985). The pretreatment of animals with retinoids enhances or potentiates the hepatotoxicity of several compounds, including carbon tetrachloride (ElSisi *et al.,* 1993a; ElSisi *et al.,* 1993b; ElSisi *et al.,* 1993c), allyl alcohol, acetaminophen and D-galactosamine

(Rosengren *et al.,* 1995). Thus factors affecting macrophage function can modify the ultimate amount of injury produced by a given xenobiotic agent.

As described above, LPS is a potent activator of macrophages. Like the retinoids, it can potentiate the hepatotoxicity of some of the same xenobiotics, including carbon tetrachloride (Chamulitrat *et al.,* 1994), D-galactosamine (Galanos *et al.,* 1979), and allyl alcohol (Sneed *et al.,* 1997).

The xenobiotic of interest for this dissertation is allyl alcohol. The hepatotoxicity of this compound in rats is increased significantly when LPS is used to stimulate the innate immune system. The following section is a brief review of the chemistry and toxicology of allyl alcohol.

1. D. Allyl Alcohol

1. D. 1. Structure, Chemistry, and Metabolism

Allyl alcohol is a three-carbon, unsaturated compound belonging to the chemical group known as the 2-alkenols. The nominative structural group,

Table 1.1

Xenobiotics that interact with LPS to cause increased liver injury

XENOBIOTIC	REFERENCE
Aflatoxin B1	Yee et al., 1998
Alpha-naphthylisothiocyanate	Calcamuggi et al., 1992
Carbon tetrachloride	Chamulitrat et al., 1994
D-galactosamine	Galanos et al., 1979
Halothane	Lind et al., 1994
Ethanol	Hansen et al., 1994
Monocrotaline	Hill et al., 1998
Trichothecene T-2 toxin	Tai and Pestka, 1988

The above compounds interact with LPS *in vivo* to produce more liver injury than either one alone. These xenobiotics produce liver injury by a variety of mechanisms and target different portions of the liver lobule.

a carbon-carbon double bond adjoined to a single carbon bond, is at the opposing end of the molecule relative to the hydroxyl group. This compound is used in the manufacture of products such as fire retardants, food flavorings, and plastics (Beauchamp, *et. al.,* 1985). Allyl alcohol is a well-known hepatotoxicant producing a characteristic periportal necrosis in the livers of laboratory rats and mice. Due to the consistent zonal location of the injury, allyl alcohol is often used in animal models of liver injury in which a periportal pattern of liver injury is desired (Thorgeirsson *et al.,* 1976; Wilson and Hart, 1981; Belinsky *et al.,* 1984a; Zieve *et al.,* 1986).

This compound itself is relatively non-toxic. It must be metabolized in the liver by alcohol dehydrogenase (ADH) to the 2-alkenal, acrolein, for toxicity to occur (Figure 1.3) (Serafini-Cessi, 1972; Reid, 1972; Butterworth, *et. al.*, 1978; Patel, *et. al.*, 1980; Beauchamp, *et. al.,* 1985). Acrolein is considered to be the most reactive member of the 2-alkenals and is, therefore, the most damaging to cellular structures. It readily binds to compounds containing sulfhydryl groups such as proteins and glutathione.

Humans risk exposure to allyl alcohol and/or acrolein by several means. Workers in industries which use allyl alcohol are at risk as well as workers who dispose of it (Beauchamp, *et. al.,* 1985). Acrolein is a byproduct of the metabolism of cyclophosphamide; thus, patients taking this drug for cancer chemotherapy are at risk for acrolein toxicity. Acrolein is also

produced during various combustion processes including cigarette smoking, ore smelting, and burning of foods, gasoline, certain plastics, etc. Most of the acrolein produced by combustion is likely sequestered in the upper respiratory tract, but some is distributed into the blood stream (Beauchamp, *et. al.,* 1985).

As mentioned above, allyl alcohol must be biotransformed into its respective aldehyde, acrolein, for hepatotoxicity to occur. This process takes place in the cytosol of hepatic parenchymal cells and is catalyzed by ADH. This reaction is Phase I and involves the oxidation of the hydroxyl group in the presence of NAD^+ to a carbonyl group. In the cytosol, acrolein is further oxidized into acrylic acid by aldehyde dehydrogenase (ALDH) in the presence of NAD^+. Metabolism may also occur in liver microsomes, producing glycidol and glycerol. In the case of acrolein, microsomal metabolism yields glycidaldehyde and glyceraldehyde (Beauchamp, *et. al.,* 1985).

It is accepted that acrolein is responsible for the hepatic lesions seen after treatment with allyl alcohol. Inhibition of ADH activity by pyrazole or one of its derivatives prevents the toxicity normally associated with allyl alcohol intoxication, whereas inhibition of ALDH activity with cyanamide or disulfiram enhances the hepatotoxicity (Rikans and Moore, 1987; Rikans,1987). In addition, the characteristic periportal lesions of allyl alcohol can be produced by direct infusion of a small dose of acrolein into

the portal vein (Butterworth *et al.*, 1978). The lesions produced are histopathologically similar to the ones produced in rats dosed orally with a larger amount of allyl alcohol.

The precise mechanism of allyl alcohol-induced hepatotoxicity is unknown. There is evidence supporting two hypotheses for the toxic mechanism, and at some point these mechanisms may not be independent. One hypothesis states that the binding of acrolein to hepatocellular proteins and their subsequent inactivation leads to cellular injury and death. The second hypothesis focuses on the role of lipid peroxidation in acrolein hepatotoxicity.

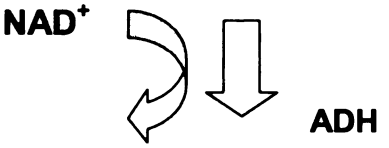
Both gender and age are factors in how allyl alcohol manifests hepatic toxicity. These differences in toxicity are due to the different levels of ADH between male and female rats and between young and old male rats. As a group, female rats are more susceptible to the hepatotoxicity of ally alcohol due to their higher rate of metabolism of allyl alcohol into acrolein (Rikans and Moore, 1987; Sasse and Maly, 1991).

Allyl alcohol is least hepatotoxic in young, male rats due to the lower levels of ADH activity in their hepatocytes. The rate of oxidation of allyl alcohol to acrolein increases with age and is fifty percent higher in old male rats versus young male rats (Rikans and Moore, 1987). This difference in toxicity is also present in hepatocytes isolated from young and old male rats

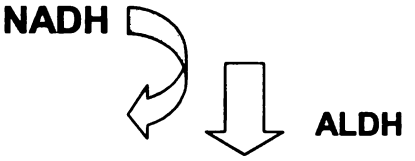
Figure 1.3. Structure and hepatic metabolism of allyl alcohol. Allyl alcohol metabolism occurs primarily in the hepatic cytosol. The alcohol is first oxidized into the toxic aldehyde, acrolein, by alcohol dehydrogenase (ADH). Acrolein is further metabolized into the non-toxic acrylic acid by aldehyde dehydrogenase (ALDH).

Metabolic Pathway

$CH_2=CHCH_2OH$ (allyl alcohol)



$CH_2=CHCHO$ (acrolein)



$CH_2=CHCOOH$ (acrylic acid)

(Rikans and Hornbrook, 1986). The rate of oxidation of acrolein to acrylic acid is independent of age, indicating that the detoxification pathway is not altered.

1. D. 2. Pathology

After exposure to a toxic dose of allyl alcohol (85 mg/kg po), pathologic changes can be seen in rat hepatocytes and Kupffer cells as early as four hours (Blyuger *et al.,* 1974). Large phagosomes and autophagous vacuoles are present at this time. By eight hours after allyl alcohol administration, all organelles show signs of injury. Maximal liver damage is seen between twenty-four and fifty-four hours, depending upon dose, and resolution of injury occurs between forty-eight and seventy-two hours (Zieve *et al.,* 1986).

The mechanism behind the characteristic periportal distribution of lesions in allyl alcohol intoxication is currently unknown. At one time researchers thought that the activity of ADH was higher in periportal regions relative to pericentral regions of the liver lobule, but this has not proven to be the case (Penttila, 1988; Yamauchi *et al.,* 1988; Sasse and Maly, 1991). Another hypothesis is that allyl alcohol requires the higher

oxygen tension that exists in the periportal region compared to the rest of the liver lobule (Badr, *et. al.*, 1986; Przybocki, *et. al.*, 1992).

In recent years, the role of nonparenchymal cells in the hepatotoxicity of allyl alcohol has been investigated. Kupffer cells have been implicated in the toxic mechanism of allyl alcohol based on the observation that gadolinium chloride ($GdCl_3$) decreases the toxicity of allyl alcohol (Przybocki *et. al.*, 1992). The results from another laboratory, however, contradicted the findings of Przybocki *et al.* (Ganey and Schultze, 1995). In the periportal region of the liver lobule, Kupffer cells tend to be larger in size, have larger lysosomes, and be more phagocytic than Kupffer cells in other regions (Jones and Summerfield, 1988), thus these cells may be a factor in the propensity for allyl alcohol to cause periportal liver necrosis.

1. D. 3. Mechanisms of toxicity

1. D. 3. 1. Binding to Protein Sulfhydryl Groups

Acrolein causes rapid depletion of hepatocellular levels of reduced glutathione (GSH) without a concomitant increase in oxidized glutathione (GSSG) (Ohno *et al.,* 1985; Silva and O'Brien, 1989). This indicates that

the reactive aldehyde is binding to GSH. Reactions between acrolein and GSH may occur spontaneously, or they may be catalyzed by glutathione-S-alkene-transferase (Kaye, 1973). The resulting adduct, found in the urine of experimental animals after further metabolism, is 3-hydroxypropylmercapturic acid (Kaye, 1973). Depletion of hepatocellular GSH with diethylmaleate enhances the toxicity of acrolein, whereas the presence of N-acetylcysteine or dithiothreitol has a protective effect (Ohno *et al.,* 1985; Jaeschke *et al.,* 1987; Hormann *et al.,* 1989; Silva and O'Brien, 1989). Both of these experimental results provide strong evidence for the role of GSH depletion in acrolein hepatotoxicity.

As mentioned above, acrolein is the toxic principle of allyl alcohol hepatotoxicity. This reactive electrophile readily forms covalent bonds with nucleophilic groups such as the sulfhydryl groups of cellular proteins, resulting in their alkylation and inactivation. After depletion of cellular GSH, acrolein proceeds to attack other sources of nucleophilic groups. The loss of protein sulfhydryl groups begins 30 minutes after exposing isolated, rat hepatocytes to allyl alcohol, coinciding with the depletion of GSH (Rikans and Cai, 1994). Dogterom and colleagues (1989b) have measured the decrease in protein sulfhydryl groups at one hour and have found similar results. Treatment of hepatocyte cultures with the sulfhydryl-rich compounds, cysteine, methionine, N-acetylcysteine, or dithiothreitol prevents or reverses the cellular damage induced by allyl alcohol

metabolism (Ohno *et al.,* 1985; Dogterom *et al.,* 1989b; Hormann *et al.,* 1989; Silva and O'Brien, 1989; Rikans and Cai, 1994). In summary, depletion of reduced GSH by acrolein leaves hepatocytes susceptible to the further toxic actions of the aldehyde.

1. D. 3. 2. Peroxidation of Lipid Membranes

Some investigators believe that peroxidation of lipid membranes is a more important mechanism of allyl alcohol toxicity compared to depletion of protein sulfhydryl groups. In this model of toxicity, peroxidation of lipid membranes follows depletion of GSH by acrolein (Jaeschke *et al.,* 1987; Dogterom *et al.,* 1988; Haenen *et al.,* 1988; Miccadel *et al.,* 1988; Pompella et al., 1988; Dogterom *et al.,* 1989b; Kyle *et al.,* 1989; Maellaro *et al.,* 1990; Pompella *et al.,* 1991). Low concentrations of disulfiram have an antioxidant effect and prevent the peroxidation of lipid membranes and resultant cell death (Kyle *et al.,* 1989). The antioxidant vitamins, ascorbic acid (vitamin C) and α-tocopherol (vitamin E), also provide protection against allyl alcohol-induced hepatotoxicity (Maellaro *et al.,* 1990; Jaeschke *et al.,* 1992; Maellaro *et al.,* 1994). Depletion of vitamin E makes mice more sensitive to allyl alcohol hepatotoxicity whereas supplementation with vitamin E protects animals from lipid

peroxidation (Dogterom *et al.*, 1989b; Maellaro *et al.*, 1990; Jaeschke *et al.*, 1992).

Results of studies examining the role of iron in allyl alcohol toxicity support the hypothesis that lipid peroxidation is important. For example, the presence of iron enhanced the toxicity of allyl alcohol in mice (Jaeschke *et al.*, 1992), and this may have been due to the Fenton reaction in which hydroxyl radicals were generated in the presence of ferric iron. In a series of experiments using male mice, Jaeschke *et al.*,. (1992) demonstrated that a subtoxic dose of ferrous sulfate (0.36mMol/kg) potentiates the lipid peroxidation produced by a subtoxic dose of allyl alcohol (0.6mMol/kg). Chelation of iron with desferrioxamine protected mice from the lipid peroxidation produced by allyl alcohol intoxication (Jaeschke *et al.*, 1987). Desferrioxamine also protected cultured hepatocytes and erythrocytes from the lipid peroxidation produced by exposure to allyl alcohol (Miccadei *et al.*, 1988; Ferrali *et al.*, 1989).

Several research groups investigated the role of calcium in the toxic mechanism. In isolated, perfused livers, the concentrations of malondialdehyde, a measure of lipid peroxidation, caused by exposure to allyl alcohol was not influenced by the levels of extracellular calcium (Strubelt *et al.*, 1986). Toxicity, measured by release of alanine amino transferase (ALT), was also reflected by increased calcium concentration. Increased levels of extracellular calcium (5 mMol/L) alone caused a mild

release of hepatic transaminase and sorbitol dehydrogenase into the medium compared to controls; however, there was no significant difference in enzyme release between groups of isolated, perfused livers in the presence of both allyl alcohol and calcium as compared to the presence of allyl alcohol alone. Extracellular calcium did have a protective effect in isolated, rat hepatocytes exposed to allyl alcohol (Dogterom *et al.,* 1989a; Dogterom and Mulder, 1993). In this case, the hypothesis was that excess calcium prevents the loss of vitamin E and thus acts indirectly to prevent lipid peroxidation (Dogterom and Mulder, 1993).

1. D. 4. Summary

Experimental evidence exists to support both current mechanisms in the hepatotoxicity of allyl alcohol. Depletion of protein sulfhydryl groups and peroxidation of lipid membranes have been detected in hepatocytes treated with allyl alcohol (Dogterom *et al.,* 1989b; Hormann *et al.,* 1989; Pompella *et al.,* 1991). The primary controversy is over the relative contribution of each mechanism.

1. E. Conclusions

The innate immune system is a powerful defense against bacterial invasion and exerts global effects on the host. These effects are normally beneficial but may become detrimental under certain circumstances. LPS, a potent activator of the innate immune system, can make a mammalian host more sensitive to xenobiotic toxicants and thus result in enhancing the toxicity of those xenobiotics. In the following chapters, results are presented of experimental designs to test this possibility for one hepatotoxicant, allyl alcohol. The hypothesis tested is that LPS stimulation of the innate immune system enhances the hepatotoxicity of allyl alcohol.

Chapter 2

CHARACTERIZATION OF LPS-INDUCED ENHANCEMENT OF ALLYL ALCOHOL HEPATOTOXICITY

2. A. Abstract

Lipopolysaccharide (LPS), or bacterial endotoxin, causes liver damage at relatively large doses in rats. Smaller doses, however, may influence the response to other hepatotoxicants. The purpose of these studies was to examine the effect of exposure to relatively small doses of LPS on the hepatotoxic response to allyl alcohol, which causes periportal necrosis in laboratory rodents through an unknown mechanism. Rats were pretreated with LPS (100 μg/kg) 2 hours before treatment with a minimally toxic dose of allyl alcohol (30 mg/kg), and liver toxicity was assessed 18 hours later from activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma and from histologic changes in liver sections. Plasma ALT and AST activities were not elevated significantly in rats treated with vehicle, LPS, or allyl alcohol alone, but pronounced increases were observed in rats treated with LPS and allyl alcohol. Significant liver injury occurred as early as two hours after allyl alcohol treatment in LPS-pretreated rats and peaked at 6 hours. LPS treatment did not affect the activity of alcohol dehydrogenase and did not affect the rate of production of NADH in isolated livers perfused with allyl alcohol; thus, LPS does not appear to increase the metabolic bioactivation of allyl alcohol to acrolein. On the other hand, pretreatment with 4-

methylpyrazole, an inhibitor of alcohol dehydrogenase, abolished the hepatotoxicity of allyl alcohol in LPS-treated rats, indicating that production of acrolein was needed for LPS enhancement of the toxicity of allyl alcohol.

2. B. Introduction

LPS, a component of gram-negative bacteria, elicits powerful responses in mammalian hosts. These responses can range from mild fever through cachexia to organ failure and death. The presence of LPS activates a cascade of biochemical events which ideally ready the body for defense against bacterial invaders but may also make the body more susceptible to injury.

At large doses, LPS itself causes overt liver damage (Hirata *et al.,* 1980; Shibayama, 1987; Jaeschke *et al.,* 1991; Hewett *et al.,* 1992; Wang *et al.,* 1995). However, administration of small doses of LPS potentiates liver injury in response to a variety of chemicals (e.g. galactosamine and ethyl alcohol) (Galanos *et al.,* 1979; Bhagwandeen *et al.,* 1987; Hansen *et al.,* 1994). In addition, it has been proposed that exposure to endogenous LPS due to increased movement across a compromised intestinal mucosa contributes to hepatotoxicity produced by some agents, for example, carbon tetrachloride and alpha-

naphthylisothiocyanate (Calcamuggi *et al.*, 1992; Czaja *et al.*, 1994). The following study was undertaken to explore the effect of small doses of LPS on the hepatotoxicity of allyl alcohol.

2. C. Materials and Methods

2. C. 1. Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12, specific activity 24 x 10^7 endotoxin units/mg), 4-methylpyrazole chloride, and Sigma Diagnostics Kits No. 59 UV and No. 58 UV for determination of activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) respectively, were purchased from Sigma Chemical Company (St. Louis, MO). Allyl alcohol was purchased from Aldrich Chemical Company (St. Louis, MO). Sterile saline was obtained from Abbott Labs (Abbott Park, IL). Histochoice fixative for the preservation of liver tissue was purchased from Amresco (Solon, OH). Protein concentration in the supernatant was determined using the bicinchoninic acid (BCA) assay kit purchased from Pierce (Rockford, IL).

2. C. 2. Animals

Male, Sprague-Dawley rats [CD-Crl:CD-(SD)BR VAF/Plus]; Charles River, Portage, MI) weighing 200-300 gm were used in these studies. The animals were allowed food (Rodent Chow, Teklad, Madison, WI) and water *ad libitum*. They were maintained on a 12-hour light and dark cycle under conditions of controlled temperature and humidity.

2. C. 3. Treatment Protocol

Rats were treated intravenously with LPS in doses of 0.01, 0.1, 0.5, 1, 5, 10, 50 and 100 μg/kg as indicated in the figure and table legends or with an equivalent volume of sterile saline vehicle. Two hours after the administration of LPS, allyl alcohol diluted in sterile saline or an equivalent volume of sterile saline was injected intraperitoneally in doses of 10, 20, or 30 mg/kg as indicated in the figure and table legends. Liver injury was assessed at 2, 4, 6, 12, 18, or 24 hours after administration of allyl alcohol.

In studies examining the effects of inhibition of the activity of alcohol dehydrogenase (ADH), animals were treated with a competitive inhibitor of ADH, 4-methylpyrazole chloride (4-MP) (77 mg/kg, ip), 15 minutes before exposure to allyl alcohol. This treatment regimen effectively inhibits ADH

activity (Rikans and Moore, 1987). The experimental protocol was a 2 x 2 x 2 design in which the factors were treatment with LPS or vehicle, with 4-MP or vehicle, and with allyl alcohol or vehicle.

2. C. 4. Assessment of Hepatotoxicity

Rats were anesthetized with sodium pentobarbital (50 mg/kg ip), and blood was collected from the abdominal aorta into syringes containing 3.8% sodium citrate (final concentration 0.38%). Activities of ALT and AST were determined in plasma. The liver was removed intact. Samples taken for microscopic examination were preserved in Histochoice fixative (Amresco, Solon, OH). Tissue sections were processed for light microscopy, cut into sections 6 microns thick, stained with hematoxylin and eosin and evaluated for lesion severity. Tissue sections were graded without knowledge of treatment group according to the scale outlined in Table 2.1.

2. C. 5. Determination of Factors Influencing the Metabolism of Allyl Alcohol

Rats were treated with LPS (100 μg/kg, iv) or saline vehicle and killed two hours later. The liver was removed and homogenized in a solution of 0.05 M HEPES (pH 8.4) and 0.33 mM dithiothreitol. The homogenate was centrifuged at 100,000 x g for 45 minutes. The supernatant fluid was collected, and activity of ADH was measured spectrophotometrically (366 nm) by monitoring the reduction of nicotinamide dinucleotide (NAD^+) using ethanol as a substrate (Krebs *et al.,* 1969). Protein concentration in the supernatant was determined using the BCA assay kit.

The rate of conversion of allyl alcohol into acrolein was determined indirectly in isolated, perfused rat livers by monitoring reduction of NAD^+ upon infusion of allyl alcohol (Belinsky *et al.,* 1984a). Rats were treated with LPS (100 μg/kg) or saline vehicle. Two hours later, the livers were removed and perfused in a nonrecirculating system with Krebs-Henseleit buffer (pH 7.4, 37° C, 95% O_2/5% CO_2). The tip of a light guide was positioned close to the surface of the liver to monitor continuously the fluorescence of NADH. The opposite end of the light guide was bifurcated, with one end attached to a light source (mercury arc lamp) and the other to a photomultiplier. Excitation and emission wavelengths were 366 nm and 450 nm, respectively. After stabilization of surface fluorescence

(approximately 10 minutes), allyl alcohol (100 μM) was infused into the liver, and the increase in fluorescence was recorded as a percent of baseline.

2. C. 6. Determination of Hepatic Concentrations of GSH and GSSG

The concentrations of reduced (GSH) and oxidized (GSSG) glutathione in the liver were determined using high performance liquid chromatography (HPLC) (Fariss and Reed, 1987; Jean *et al.,* 1995). Liver samples were quick frozen in liquid nitrogen to prevent oxidation of GSH. Frozen livers were weighed and then homogenized in 4 ml 10% perchloric acid (J. T. Baker, Phillipsburg, NJ) containing 1 mM bathophenathroline disulfonic acid (Aldrich Chemical Company, St. Louis, MO) using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The homogenate was spun in a centrifuge at 400 x g for 10 minutes. The supernatant fluid was collected, and 0.3 ml was mixed with 50 μl of iodoacetic acid (20 mg/ml) (Sigma Chemical Company, St. Louis, MO) in 0.2 mM of *m*-cresol purple and 0.2 ml of 0.5 mM gamma-glutamylglutamate (internal standard) (Sigma Chemical Company). This was followed by the addition of 0.4 ml of 2M KOH/2.4M $KHCO_3$. After 45 minutes, 0.5 ml of 1-fluoro-2,4-dinitrobenzene (Sigma Chemical Company, St. Louis, MO) (1% in absolute

ethanol) was added, and the samples were stored in the dark at room temperature for 18 to 24 hours prior to analysis.

The HPLC system consisted of two Waters HPLC 510 pumps, a 717 WISP autosampler, and a Waters 486 variable wavelength detector. Millennium 2010 Chromatography Manager software (Millipore Corporation, Milford, MA) was used to control instrument operation and peak integration. Samples were diluted with 0.8 mM acetic acid in 80% methanol. Fifty microliters of this solution were injected onto a 3-aminopropyl column in the presence of 80% methanol. The sample was eluted from the column with 0.8 M sodium acetate in 80% methanol. The amounts of GSH and GSSG were calculated from the area under the UV absorption curves and expressed as mM per gram of liver.

2. C. 7. Statistical Analysis

Data are expressed as means $\pm$ S.E.M. For all results presented, N represents the number of individual animals. Homogeneous data were analyzed by one-way or two-way analysis of variance (ANOVA). Individual means were compared using Tukey's omega test. When variances were not homogeneous, data were analyzed using the Mann-Whitney U test for comparison of two groups and Kruskal-Wallis ANOVA on ranks for more

than two groups. In the latter case, Dunn's test was used to assess significance. The criterion for statistical significance was $p \leq 0.05$.

2. D. Results

2. D. 1. Effect of LPS Pretreatment on Allyl Alcohol-Induced Hepatotoxicity

ALT activity was low in the plasma 18 hrs after treatment of rats with vehicle, LPS, or allyl alcohol (Figure 2.1.A). Those animals cotreated with LPS and allyl alcohol had pronounced elevation of plasma ALT activity. Similar results were observed for plasma AST activity: only cotreatment with LPS and allyl alcohol caused a significant increase in this marker of liver injury (Figure 2.1.B).

Histologically, livers from animals treated with vehicle had normal lobular architecture and no necrosis. Livers from animals treated only with allyl alcohol showed a range of responses including no visible damage, lesions confined to periportal regions, and ocassionallyl more severe and extensive lesions. The lesions typically consisted of a necrotizing, coagulative hepatitis with hemorrhage. In some livers from cotreated animals, necrosis originated as a periportal lesion and coalesced to a

panlobular distribution (Table 2.1). Inflammatory infiltrates, including neutrophils, macrophages, and a few small lymphocytes, were present within areas of necrosis and sinusoids. Periportal edema and triaditis were commonly observed. Bile ducts were largely unaffected. Although lesions in livers of animals cotreated with LPS and allyl alcohol were qualitatively similar to those seen in rats which received allyl alcohol alone, lesions were more numerous and involved a greater area of the liver.

Liver damage developed early in animals cotreated with LPS and allyl alcohol (Figure 2.2). In these rats, plasma ALT activity was significantly elevated two hours after allyl alcohol treatment. The rise in plasma ALT activity was maximal six hours after allyl alcohol treatment. There was no significant increase in plasma ALT activity in animals treated with vehicle, LPS, or allyl alcohol alone.

To examine the effects of LPS dosage on the enhancement of allyl alcohol hepatotoxicity, LPS was administered at nonlethal doses ranging from 0.01 μg/kg to 100 μg/kg (Figure 2.3.A). ALT activity was significantly increased in the plasma of allyl alcohol-treated rats pretreated with doses of LPS >0.01 μg/kg. Histological analysis of livers revealed prominent Kupffer cells in sinusoids of rats treated with doses of LPS >5 μg/kg and edema and mild neutrophilic infiltration at doses of 10-50 μg/kg LPS. A limited number of animals was pretreated with 500 μg/kg LPS

Figure 2.1. LPS enhancement of the hepatotoxicity of allyl alcohol. Animals were treated with LPS (100 μg/kg iv) or vehicle two hours before administration of allyl alcohol (30 mg/kg ip) or vehicle. ALT (A) and AST (B) activities in plasma were measured as markers of liver toxicity 18 hrs after treatment with allyl alcohol. Data are expressed as mean ± S.E.M. a, significantly different from respective value in the absence of allyl alcohol. b, significantly different from respective value in the absence of LPS. N = 5 -13.

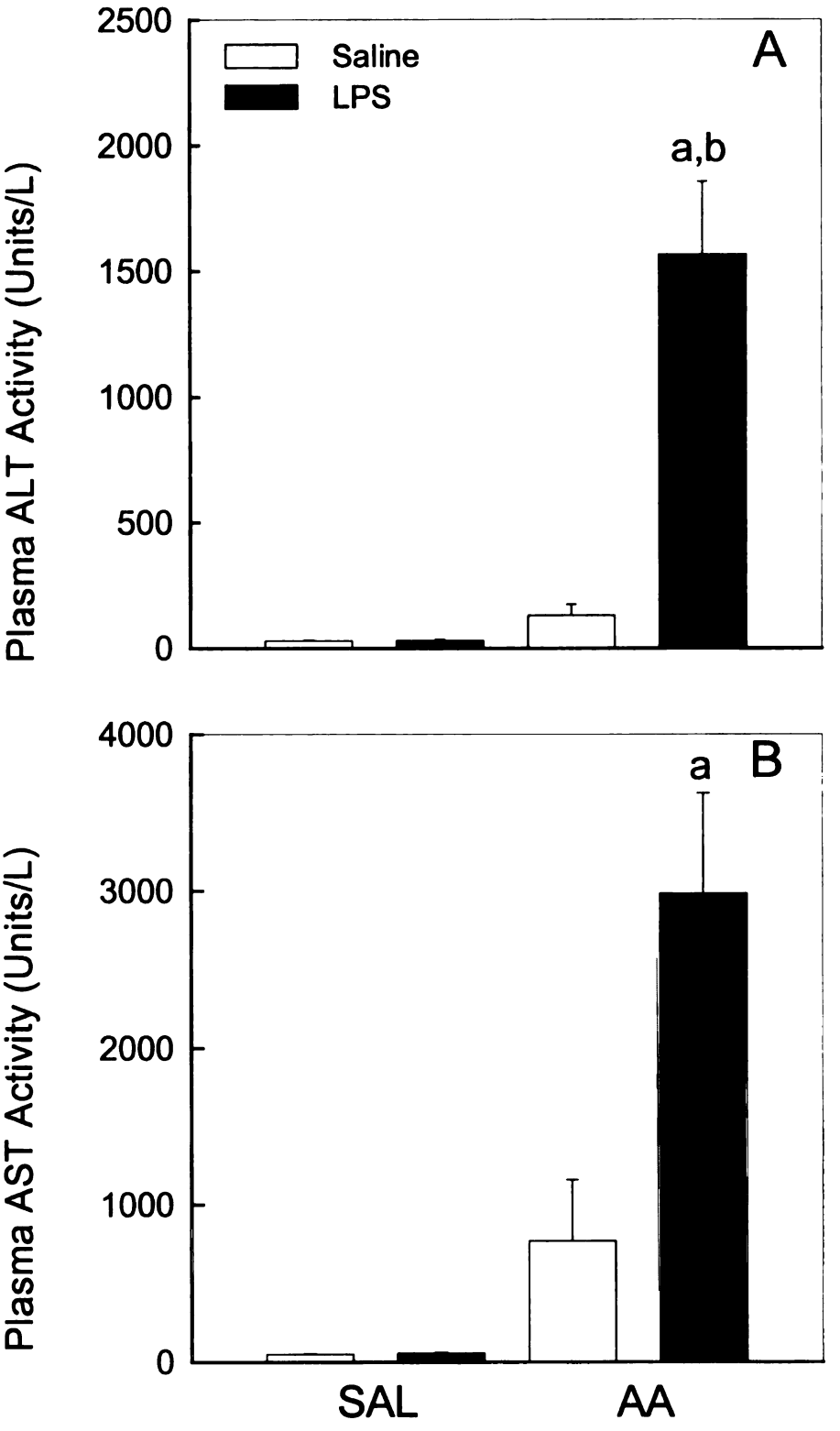

Saline
LPS
A
a,b
Plasma ALT Activity (Units/L)
2500
2000
1500
1000
500
0
B
a
Plasma AST Activity (Units/L)
4000
3000
2000
1000
0
SAL
AA

Table 2.1

Pathological changes in the livers of rats

TREATMENT	HEPATOCYTIC NECROSIS	PERIPORTAL EDEMA	HEPATIC PARENCHYMAL INFLAMMATORY INFILTRATE	SINUSOIDAL INFLAMMATORY INFILTRATE
Sal/Sal	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LPS/Sal	0.1 ± 0.09	0.3 ± 0.2	0.1 ± 0.09	0.1 ± 0.09
Sal/AA	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3
LPS/AA	2.9 ± 0.3	2 ± 0	2.2 ± 0.1	2 ± 0

Rats were treated as described in Materials and Methods. Liver sections were taken, fixed, and lesions were evaluated. Microscopic lesions were assigned a grade using the following scale: 0, no lesions; 1, mild; 2, moderate; 3, marked; 4, severe. See text for detailed description of lesions. N = 4 for Sal/Sal group; 9–10 for all other groups.

Figure 2.2. Time course of development of LPS-enhanced allyl alcohol hepatotoxicity. Animals were treated as described in the legend to Figure 1, and plasma ALT activity was measured at the indicated times. a, significantly different from respective value in the absence of allyl alcohol. b, significantly different from respective value in the absence of LPS. N = 5 -14.

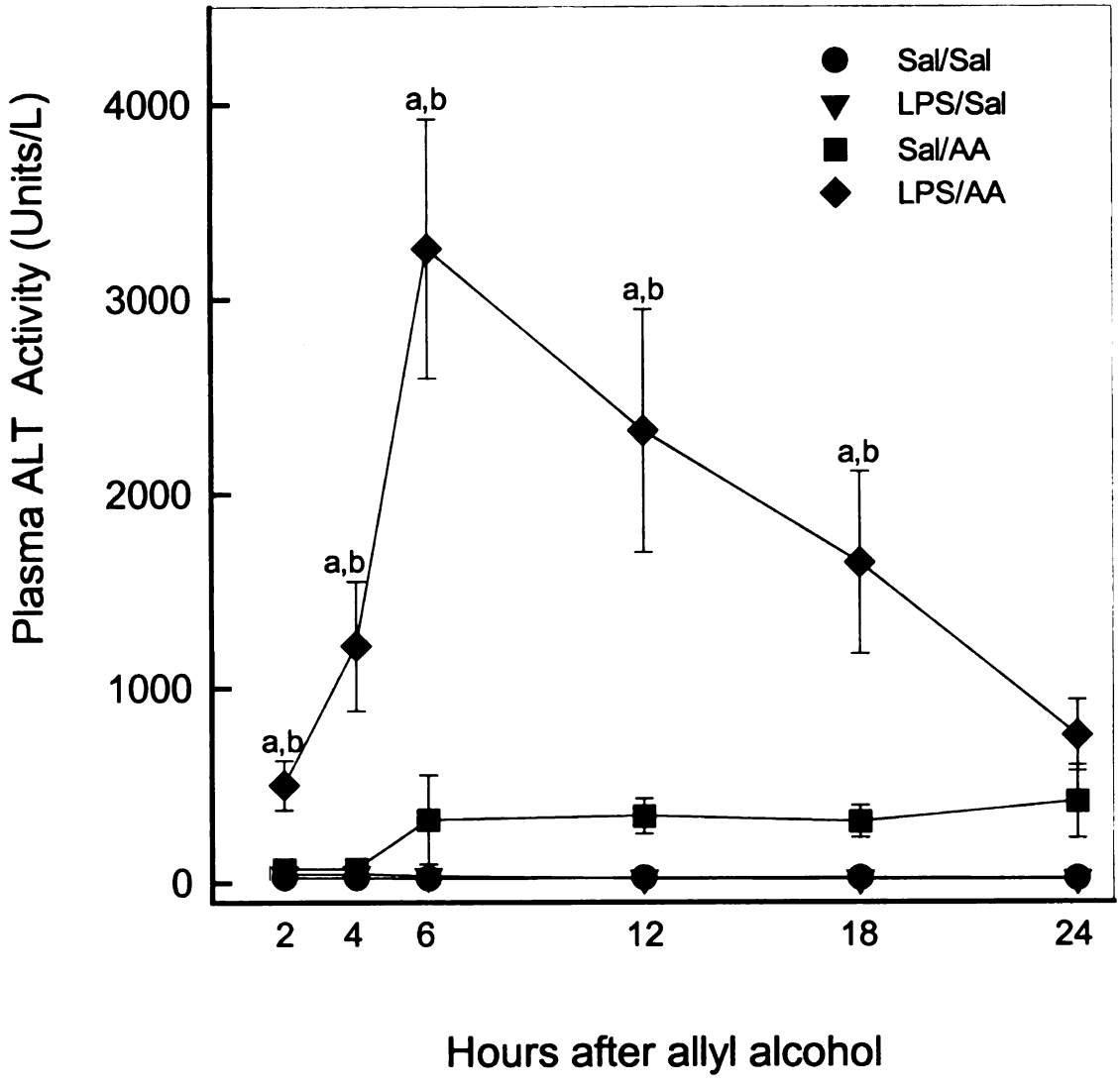
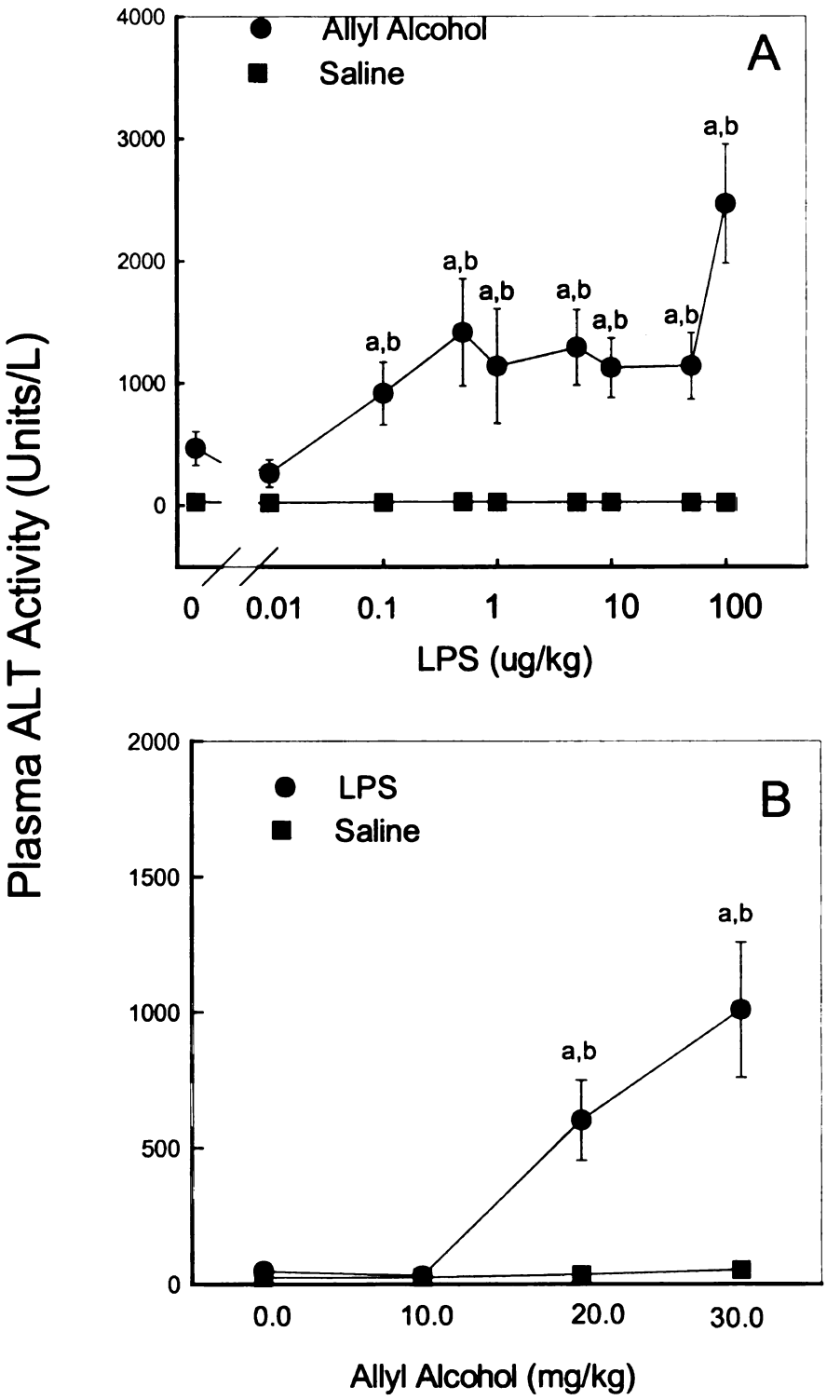

Plasma ALT Activity (Units/L)
4000
3000
2000
1000
0
2
4
6
12
18
24
Hours after allyl alcohol
Sal/Sal
LPS/Sal
Sal/AA
LPS/AA
a,b
a,b
a,b
a,b
a,b

Figure 2.3. Dose-response relations for plasma ALT activity after treatment with LPS and/or allyl alcohol. A) Animals were treated with the indicated doses of LPS and with either 30 mg/kg of allyl alcohol or vehicle. B) Animals were treated with the indicated doses of allyl alcohol and with either 100 μg/kg of LPS or vehicle. Plasma ALT activity was measured eighteen hours after allyl alcohol administration. Data are expressed as mean ± S.E.M. a, significantly different from respective value in the absence of allyl alcohol. b, significantly different from respective value in the absence of LPS. N = 3-14.


Plasma ALT Activity (Units/L)
4000
3000
2000
1000
0
Allyl Alcohol
Saline
A
a,b
0
0.01
0.1
1
10
100
LPS (ug/kg)
2000
1500
1000
500
0
LPS
Saline
B
a,b
0.0
10.0
20.0
30.0
Allyl Alcohol (mg/kg)

prior to administration of allyl alcohol: most (> 75%) rats in that group died within 18 hrs.

The dose-response relationship for allyl alcohol is shown in Figure 2.3.B. In the absence of LPS, no significant increase in plasma ALT activity was seen at doses of allyl alcohol ranging from 0-30 mg/kg. LPS (100 μg/kg) cotreatment increased plasma ALT activity at doses of 20 and 30 mg/kg allyl alcohol. Significant mortality occurred at allyl alcohol doses greater than 30 mg/kg.

2. D. 2. Role of Metabolism of Allyl Alcohol

ALT activity was low in plasma of rats treated only with saline or LPS and was not affected by administration of the ADH inhibitor, 4-MP (Figure 2.4). In allyl alcohol-treated rats pretreated with LPS, ALT activity in plasma was markedly increased. This increase was abolished in rats pretreated with 4-MP. ALT activity in rats treated with allyl alcohol only was also decreased by pretreatment with 4-MP.

Allyl alcohol requires bioactivation by ADH to acrolein, a reactive aldehyde that binds rapidly to glutathione and other sulfhydryl-containing molecules (Beauchamp, *et al.*, 1985; Serafini-Cessi, 1971; Reid, 1972; Butterworth *et al.*, 1978; Patel *et al.*, 1980; Ohno *et al.*, 1985). Allyl

alcohol is metabolized rapidly after administration *in vivo* as evidenced by a dramatic decrease in hepatic reduced glutathione within 12-20 minutes (Penttilä, 1987; Pompella *et al.*, 1988). Accordingly, the activity of ADH in liver cytosolic fractions was examined 2 hours after administration of LPS, the time at which allyl alcohol was given in experiments described above. ADH activity in livers from LPS-treated rats was not significantly different from activity in cytosolic fractions from control rats (Table 2.2). Perfusion of isolated livers with allyl alcohol caused an increase in fluorescence of NADH. This was used as an indirect measure of oxidation of allyl alcohol to acrolein (allyl alcohol + NAD^+ yielded acrolein and NADH). LPS pretreatment did not affect the increase in fluorescence of NADH upon perfusion of allyl alcohol into isolated livers.

2. D. 3. Effect of LPS Treatment on Hepatocellular Concentrations of GSH and GSSG

To assess GSH status in the liver prior to administration of allyl alcohol, the concentrations of both GSH and GSSG were determined 1.5 hr after administration of LPS or saline vehicle. The concentration of GSH was not different in livers of LPS-treated and control rats (Table 2.3). Similarly,

administration of LPS to rats did not affect the concentration of GSSG in the liver.

2. E. Discussion

Pretreatment of rats with a nontoxic dose of LPS (100 μg/kg) significantly increased liver damage, as measured by both biochemical assays and pathological examination in rats exposed to allyl alcohol. A significant increase in plasma ALT activity was detected as early as two hours after allyl alcohol treatment. This biochemical marker of liver damage continued to increase through 6 hrs and then declined, indicating that injury occurred within the first 6 hrs after administration of allyl alcohol. The decrease in ALT activity after 6 hrs may be due to clearance of this enzyme from the circulating blood. In contrast to the situation in LPS-pretreated rats, plasma ALT activity in rats treated only with allyl alcohol did not increase significantly over a 24 hour period.

The results of histological evaluation were consistent with changes in aminotransferase activities. Based on the histopathologic lesions, it appears that LPS enhanced the toxic effects of allyl alcohol, rather than

Figure 2.4. Protection from LPS-induced enhancement of allyl alcohol hepatotoxicity by 4-methylpyrazole. Animals were treated with 4-MP (77 mg/kg ip) 15 minutes before administration of allyl alcohol. The remainder of the experimental protocol was the same as in Figure 1 except that animals were killed 6 hours after allyl alcohol administration. Data are expressed as mean $\pm$ S.E.M. a, significantly different from respective value in the absence of allyl alcohol. b, significantly different from respective value in the absence of LPS. c, significantly different from respective value in the absence of 4-MP. N = 4-8.

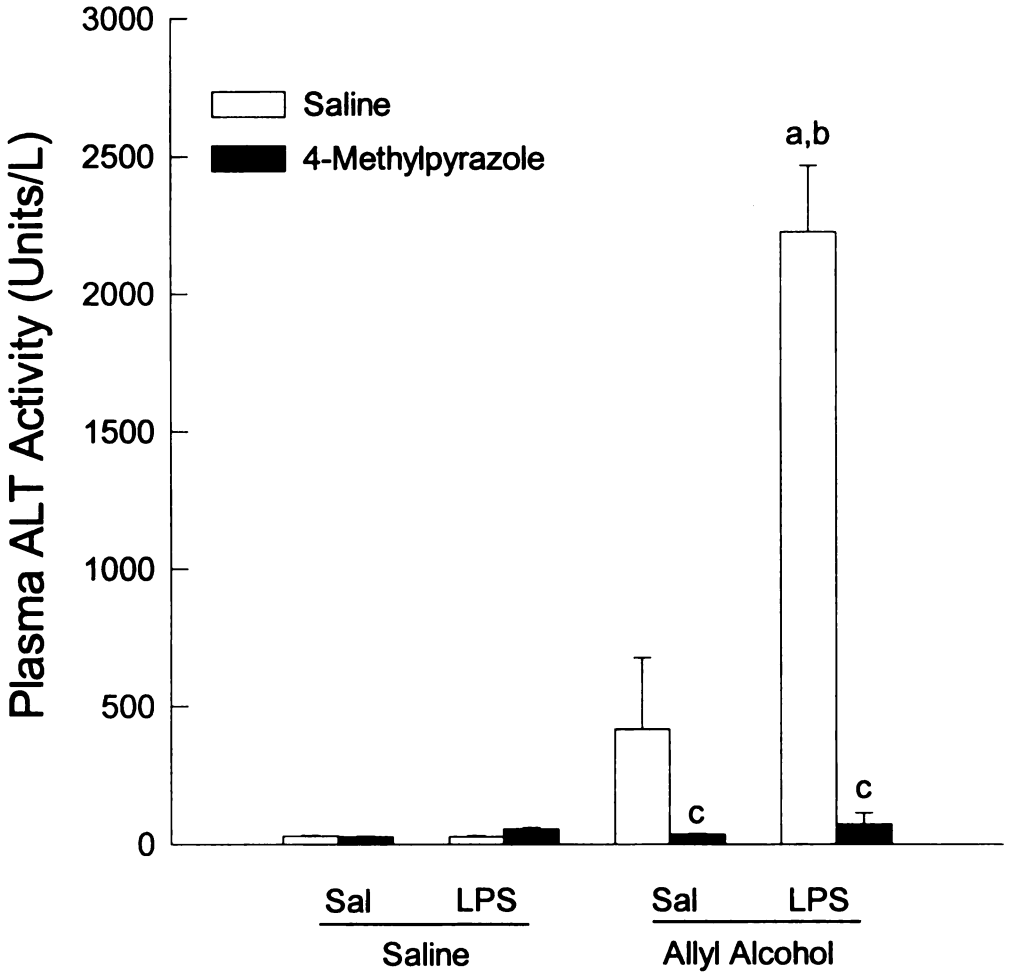

Plasma ALT Activity (Units/L)
3000
2500
2000
1500
1000
500
0
Saline
4-Methylpyrazole
a,b
c
c
Sal
LPS
Saline
Sal
LPS
Allyl Alcohol

Table 2.2

Factors influencing or reflecting the metabolism of allyl alcohol

Treatment[A]	Alcohol Dehydrogenase[B] (mM/min/gm of liver)	Fluorescence[C] (% increase from baseline)
Saline	0.19 ± 0.01	13.5 ± 7.3
LPS	0.24 ± 0.05	14.5 ± 3.5

A, Rats were treated with LPS (100 μg/kg) or saline two hours prior to measurements. B, Activity of alcohol dehydrogenase was measured in cytosolic fractions prepared from liver (N = 6). C, Livers were isolated and perfused in a nonrecirculating system. Fluorescence of NADH from the surface of the liver was monitored using a light guide (366 nm excitation, 450 nm emission). Increase in fluorescence during perfusion with allyl alcohol (100 μM) was expressed as a percentage of baseline fluorescence (n = 3). Statistically significant differences between the saline and LPS exposure groups were not seen for either parameter.

Table 2.3

Comparison of hepatocellular levels of reduced and oxidized glutathione in rats pretreated with saline

Treatment	GSH μM/g liver	GSSG μM/g liver
Sal	6.6 + 0.2	0.42 + 0.02
LPS	5.7 + 0.5	0.48 + 0.09

Female, Sprague-Dawley rats received saline 24 hours before receiving either LPS (4 mg/kg) or saline. Animals were killed 1.5 hours after LPS or saline exposure, and liver GSH and GSSG concentrations were determined as described in Materials and Methods. N = 4-7. No significant differences were observed.

allyl alcohol increasing LPS-induced liver damage. Hepatic lesions caused by administration of LPS at doses larger than those used in these studies are confined to midzonal areas of the lobule and have as a prominent feature neutrophils in those areas (Jaeschke *et al.,* 1991; Hewett *et al.,* 1992; Pearson *et al.,* 1995). On the other hand, injury caused by exposure to allyl alcohol alone at doses larger than those used in these studies produces predominantly periportal hepatic lesions (Thorgeirsson *et al.,* 1976; Gumucio *et al.,* 1978). The lesions observed in livers of rats cotreated with small doses of LPS and allyl alcohol in these studies were predominantly periportal, suggesting a histological picture more like allyl alcohol than LPS. The inflammatory infiltrate was moderate and may either be a response to LPS or to hepatic injury.

Intravenous doses of LPS exceeding 1 mg/kg are required to produce liver injury in Sprague-Dawley rats (Hewett *et al.,* 1992). Doses of LPS 4 orders of magnitude smaller than this significantly potentiated the hepatotoxicity of allyl alcohol. This effect of LPS to enhance the hepatotoxicity of allyl alcohol does not appear to be the result of increased production of acrolein, the reactive metabolite of allyl alcohol. There was no significant difference between the activities of ADH in liver cytosolic fractions from LPS- and vehicle-treated rats. Moreover, LPS did not affect the rate of conversion of allyl alcohol into acrolein in isolated, perfused livers. As with hepatotoxicity caused by allyl alcohol alone, bioactivation to

acrolein was necessary for LPS-induced potentiation of injury: inhibition of acrolein production with 4-MP prevented toxicity in cotreated rats.

Acrolein readily forms adducts with GSH, causing a loss of hepatocellular GSH without a concomitant increase in GSSG (Silva and O'Brien, 1989; Dogterom *et al.,* 1989b; Rikans *et al.,* 1994). This loss occurs very rapidly, e.g., within 20 minutes after administration of allyl alcohol (Penttilä *et al.,* 1987; Pompella *et al.,* 1988). GSH serves a protective role against allyl alcohol toxicity, and agents which diminish hepatocellular GSH increase toxicity of allyl alcohol (Maellaro *et al.,* 1990). The possibility existed that LPS augmented the response to allyl alcohol by diminishing the availability of hepatocellular GSH; however, this does not appear to be the case because LPS pretreatment did not affect the hepatic concentrations of either GSH or GSSG.

LPS has been shown to potentiate the hepatotoxicity of other compounds in experimental animals. Administration of exogenous LPS at doses similar to those used in this study (i.e., > 0.05 mg/kg) increases the hepatotoxicity of carbon tetrachloride (Chamulitrat *et al.,* 1994), galactosamine (Galanos *et al.,* 1979), and halothane (Lind *et al.,* 1984). Endogenous LPS has been associated with the hepatotoxicity of galactosamine and alpha-naphthylisothiocyanate (Calcamuggi *et al.,* 1992; Czaja *et al.,* 1994). For example, Czaja and coworkers (1994) prevented galactosamine-induced hepatotoxicity by administration of an

antibody to LPS. The mechanism by which LPS enhances hepatotoxicity of these xenobiotics is unknown.

In summary, LPS is able to potentiate the hepatotoxicity of allyl alcohol and is able to do so at remarkably small doses. Allyl alcohol must be converted into the reactive metabolite acrolein for the potentiation to occur. LPS does not affect the hepatic metabolism of allyl alcohol into acrolein nor does it affect the protective mechanism for removal of acrolein. At present, the mechanism by which LPS can enhance the potentiation of allyl alcohol hepatotoxicity is unknown. In the following chapter, we will discuss the role of the innate immune system in this model of liver injury.

Chapter 3

THE ROLE OF CELLS OF THE INNATE IMMUNE SYSTEM IN LPS-INDUCED ENHANCEMENT OF ALLYL ALCOHOL HEPATOXICITY

3. A. Abstract

We have shown previously that small doses of LPS are able to enhance the hepatotoxicity of allyl alcohol in rats. Due to the important role of cells of the innate immune system (i.e. Kupffer cells and neutrophils) in mediating the hepatotoxicity of large doses of LPS, we examined the role of these cell populations in the model of low dose LPS enhancement of the hepatotoxicity of allyl alcohol. Male, Sprague-Dawley rats were treated with gadolinum chloride ($GdCl_3$) (10 mg/kg, iv) or saline vehicle 22 hours prior to treatment with LPS (100 μg/kg, iv) or saline vehicle followed by allyl alcohol (30 mg/kg, ip) or saline vehicle. Gadolinium chloride inactivates Kupffer cells. The inactivation of Kupffer cell function provided the rats significant protection from the ability of LPS to potentiate the hepatotoxicity of allyl alcohol as measured by both ALT activity and histopathological examination. In a separate series of experiments, circulating neutrophils were eliminated with a polyclonal anti-neutrophil Ig (1 ml/dose) administered 16 and 4 hours prior to treatment with LPS. The rats received LPS and allyl alcohol as described above. Removal of neutrophils from the model also significantly protected animals from LPS-enhanced allyl alcohol hepatotoxicity. These data suggest that both Kupffer cell-dependent and neutrophil-dependent mechanisms are

involved in this model and that each of these cell populations has a distinct contribution to the ability of LPS to potentiate the hepatotoxicity of allyl alcohol.

3. B. Introduction

LPS, a component of the outer leaflet of the cell membrane of Gram-negative bacteria, is a powerful stimulant for the innate immune system. It elicits a number of biochemical events such as the release of proteases, free radicals, eicosanoids, cytokines, etc., which are ideally present to protect the host from bacterial infection but may actually cause tissue damage to the host. LPS may also be involved in another source of damage to a host mammal: acting synergistically with a xenobiotic to produce more damage to the host than either toxicant alone. In Chapter 2, results of experiments in which a nontoxic dose of LPS enhanced liver damage from a subtoxic dose of allyl alcohol were presented. In this chapter, the role of two important cell types of the innate immune system, Kupffer cells and neutrophils will be examined. The hypothesis to be addressed is as follows: removal of functional populations of either Kupffer cells or neutrophils protects rats from the synergistic effects of LPS and allyl alcohol.

3. C. Materials and Methods

3. C. 1. Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12, specific activity 12 x 10^7 endotoxin units/mg for Kupffer cell inactivation studies and 7 x 10^7 endotoxin units/mg for neutrophil depletion studies) and Sigma Diagnostics Kits No. 59 UV and No. 58 UV for determination of activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) respectively, were purchased from Sigma Chemical Company (St. Louis, MO). Allyl alcohol and $GdCl_3$ were purchased from Aldrich Chemical Company (St. Louis, MO). Sterile saline was obtained from Abbott Labs (Abbott Park, IL). The Vectastain ABC-AP and Vector Red kits used for the immunohistochemical staining of neutrophils were purchased from Vector Laboratories (Burlingame, CA).

3. C. 2. Production of Anti-Neutrophil Ig

The immunoglobulin G fraction of rabbit blood serum, both anti-neutrophil and control, was produced in our laboratory using New Zealand

White and nonpedigreed rabbits respectively (Hewett *et al.*, 1992). Glycogen-elicited, peritoneal neutrophils were obtained from Sprague-Dawley rats. These were washed and homogenized using a Dounce homogenizer. For initial injections, the neutrophil homogenate was mixed (1:1) with Freund's complete adjuvant. Rabbits were tranquilized with acepromazine and restrained. The skin on the back of the neck area was disinfected, and the neutrophil/adjuvant solution (0.1 ml) was injected subcutaneously into six areas on either side of the spine. The initial injection was followed by boost injections every two weeks. The boost injections were identical to the initial injection except that Freund's incomplete adjuvant was used in place of Freund's complete adjuvant. Blood was collected from rabbits (ear artery) on weeks in which the animals did not receive boost injections. Blood was also collected from naïve rabbits for production of control Ig.

The blood was allowed to clot at room temperature for collection of serum. The Ig fraction of the serum was prepared by ammonium sulfate precipitation followed by extensive dialysis to remove excess ammonium sulfate. The neutrophil-depleting activity of the IgG fraction was determined by peripheral white cell counts of blood smears from rats given various doses of IgG. The dose of 1 ml/rat resulted in 97% reduction in the absolute numbers of circulating neutrophils compared to controls.

3. C. 3. Animals

Male, Sprague-Dawley rats [CD-Crl:CD-(SD)BR VAF/Plus]; Charles River, Portage, MI) weighing 200-300 g were used in all studies except for the determination of GSH levels. Female, Sprague-Dawley rats were used for those experiments. The animals were allowed food (Rodent Chow, Teklad, Madison, WI) and water *ad libitum*. They were maintained on a 12-hour light and dark cycle under conditions of controlled temperature and humidity.

3. C. 4 Treatment Protocols

3. C. 4. 1. Inactivation of Kupffer Cells

In experiments to examine the role of Kupffer cells, animals were pretreated with $GdCl_3$ (10 mg/kg iv) or vehicle 22 hours before administration of LPS or its vehicle. Animals were given 100 μg/kg iv of LPS or an equivalent volume of saline vehicle 2 hours before treatment with allyl alcohol (30 mg/kg ip). The experimental protocol was a 2 x 2 x 2 design. Treatment of rats with $GdCl_3$ using this dosing regimen resulted in

inhibition of Kupffer cell phagocytic activity as assessed by clearance of colloidal carbon from blood (Husztik *et al.,* 1980; Roland *et al.,* 1993; Ganey and Schultze, 1995). Animals were killed 18 hours after treatment with allyl alcohol or its saline vehicle.

3. C. 4. 2. Depletion of Circulating Neutrophils

Rats received anti-neutrophil Ig (1 ml/dose) 16 and 4 hours before injection with LPS. Animals were given 100 μg/kg iv of LPS or an equivalent volume of saline vehicle 2 hours before treatment with allyl alcohol (30 mg/kg ip). The experimental protocol was a 2 x 2 x 2 design. Animals were killed 6 hours after treatment with allyl alcohol or saline control. The extent of neutrophil depletion was determined by differential counts of stained, peripheral blood smears.

3. C. 5. Immunohistochemical Staining of Neutrophils in Liver Sections

In order to determine the effect of the anti-neutrophil Ig on the accumulation of neutrophils into the liver, the liver was removed intact.

Samples taken for microscopic examination were preserved in ten percent buffered formalin. Tissue sections (6 microns thick) were fixed to glass slides and deparaffinized using a reverse xylene gradient to properly hydrate the tissue. The deparaffinized sections were then treated with proteinase K (Sigma, St Louis, MO). A polyclonal rabbit Ig fraction was used as the primary antibody and a biotinylated anti-rabbit Ig was used as the secondary antibody (Vector Laboratories, Burlingame, CA). A substrate was then added to the tissues to produce a red precipitate (Vector Red) on the antibody-labeled neutrophils. The tissues were counterstained with hematoxylin (Gill No.3) (Sigma, St Louis, MO). The number of neutrophils was counted in thirty high-power fields per slide (ten fields per tissue section).

3. C. 6. Assessment of Hepatotoxicity

Rats were anesthetized with sodium pentobarbital (50 mg/kg ip), and blood was collected from the abdominal aorta into syringes containing 3.8% sodium citrate (final concentration 0.38%). Activities of ALT and AST were determined in plasma using Sigma Diagnostics Kits No. 59 UV and No. 58 UV, respectively. The liver was removed intact. Samples taken for microscopic examination were preserved in Histochoice fixative (Amresco,

Solon, OH) or 10% buffered formalin. Tissue sections were processed for light microscopy, cut at 6 microns, stained with hematoxylin and eosin and evaluated for lesion severity.

3. C. 7. Statistical Analysis

Data are expressed as means $\pm$ S.E.M. For all results presented, N represents the number of individual animals. Homogeneous data were analyzed by two-way analysis of variance (ANOVA). Individual means were compared using Tukey's omega test. When variances were not homogenous, data were analyzed using the Kruskal-Wallis ANOVA on ranks for more than two groups. In the latter case, Dunn's or Tukey's test was used to assess significance. The criterion for statistical significance was $p \leq 0.05$.

3. D. Results

3. D. 1. Protective Effect of $GdCl_3$.

To test the hypothesis that LPS activation of Kupffer cells is important in the enhanced response to allyl alcohol, rats were treated with $GdCl_3$ before exposure to LPS. Plasma ALT activity was low in animals exposed to saline, LPS or allyl alcohol alone and was unaffected by pretreatment with $GdCl_3$ (Figure 3.1.A). Plasma ALT activity was significantly elevated in animals cotreated with LPS and allyl alcohol, and $GdCl_3$ pretreatment prevented this increase. Similar results were observed for activity of AST in plasma (Figure 3.1.B). $GdCl_3$ did not alter the hepatocellular concentration of GSH or GSSG (Table 3.1) as compared to the concentrations of GSH and GSSG in rats pretreated with saline (Table 2.2).

Consistent with the histopathological observations described in Chapter 2, examination of liver sections from rats cotreated with LPS and allyl alcohol revealed a necrotizing, coagulative hepatitis. In most cases the lesions were not confined to the periportal region but had spread to other regions of the lobule, becoming panlobular. The lesions observed in livers of cotreated rats pretreated with $GdCl_3$ were qualitatively similar to

Figure 3.1. Protection from LPS-induced potentiation of allyl alcohol hepatotoxicity by $GdCl_3$. Animals were treated with $GdCl_3$ (10 mg/kg iv) 22 hours before administration of LPS. Animals were treated with LPS (100 μg/kg iv) or vehicle two hours before administration of allyl alcohol (30 mg/kg ip) or vehicle. (A) ALT and (B) AST activities in plasma were measured as markers of liver toxicity 18 hrs after treatment with allyl alcohol. Data are expressed as mean ± S.E.M. a, significantly different from respective value in the absence of allyl alcohol. b, significantly different from respective value in the absence of LPS. c, significantly different from respective value in the absence of $GdCl_3$. N = 4-12.

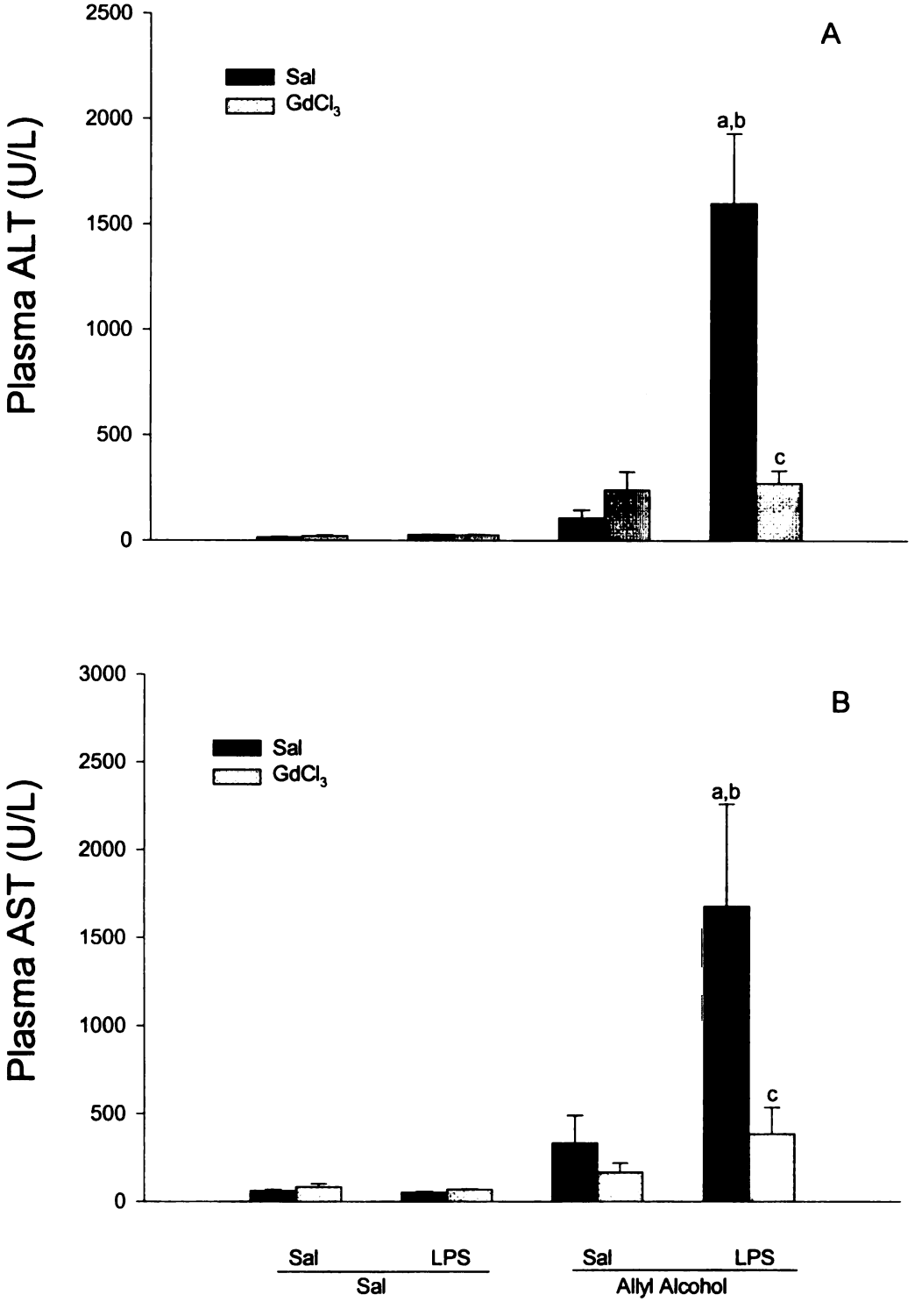

A
Plasma ALT (U/L)
2500
2000
1500
1000
500
0
Sal
GdCl3
a,b
c
B
Plasma AST (U/L)
3000
2500
2000
1500
1000
500
0
Sal
GdCl3
a,b
c
Sal
LPS
Sal
Sal
LPS
Allyl Alcohol

Table 3.1

Comparison of hepatocellular levels of reduced and oxidized glutathione in rats pretreated with $GdCl_3$

Treatment	GSH μM/g liver	GSSG μM/g liver
Sal	7.5 ± 0.4	0.47 ± 0.03
LPS	6.6 ± 0.5	0.48 ± 0.04

Female, Sprague-Dawley rats received saline 24 hours before receiving either LPS (4 mg/kg) or saline. Animals were killed 1.5 hours after LPS or saline exposure, and liver GSH and GSSG concentrations were determined as described in Materials and Methods.

those seen in cotreated animals pretreated with saline; however, the extent of the necrosis was less severe in nature. $GdCl_3$ did not affect the inflammatory infiltrate observed in livers of cotreated rats. Livers from rats treated with allyl alcohol in the absence of LPS were also less severely damaged. Sections of livers from rats treated with saline or LPS only were histologically normal with no evidence of hepatocellular necrosis or edema.

3. D. 2. Protection by Depletion of Circulating Neutrophils

To ascertain that the anti-PMN Ig had successfully depleted animals of circulating neutrophils, differential leukocyte counts were performed on blood smears from all animals. The number of circulating neutrophils was significantly lower in those animals pretreated with anti-PMN Ig compared to animals pretreated with control Ig, indicating that the circulating population of neutrophils was reduced by the anti-PMN Ig (Table 3.2).

Depletion of the circulating neutrophil pool by an anti-neutrophil IgG fraction afforded protection to rats treated with both LPS and allyl alcohol (Figure 3.2). Plasma ALT activity was significantly lower in cotreated animals pretreated with the anti-neutrophil Ig compared to those pretreated with the control Ig fraction. In rats treated with allyl alcohol alone, there was no significant difference in the plasma ALT activity between groups

Table 3.2

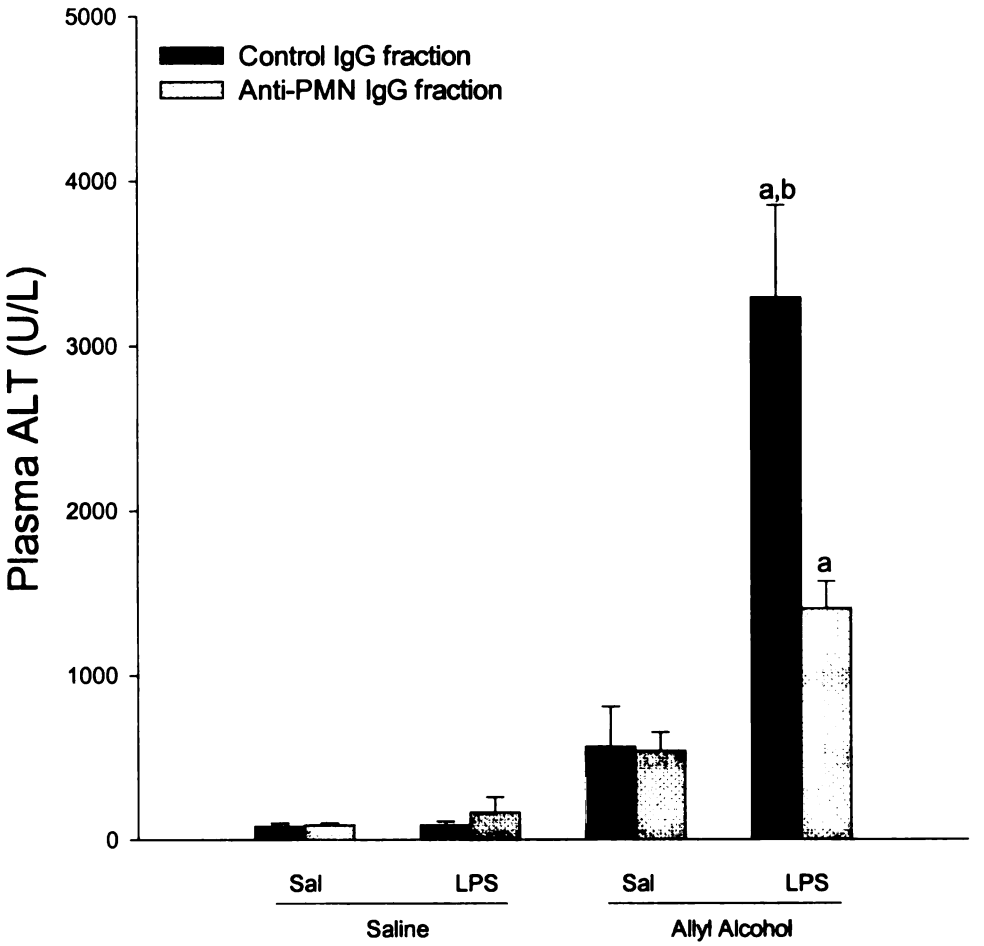
Numbers of circulating leukocytes in control rats versus rats treated with anti-PMN IgG (cells per cubic millimeter).

	TOTAL WHITE CELL COUNT	NEUTROPHILS	MONOCYTES	LYMPHOCYTES
C/Sal/AA	9400 ± 1754	1472 ± 483	1948 ± 299	5232 ± 1432
P/Sal/AA	3167 ± 547	15 ± 10[a]	245 ± 56[a]	2906 ± 497
C/LPS/AA	6720 ± 820	2084 ± 378	1415 ± 175	3413 ± 479
P/LPS/AA	4440 ± 1307	36 ± 24[a]	141 ± 30[a]	4344 ± 1265

Rats were treated with anti-PMN Ig (1 ml/animal) or control Ig 16 and 4 hours prior to administration of LPS (100 μg/kg). Rats were treated with allyl alcohol (30 mg/kg ip) 2 hrs after LPS (100 μg/kg iv).

a, significantly different from respective control Ig group

Figure 3.2 Protection from LPS-induced potentiation of allyl alcohol hepatotoxicity by depletion of circulating neutrophils. Animals were treated with an anti-PMN IgG fraction or a control IgG fraction (1 ml/animal iv) 16 and 4 hours prior to administration of LPS. Animals were treated with LPS (100 μg/kg iv) or vehicle two hours before administration of allyl alcohol (30 mg/kg ip) or vehicle. Plasma ALT activity was measured 6 hours after allyl alcohol treatment. Data are expressed as mean ± S.E.M. a, significantly different from respective value in the absence of LPS. b, significantly different from respective value in the presence of anti-PMN IgG. N = 2-12.


5000
4000
3000
2000
1000
0
Plasma ALT (U/L)
Control IgG fraction
Anti-PMN IgG fraction
a,b
a
Sal
LPS
Sal
LPS
Saline
Allyl Alcohol

that received the anti-neutrophil Ig fraction or control Ig. These data indicate that Ig alone does not affect the baseline hepatotoxicity of allyl alcohol.

Examination of liver sections by light microscopy revealed liver lesions similar to those described above. In animals cotreated with LPS and allyl alcohol, there was extensive coagulative necrosis of the hepatic parenchyma which ranged from lesions confined to the periportal regions of the liver lobules to lesions encompassing several adjacent lobules. A prominent inflammatory infiltrate consisting primarily of neutrophils was present in the necrotic sinusoids of those cotreated animals that had been pretreated with control Ig. In contrast, cotreated animals pretreated with anti-PMN Ig had markedly fewer neutrophils present in the necrotic areas. There was no accumulation of inflammatory cells in areas of healthy tissue in either group. Hepatic lesions were also noted in animals treated with allyl alcohol only. These lesions were similar to those observed in cotreated animals but they tended to be less severe.

The infiltration of neutrophils into the livers of animals was also quantified. There was a significant decrease in the number of neutrophils present in the liver lesions of cotreated animals pretreated with the anti-PMN Ig as compared to the liver lesions of cotreated animals pretreated with control Ig (Table 3.3).

Table 3.3

Infiltration of neutrophils into hepatic sinusoids

(number of cells per 30 high power fields)

	Control Ig Fraction	Anti-PMN Ig Fraction
Sal/Sal	6 ± 2	7 ± 4
LPS/Sal	72 ± 9	12 ± 3
Sal/Allyl Alcohol	115 ± 44	24 ± 9[a]
LPS/Allyl Alcohol	276 ± 63	19 ± 6[a]

Rats were treated with anti-PMN IgG (1 ml/animal) 16 and 4 hours prior to administration of LPS (100 μg/kg). Liver sections (6 microns thick) were immunostained for neutrophils.

a, significantly different from values in the absence of neutrophil depletion.

3. E. Discussion

Pretreatment of rats with nontoxic doses of LPS (100 μg/kg) significantly increased liver damage in rats exposed to a nonlethal dose of allyl alcohol as measured by both biochemical assays and pathological examination. This increase in liver injury was significantly attenuated by either inactivation of Kupffer cell phagocytosis by $GdCl_3$ or depletion of circulating neutrophils with an anti-neutrophil Ig fraction. These data strongly suggest that both of these cellular components of the innate immune system play important roles in the ability of small amounts of LPS to potentiate allyl alcohol hepatotoxicity.

In mammals, a small amount of LPS normally escapes the intestinal tract and enters the portal circulation (Jones and Summerfield, 1988; Fox *et al.*, 1989; Nakao *et al.*, 1994). This LPS is removed by Kupffer cells before it can reach the general circulation and potentially elicit a systemic inflammatory response. Kupffer cells and hepatocytes are believed to work together in the chemical detoxification of LPS (Fox *et al.*, 1989). Kupffer cells perform the first part of the detoxification process by removing some of the polysaccharide moieties; the modified LPS is then passed onto hepatocytes for further metabolism.

Despite the fact that Kupffer cells serve this beneficial role in removal of LPS, inhibition of Kupffer cell function affords protection from the lethal effects and liver injury caused by large doses of LPS (Iimuro *et al.*,, 1994; Pearson *et al.*, 1996; Brown *et al.*, 1997). This apparent paradox may be explained in part by activation of Kupffer cells under conditions in which there are large concentrations of LPS in the portal circulation which overload the detoxification mechanism of Kupffer cells; for example, during overgrowth of gram-negative bacteria or after intravenous administration of LPS (Biliar *et al.*, 1988). In response to activation by LPS, Kupffer cells release a number of chemical mediators of inflammation including cytokines, prostaglandins, leukotrienes, reactive oxygen species (ROS), platelet activating factor, and nitric oxide (Van Bossuyt *et al.*, 1988; Van Bossuyt and Wisse, 1988; Decker, 1990; Spolarics *et al.*, 1993; Portoles *et al.*, 1994; Brouwer *et al.*, 1995). These mediators may have adverse effects on neighboring cell types and contribute to the development of tissue injury. Thus, the mechanism by which inhibition of Kupffer cell function protects animals from adverse effects of LPS may involve attenuated release of potentially toxic mediators. Indeed, although inhibition of Kupffer cell activity with $GdCl_3$ decreased liver injury in response to large doses of LPS, hepatic accumulation of neutrophils was not attenuated (Brown *et al.*, 1997) and accumulation of platelets was decreased only slightly (Pearson *et al.*, 1996). These results suggest that

Kupffer cells are not responsible for signaling the initiation of inflammation in the liver during hepatic injury caused by large doses of LPS, but rather contribute to subsequent events such as release of soluble mediators that lead ultimately to tissue damage.

Results similar to those in animals exposed to large doses of LPS were observed in rats cotreated with small doses of LPS and allyl alcohol: $GdCl_3$ decreased the severity of hepatic necrosis but did not alter the inflammatory infiltrate. Although Kupffer cell activation was not measured directly, at the doses of LPS used in these studies TNF activity in plasma is increased (see Chapter 4). Kupffer cells are hypothesized to be the primary source of this TNF, although this hypothesis has been questioned. Furthermore, histological analysis revealed unusually prominent Kupffer cells in the sinusoids of rats treated with small doses of LPS, consistent with modification of these cells. Thus, low doses of LPS may increase susceptibility of hepatic parenchymal cells to injury from allyl alcohol through some of the same, Kupffer cell-dependent events that ultimately lead to liver injury at larger doses of LPS.

It is likely that the protective effect of $GdCl_3$ on LPS enhancement of allyl alcohol hepatotoxicity is mediated through abrogation of LPS effects rather than allyl alcohol effects. In support of this, it has been demonstrated that treatment with $GdCl_3$ does not affect activity of ADH (Przybocki *et al.*, 1992), ruling out decreased bioactivation of allyl alcohol

as a mechanism by which $GdCl_3$ affords protection. Furthermore, inhibition of Kupffer cell function did not prevent the hepatotoxicity of allyl alcohol in the absence of LPS in our studies (Ganey and Schultze, 1995). These results suggest that, although functional Kupffer cells are not required for liver damage from larger doses of allyl alcohol, LPS-induced augmentation of allyl alcohol hepatotoxicity occurs through a Kupffer cell-dependent mechanism.

Another suggestion from this study is that the activation of Kupffer cells may be important in their augmentation of the hepatotoxicity of allyl alcohol. These data are consistent with the findings of other researchers using different hepatotoxicants. For example, activation of Kupffer cells with vitamin A increased the hepatotoxicity of carbon tetrachloride (ElSisi *et al.,* 1993a; ElSisi *et al.,* 1993b; ElSisi *et al.,* 1993c; Rosengren *et al.,* 1995). Release of reactive oxygen species by vitamin A-stimulated Kupffer cells is believed to be a contributing factor in this increased liver damage (ElSisi *et al.,* 1993a). Inflammatory mediators are likely to be important in the ability of LPS-stimulated Kupffer cells to potentiate the hepatotoxicity of allyl alcohol, and in Chapter 4 we begin to explore this possibility.

In addition to Kupffer cells, the other major cell type of innate immunity, the neutrophil, is implicated in the mechanism by which LPS enhances allyl alcohol hepatotoxicity. Neutrophils play a central role in the acute

inflammatory response. These cells are among the first to arrive at a focus of infection and begin killing bacteria by phagocytosis and release of free radicals and proteases. A certain amount of tissue damage occurs during this process but is usually repaired during the healing phase of inflammation.

As mentioned in Chapter 1, neutrophils are a critical component of liver injury induced by large doses of LPS. Large numbers of neutrophils accumulate in the liver sinusoids and release mediators of inflammation which are toxic to adjacent hepatocytes. Removal of neutrophils with an anti-neutrophil antibody (Hewett *et al.,* 1992; Sato *et al.,* 1993) or prevention of neutrophil migration with an antibody to leukocyte adhesion molecules (Jaeschke *et al.,* 1991) protected animals from the toxic effects of LPS, confirming the important role neutrophils play in organ damage during exposure to large doses of LPS.

A marked feature of the histopathology from the initial studies of the LPS/allyl alcohol model presented in Chapter 2 was the presence of large numbers of neutrophils in the damaged and necrotic sinusoids. The exact role of the neutrophils was unclear: were they attracted by the damaged tissue or did they have an active role in causing the damage? Depletion of circulating neutrophils in cotretreated animals reduced ALT release and liver injury as seen histologically to a level that was significantly different from that of cotreated controls. These results suggest that neutrophils

attracted into the sinusoids by LPS contribute to the liver injury when LPS potentiates the hepatotoxicity of allyl alcohol. However, neutrophils were seen in the necrotic hepatic sinusoids of rats treated with allyl alcohol alone. The role of these cells is unclear. Work by Ganey and Schulze (1995) has demonstrated that neutrophil depletion did not protect rats from a toxic dose of allyl alcohol.

In summary, LPS appears to potentiate the hepatotoxicity of allyl alcohol by activating the host's innate immune system, specifically Kupffer cells and neutrophils. These cells are the source of many powerful biochemical mediators during inflammation and it is possible that some of these affect adjacent hepatic parenchymal cells, making them more sensitive to the toxic properties of allyl alcohol given alone. One possible mediator will be explored in Chapter 4.

Chapter 4

PENTOXIFYLLINE ATTENUATES BACTERIAL LIPOPOLYSACCHARIDE-INDUCED ENHANCEMENT OF ALLYL ALCOHOL HEPATOTOXICITY

4. A. Abstract

Small amounts of exogenous lipopolysaccharide (LPS) (10 ng/kg-100 μg/kg) enhance the hepatotoxicity of allyl alcohol in male, Sprague-Dawley rats. This augmentation of allyl alcohol hepatotoxicity appears to be linked to Kupffer cell function, but the mechanism of Kupffer cell involvement is unknown. Since Kupffer cells produce tumor necrosis factor-alpha (TNF) upon exposure to LPS and this cytokine has been implicated in liver injury from large doses of LPS, we tested the hypothesis that TNF contributes to LPS enhancement of allyl alcohol hepatotoxicity. Rats were treated with LPS (10-100 μg/kg iv) 2 hours before allyl alcohol (30 mg/kg ip). Cotreatment with LPS and allyl alcohol caused liver injury as assessed by an increase in activity of alanine aminotransferase in plasma. Treatment with LPS caused an increase in plasma TNF concentration which was prevented by administration of either pentoxifylline (PTX) (100 mg/kg iv) or anti-TNF serum (1ml/rat iv) one hour prior to LPS. However, only PTX protected rats from LPS-induced enhancement of allyl alcohol hepatotoxicity; anti-TNF serum had no effect. Exposure of cultured hepatocytes to LPS (1-10 μg/ml) or to TNF (15-150 ng/ml) for 2 hours did not increase the cytotoxicity of allyl alcohol (0.01-200 μM). These data suggest that neither LPS nor TNF alone is sufficient to increase the sensitivity of isolated hepatocytes to allyl alcohol. Furthermore,

hepatocytes isolated from rats treated 2 hours earlier with LPS (i.e. hepatocytes which were exposed *in vivo* to TNF and other inflammatory mediators) were no more sensitive to allyl alcohol-induced cytotoxicity than hepatocytes from naïve rats. These data suggest that circulating TNF is not involved in the mechanism by which LPS enhances hepatotoxicity of allyl alcohol and that the protective effect of PTX may be due to another of its biological effects.

4. B. Introduction

We have recently demonstrated that the hepatotoxicity of allyl alcohol is enhanced by pretreatment with quite small doses of LPS and that this augmented response is prevented by inhibition of the function of another cellular mediator of inflammation, Kupffer cells (Sneed *et al.,* 1997). These results indicate that properly functioning Kupffer cells are important in the mechanism of LPS-induced enhancement of allyl alcohol hepatotoxicity and evoke interest in whether inflammatory mediators released by these cells participate in augmenting toxicity.

Kupffer cells are the resident macrophages of the hepatic sinusoids (Jones and Summerfield, 1988; Bouwens and Wisse, 1992; Wisse *et al.,* 1996) and have a major role in clearing the hepatic portal blood of

intestinally derived LPS (Fox *et al.,* 1989; Toth *et al.,* 1992). These macrophages respond to LPS with production of mediators such as cytokines (e.g. tumor necrosis factor-alpha (TNF), interleukin-1, interleukin-6), reactive oxygen species, and prostaglandins (Decker *et al.,* 1990). Kupffer cells play a critical role in liver injury from large doses of LPS as evidenced by the observation that inhibition of their function with gadolinium chloride ($GdCl_3$) affords protection (Iimuro *et al.,* 1994; Pearson *et al.,* 1996; Brown *et al.,* 1997). Cytokines are also essential to LPS-induced responses. For example, inhibition of TNF synthesis or activity attenuates liver injury and lethality in baboons (Tracey *et al.,* 1987), mice (Beutler *et al.,* 1985) and rats (Hewett *et al.,* 1993). These results indicate that TNF is important in the pathogenesis of tissue injury from large doses of LPS and raise the possibility that TNF may be a factor in the ability of LPS to enhance the hepatotoxicity of xenobiotics. The present study was undertaken to test the hypothesis that TNF participates in the potentiation of allyl alcohol hepatotoxicity by LPS. Two approaches were taken to inhibit the effects of TNF in animals treated with LPS and allyl alcohol: pentoxifylline (PTX) was given to inhibit synthesis of TNF, and an antiserum directed against TNF was administered to neutralize TNF activity.

4. C. Materials and Methods

4. C. 1. Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12. Specific activity 1 x 10^7 endotoxin units/mg for the *in vitro* studies and 7 x 10^7 endotoxin units/mg for the *in vivo* studies), and Sigma Diagnostics Kit No. 59 UV for determination of activity of alanine aminotransferase (ALT) were purchased from Sigma Chemical Company (St. Louis, MO). Collagenase and pentoxifylline were obtained from Sigma Chemical Company (St. Louis, MO). Allyl alcohol was purchased from Aldrich Chemical Company (St. Louis, MO). Williams' medium E was purchased from Gibco (Gaithersburg, MD).

4. C. 2. Production of Anti-TNF Serum

The rabbit blood serum, both anti-TNF and control, was produced in our laboratory using New Zealand White and nonpedigree rabbits respectively (Hewett *et al.,* 1992). Recombinant murine TNF (50 μl) (R & D Systems, Minneapolis, MN) was compounded with 1 ml of saline and 1 ml of

Freund's complete adjuvant. Rabbits were tranquilized with acepromazine and restrained. The skin on the back of the neck area was disinfected, and 30 μl of the TNF/adjuvant solution was injected intradermally into six areas on either side of the spine. A separate injection (0.5 ml) was given intraperitoneally. The initial injection was followed by boost injections 14 and 28 days later. The boost injections were identical to the initial injection except that Freund's incomplete adjuvant was used in place of Freund's complete adjuvant.

Blood was drawn from the ear artery or vein at two week intervals after the last boost series. The blood was allowed to clot for two hours at room temperature then overnight at 4°C. Serum was harvested and tested for anti-TNF titer using the WEHI subclone 13 bioasaay (Espevik and Nissen-Meyer, 1986; Eskandari *et al.,* 1990). The serum was stored at –20°C.

4. C. 3. Animals

Male, Sprague-Dawley rats [CD-Crl:CD-(SD)BR VAF/Plus]; Charles River, Portage, MI) weighing 200-300 gm were used in all studies. The animals were allowed food (Rodent Chow, Teklad, Madison, WI) and water *ad libitum*. They were maintained on a 12-hour light and dark cycle under conditions of controlled temperature and humidity.

4. C. 4. Isolation of Hepatocytes

Hepatocytes were isolated by collagenase digestion (Seglen, 1973; Klaunig *et al.,* 1981), placed in Williams' medium E supplemented with 10% fetal calf serum and 0.1% gentamicin. Hepatocytes from isolations with a cell viability of 90% or greater were plated in 6-well primaria plates (Falcon Laboratories) at a density of 5 x 10^5 cells per well. In some experiments, hepatocytes were obtained from the livers of rats treated 2 hours earlier with LPS (4 mg/kg iv). The hepatocytes were allowed to stabilize in culture for 3 hours, the medium was removed, and the cells were washed once with Williams' medium E supplemented only with 0.1% gentamicin. A final volume of 2 ml per well of the latter medium was used in the remainder of the study. Allyl alcohol was added to the hepatocyte cultures at the concentrations of 0, 0.1, 1, 25, 30, 50, 60, 100, or 200 mM as indicated in the Figures and Results. Hepatocyte injury was assessed 90 or 180 minutes after addition of allyl alcohol. In studies in which hepatocyte cultures were incubated with LPS or TNF, these agents were added 2 hours prior to treatment with allyl alcohol.

4. C. 5. Treatment Protocols

4. C. 5. 1. Treatment of Animals with Anti-TNF Serum

. In preliminary experiments to determine the effective dose of serum, a limited number of rats were pretreated intravenously with 0, 0.25, 0.50, or 1.0 ml of anti-TNF serum diluted 1:1 with saline 1 hour before treatment with LPS (100 μg/kg iv) (Hewett *et al.*, 1993). Ninety minutes after administration of LPS animals were anesthetized with pentobarbital (50 mg/kg ip) and blood was drawn from the abdominal aorta into syringes containing 3.8% sodium citrate (final concentration 0.38%) allyl alcohol (30 mg/kg) or sterile saline was injected intraperitoneally. The activity of TNF was determined using the WEHI subclone 13 bioassay (Figure 4.1). Administration of either 0.25 or 0.5 ml of anti-TNF serum neutralized TNF to undetectable levels in 3 out of 4 rats; however, I ml of serum effectively eliminated TNF activity in all rats tested. This dose was chosen for the remaining studies.

4. C. 5. 2. Treatment of Animals with Pentoxifylline

Rats were treated intravenously with PTX (100 mg/kg) or with an equivalent volume of sterile saline (Abbott Laboratory, Abbott Park, IL) 1 hour prior to treatment with LPS (100 μg/kg). This treatment protocol for PTX has been shown to prevent the LPS-induced rise in plasma TNF activity (Hewett *et al.,* 1993). Two hours after administration of LPS, allyl alcohol or its sterile saline vehicle was injected intraperitoneally. Liver injury was assessed six hours later.

4. C. 6. Assessment of Hepatocyte Cytotoxicity

Cytotoxicity was assessed from release of ALT into the medium. The medium was collected from the wells, and 2 ml of 1% TritonX-100 were added to each well and allowed to remain for at least 5 minutes at room temperature. The wells were scraped thoroughly with a rubber policeman to remove all cells, and the resulting solution was sonicated to disperse subcellular components. All samples (both medium and lysate) were centrifuged for 10 minutes at 600 x g. The activity of ALT in all cell-free supernatant fluids was determined with Sigma Diagnostics Kit No. 59-UV. The activity in the medium (i.e., ALT released) was expressed as a

percentage of the total (medium plus lysate) activity (Ganey *et al.*, 1994; Ho *et al.*, 1996).

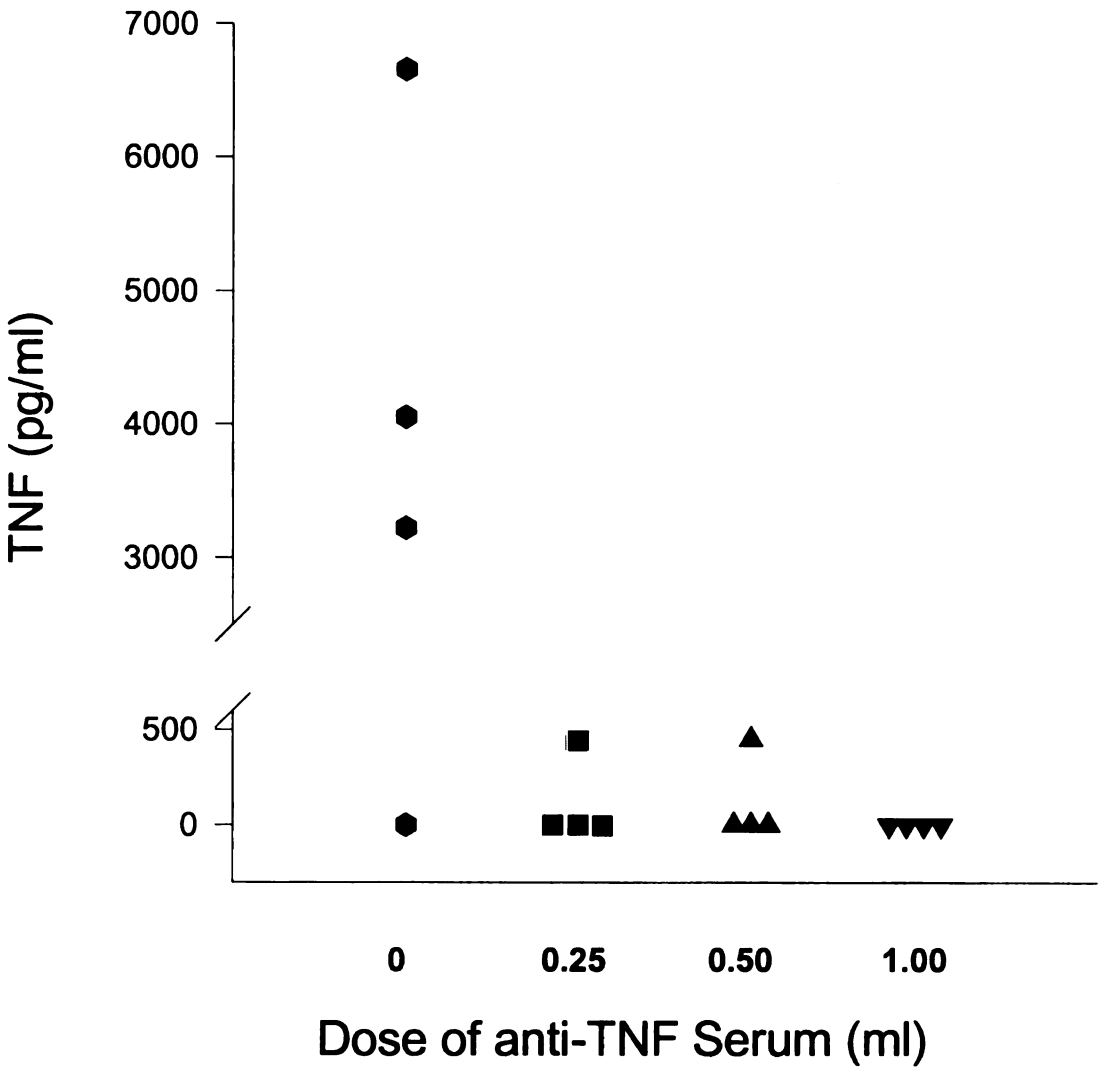
4. C. 7. Assessment of Hepatotoxicity.

Rats were anesthetized with sodium pentobarbital (50 mg/kg ip), and blood was collected from the abdominal aorta into syringes containing sodium citrate (final concentration, 0.38%). ALT activity was determined in plasma.

4. C. 8. Determination of Activity of TNF

The activity of TNF in the blood of rats was determined 90 minutes after administration of LPS or its saline vehicle. Rats were anesthetized with sodium pentobarbital (50 mg/kg ip), and blood was collected from the abdominal aorta into syringes containing sodium citrate (final concentration, 0.38%). Plasma was collected, diluted serially and

Figure 4.1. Dose-response for anti-TNF serum. In order to determine the amount of anti-TNF serum needed to completely neutralize circulating levels of plasma TNF, rats were treated with either 0.25, 0.50, or 1.0 ml of anti-TNF serum diluted 1:1 in saline or a saline control. The animals were given LPS (100 μg/kg) one hour later. Plasma was collected 90 minutes after injection with LPS, and activity of TNF was determined as described in Materials and Methods. N = 4.


7000
6000
5000
4000
3000
500
0
TNF (pg/ml)
0
0.25
0.50
1.00
Dose of anti-TNF Serum (ml)

incubated for 22 hours in the presence of the TNF-sensitive fibrosarcoma cell line, WEHI 164 clone 13 (Espevik and Nissen-Meyer, 1986; Eskandari *et al.,* 1990). The extent of cell lysis was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) using a Bio-Tek plate reader, and the amount of TNF was calculated from a standard curve using a serial dilution of a recombinant murine TNF standard (R & D Systems, Minneapolis, MN).

4. C. 9. Assessment of Alcohol Dehydrogenase Activity in Liver Homogenates

Rats were treated with PTX (100 mg/kg) or saline vehicle 90 minutes prior to treatment with LPS (10 μg/kg). Two hours after treatment with LPS or saline vehicle, animals were killed. The liver was removed and homogenized in a solution of 0.05 M HEPES (pH 8.4) and 0.33 mM dithiothreitol. The homogenate was centrifuged at 100,000 x g for 45 min. The supernatant fluid was collected, and activity of ADH was measured spectrophometrically (366 nm) by monitoring the reduction of nicotinamide adenine dinucleotide (NAD) using ethanol as a substrate (Krebs *et al.,* 1969). Protein concentration in the supernatant was determined using the bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL).

4. C. 10. Statistical Analysis

Data are expressed as means ± S.E.M. For all results presented, N represents the number of individual animals used for *in vivo* or *in vitro* studies. Homogenous data were analyzed by one-way or two-way analysis of variance (ANOVA). Individual means were compared using Tukey's omega test. When variances were not homogenous, data were analyzed using Kruskal-Wallis ANOVA on ranks, and Dunn's test was used to assess significance. Data expressed as percentages were transformed by the arc sine square root method prior to analysis. The criterion for statistical significance was $p \leq 0.05$.

4. D. Results

4. D. 1. Effect of *In Vitro* Exposure to LPS on Allyl Alcohol-Induced Cytotoxicity in Isolated Hepatotcytes

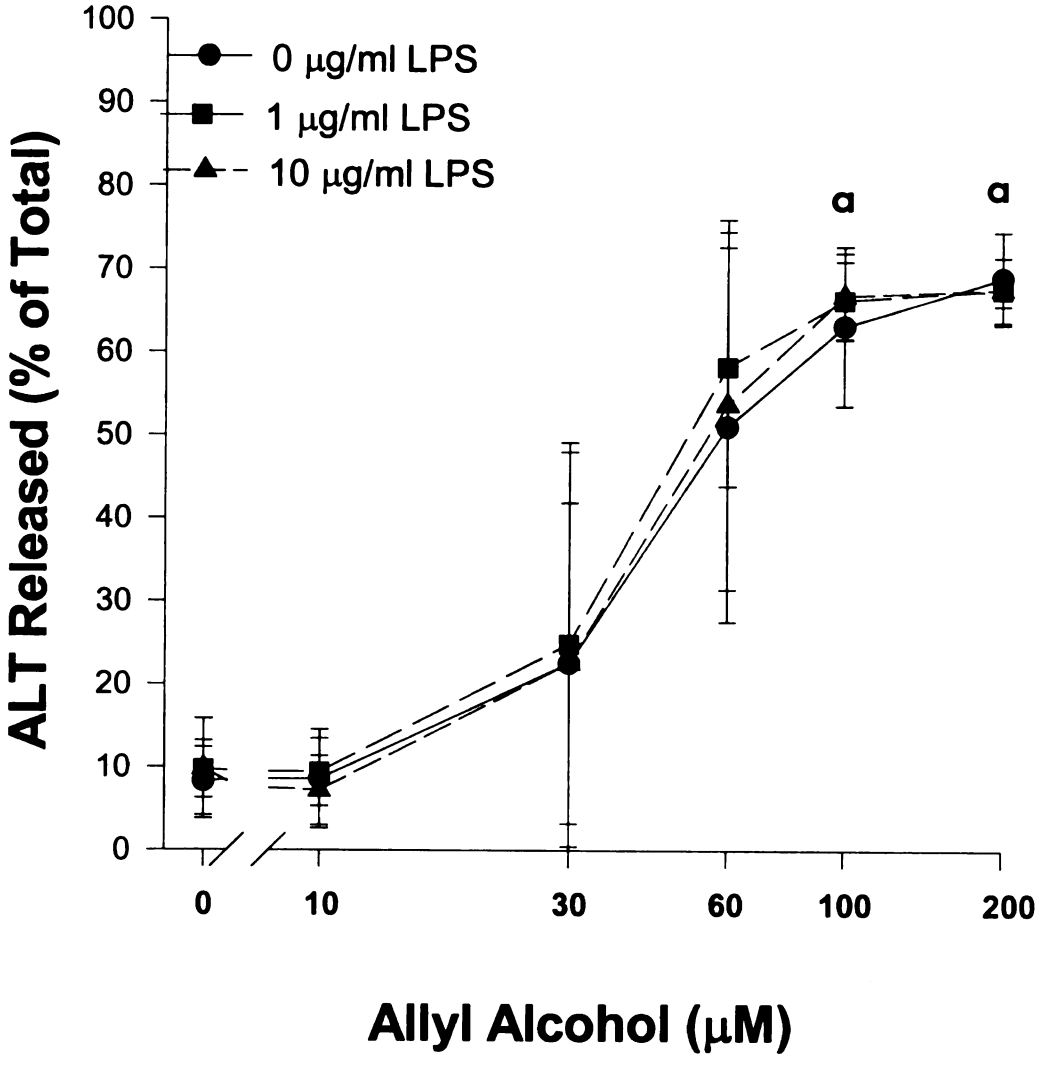
To test whether LPS has direct effects on hepatocytes that contribute to enhancement of allyl alcohol toxicity, isolated hepatocytes were pretreated with LPS for 2 hours, then exposed to allyl alcohol (Figure 4.2). This

experimental design was selected to mimic the dosing regimen for LPS and allyl alcohol that results in LPS enhancement of allyl alcohol toxicity *in vivo* (Sneed *et al.*, 1997). In hepatocytes not exposed to LPS, allyl alcohol caused a concentration-related increase in ALT release at 90 minutes; significant differences were observed at concentrations of allyl alcohol $\geq$ 100 μM. In hepatocytes exposed to LPS, a significant rise in ALT release was also seen at concentrations of allyl alcohol $\geq$ 100 μM, and there were no significant differences in ALT release among groups at any concentration of allyl alcohol. LPS was not cytotoxic in the absence of allyl alcohol (Figure 4.2). Similar experiments were performed in which cytotoxicity was assessed at 180 minutes after addition of allyl alcohol. No further increase in toxicity was observed at this time, and no differences were observed among the LPS-treated groups (data not shown).

4. D. 2. Protection from LPS-Induced Enhancement of Allyl Alcohol Hepatotoxicity by PTX

PTX decreases the synthesis of TNF at the mRNA level (Dezube *et al.*, 1993). Control animals treated with saline only or with PTX and saline only did not have detectable plasma activity of TNF (Table 4.1). TNF

Figure 4.2. Lack of effect of LPS exposure *in vitro* on allyl alcohol cytotoxicity towards hepatic parenchymal cells. Rat hepatocytes were cultured in medium containing 0, 1, or 10 μg/ml LPS for 2 hours. Allyl alcohol was added at the indicated concentrations, and cytotoxicity was assessed 90 minutes later from release of ALT activity into the medium. Data are expressed as mean ± S.E.M. a, all values at these points, irrespective of LPS concentration, are significantly different from respective controls not exposed to allyl alcohol. N=5.


100
90
80
70
60
50
40
30
20
10
0
ALT Released (% of Total)
0 µg/ml LPS
1 µg/ml LPS
10 µg/ml LPS
a
a
0
10
30
60
100
200
Allyl Alcohol (µM)

activity was increased significantly in the plasma of animals treated 90 minutes earlier with LPS. Pretreatment with PTX significantly reduced the circulating activity of TNF.

Plasma ALT activity was low in rats in the saline/saline or LPS/saline groups irrespective of PTX pretreatment (Figure 4.3). In animals that received the vehicle for PTX and then were treated with saline and allyl alcohol there was an increase in plasma ALT activity, but this increase was not statistically significant. Animals treated with the vehicle for PTX and then cotreated with LPS and allyl alcohol had significantly elevated plasma ALT activity. Pretreatment with PTX attenuated the increase in plasma ALT activity in cotreated animals.

Allyl alcohol hepatotoxicity requires bioactivation by ADH to acrolein. Accordingly, we examined the effect of PTX on the activity of ADH in the livers of rats pretreated with LPS to determine if PTX afforded protection by inhibition of the bioactivation of allyl alcohol. As shown in Table 4.2, pretreatment with PTX did not affect the activity of ADH in rat liver.

Table 4.1

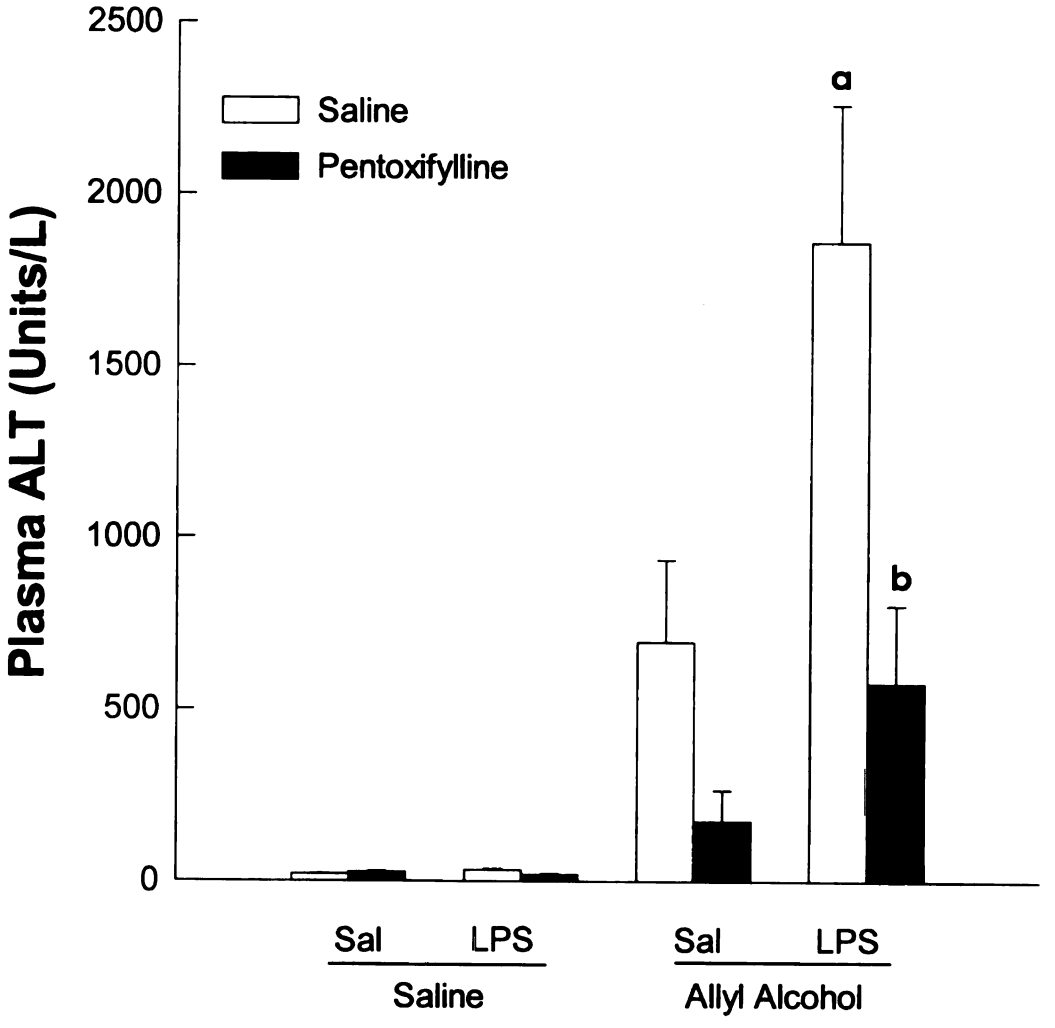
Effect of PTX on the LPS-induced increase in plasma TNF activity

	TNF (ng/ml)	
Treatment	Saline	PTX
Saline	ND	ND
LPS	15.0 ± 3.2	0.04 ± 0.01[a]

Rats were treated with PTX (100 mg/kg, iv) or saline vehicle one hour before treatment with LPS (0.1 mg/kg, iv). The activity of TNF was measured in plasma collected 90 minutes after LPS treatment. a, significantly different from respective value in the absence of PTX.

ND, Not Detected.

Figure 4.3. Protection by PTX from LPS-enhancement of allyl alcohol hepatotoxicity. Animals were pretreated with PTX (100 mg/kg) 1 hour prior to treatment with LPS (100 μg/kg). Allyl alcohol (30 mg/kg) was given 2 hours after LPS; liver damage was measured 6 hours after allyl alcohol treatment. Data are expressed as mean ± S.E.M. a, significantly different from values in absence of LPS . b, significantly different from respective value in the absence of PTX. N = 6.


2500
2000
1500
1000
500
0
Plasma ALT (Units/L)
Saline
Pentoxifylline
a
b
Sal
LPS
Sal
LPS
Saline
Allyl Alcohol

4. D. 3. Lack of Effect of Inactivation of TNF on LPS-Induced Potentiation of Allyl Alcohol Hepatotoxicity

To verify that reduction of circulating TNF protects rats from LPS-potentiated allyl alcohol hepatotoxicity, animals were treated with an anti-serum specific for TNF prior to treatment with LPS. In preliminary studies, the efficacy of the anti-TNF antibody was determined. The administration of 1 ml of anti-TNF serum prevented the rise in plasma TNF such that TNF activity in all samples was below the limit of detection.

There was no significant elevation in plasma ALT activity in animals in the saline/saline, LPS/saline or saline/allyl alcohol groups irrespective of pretreatment with control or anti-TNF serum (Figure 4.4). Plasma ALT activity was significantly elevated in animals cotreated with LPS and allyl alcohol compared to animals treated with LPS alone or allyl alcohol alone. There was no significant difference in ALT activity between cotreated animals pretreated with control serum and anti-TNF serum.

Table 4.2

Effect of PTX on the activity of alcohol dehydrogenase in LPS-treated rats

	ALCOHOL DEHYDROGENASE ACTIVITY (μmol/min/g of liver)	
Treatment	Saline	PTX
Saline	7.32 ± 1.96	7.44 ± 2.31
LPS	7.31 + 2.19	8.07 ± 0.54

Rats were treated with PTX (100 mg/kg, iv) or saline vehicle one hour before treatment with LPS (0.1 mg/kg, iv). The activity of alcohol dehydrogenase was measured in homogenates collected 90 minutes after LPS treatment.

There were no significant differences in the activity of hepatic alcohol dehydrogenase among any of the treatment groups.

Figure 4.4. Lack of protection from LPS enhancement of allyl alcohol hepatotoxicity by anti-TNF serum. Animals were pretreated with control or anti-TNF serum 1 hour prior to treatment with LPS (10 μg/kg). Allyl alcohol (30 mg/kg) was given 2 hours after LPS; liver damage was measured 6 hours after allyl alcohol treatment. Data are expressed as mean ± S.E.M. a, significantly different from respective value in absence of LPS. b, significantly different from respective values in the absence of allyl alcohol. N = 3-13.

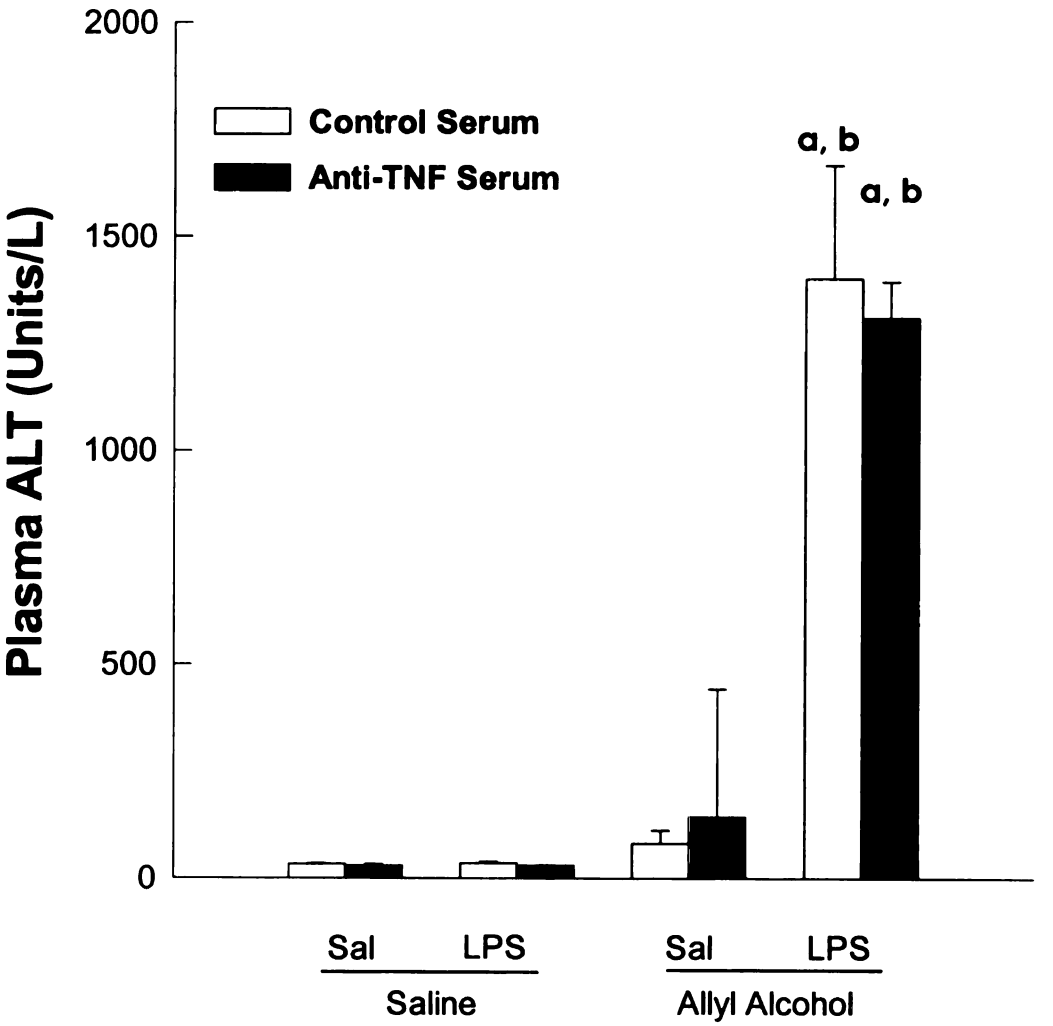
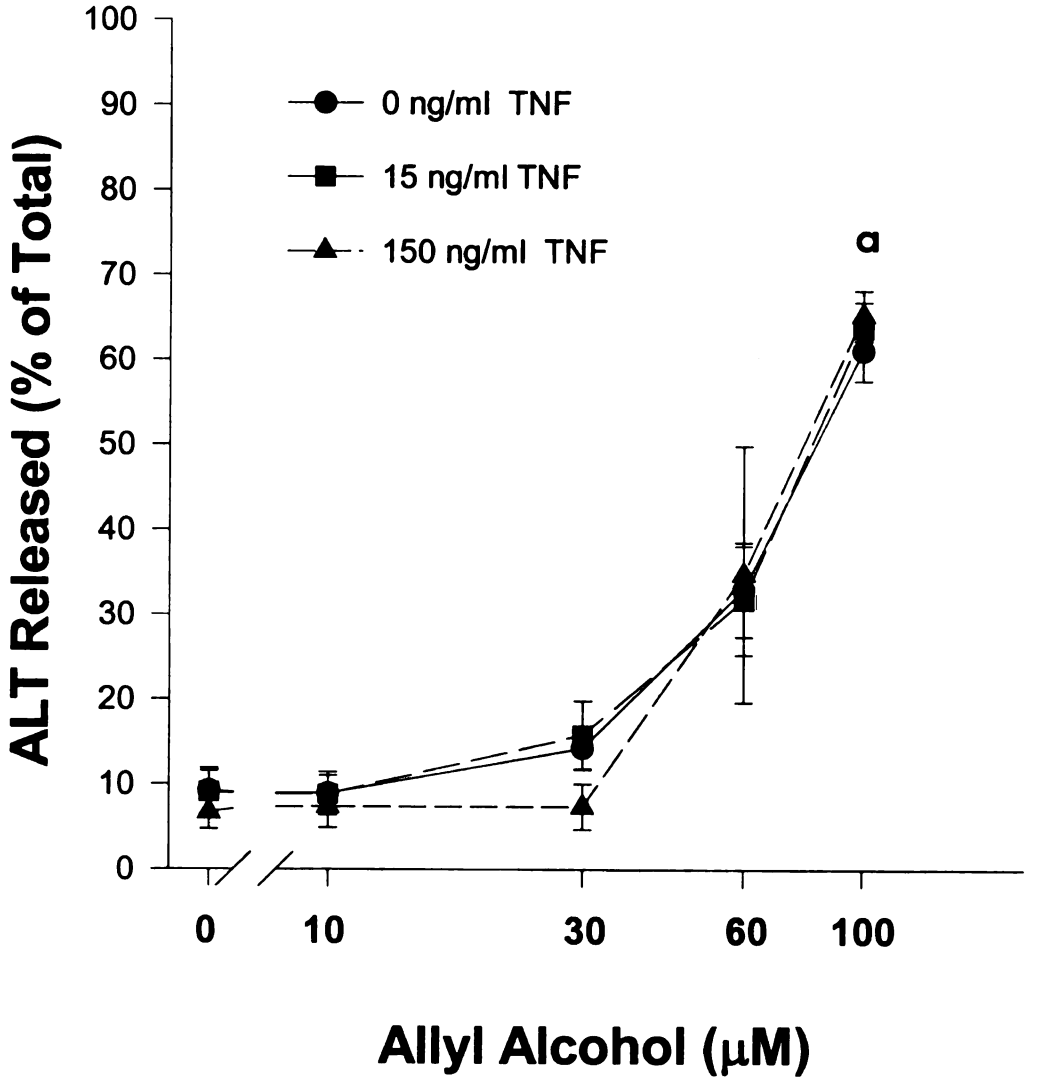

2000
1500
1000
500
0
Plasma ALT (Units/L)
Control Serum
Anti-TNF Serum
a, b
a, b
Sal
LPS
Sal
LPS
Saline
Allyl Alcohol

Figure 4.5. Lack of effect of TNF exposure *in vitro* on allyl alcohol cytotoxicity towards hepatic parenchymal cells. Rat hepatocytes were cultured in medium containing 0, 15, or 150 ng/ml TNF for 2 hours. Allyl alcohol was added at the indicated concentrations, and cytotoxicity was assessed 90 minutes later. Data are expressed as mean $\pm$ S.E.M. a, all values at these points, irrespective of TNF concentration, are significantly different from respective controls not exposed to allyl alcohol. N = 3-5.


100
90
80
70
60
50
40
30
20
10
0
ALT Released (% of Total)
0 ng/ml TNF
15 ng/ml TNF
150 ng/ml TNF
a
0
10
30
60
100
Allyl Alcohol (μM)

4. D. 4. Effect of *In Vitro* Exposure to TNF on Allyl Alcohol-Induced Cytotoxicity in Isolated Hepatocytes

To test whether TNF alone can enhance the hepatotoxicity of allyl alcohol in isolated hepatocytes, cells were pretreated with TNF for 2 hours before exposure to allyl alcohol. Two concentrations of TNF were used: 15 ng/ml to replicate the TNF activity found in peripheral plasma of rats treated with LPS (Table 4.1) and 150 ng/ml to estimate a greater TNF activity potentially found in the liver sinusoids after treatment with LPS . As in experiments depicted in Figure 1, allyl alcohol caused a concentration-dependent increase in release of ALT (Figure 4.5). Cytotoxicity of allyl alcohol was unaffected by pretreatment with TNF.

4. D. 5. Allyl Alcohol-Induced Cytotoxicity in Isolated Hepatocytes from LPS-Treated Rats

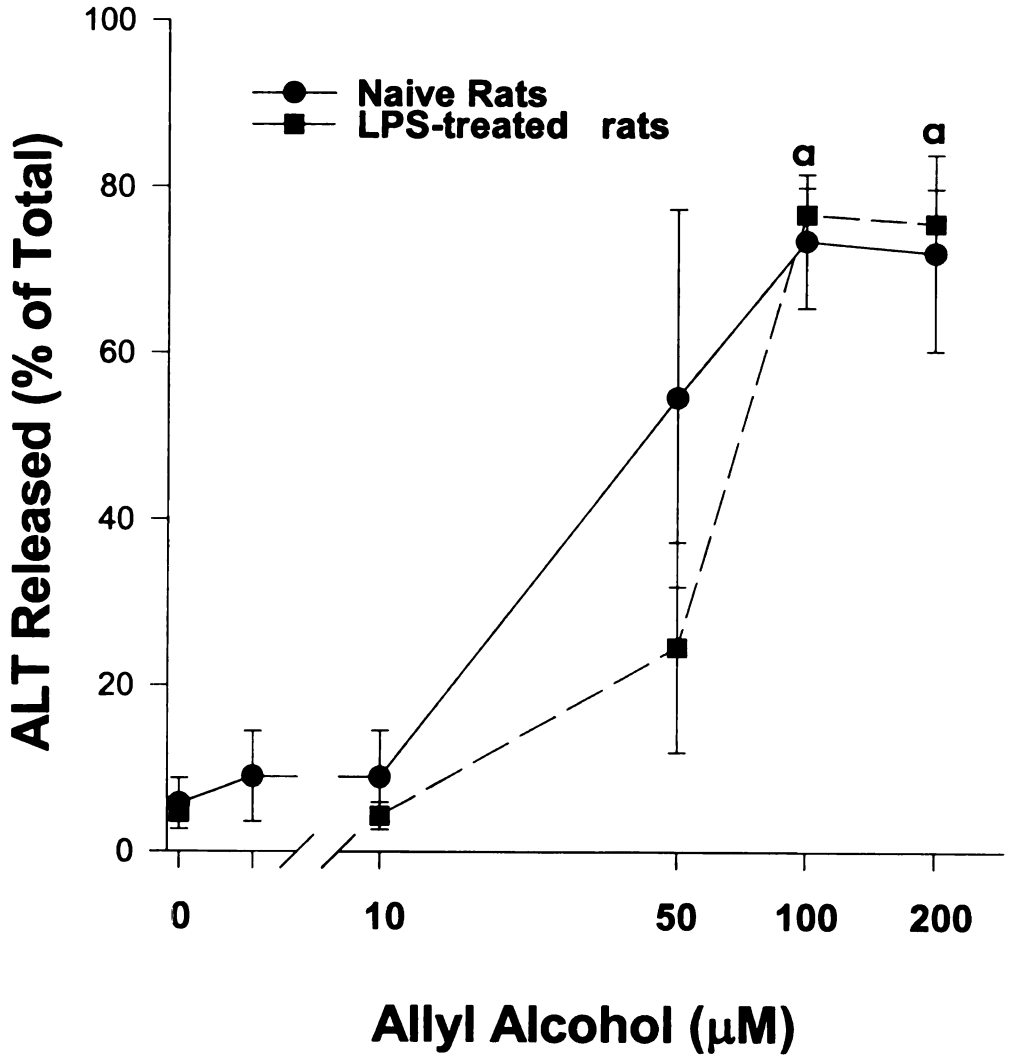
TNF reaches a maximal concentration in plasma 90 minutes after treatment with LPS *in vivo*. To examine whether exposure *in vivo* to TNF increased sensitivity of hepatocytes to allyl alcohol, hepatocytes were isolated from rats treated with LPS 2 hours earlier and were exposed to ally alcohol as described in Figure 4.1. Allyl alcohol caused a concentration-dependent increase in ALT release in hepatocytes isolated

from LPS-treated rats (Fig 4.6); however there was no significant difference in allyl alcohol-induced toxicity.

4. E. Discussion

We have reported previously that very small amounts (10 ng/kg – 100 μg/kg) of LPS potentiate the hepatotoxicity of allyl alcohol (Sneed *et al.*, 1997), and the studies presented here were performed to begin to explore the mechanism of potentiation. Hepatic injury resulting from exposure to relatively large doses of LPS is dependent upon several factors. These factors include, but are not limited to, the release of inflammatory mediators by activated macrophages and the influx of inflammatory cells into the liver. Blockade or inhibition of any one of these factors prevents the hepatic injury associated with large doses of LPS (Tracey *et al.*, 1987; Jaescshke *et al.*, 1991; Sato *et al.*, 1993; Chang *et al.*, 1993; Hewett *et al.*,1993; Imuro *et al.*, 1994). Although LPS damages the liver through indirect means via inflammatory cells and soluble mediators, direct effects of LPS on hepatocytes have been reported. For example, LPS decreases bile formation (Utili *et al.*, 1977) and increases fatty acid synthesis in the liver (Feingold *et al.*, 1992). It is unlikely that the direct effects of LPS

Figure 4.6. Cytotoxicity of allyl alcohol toward hepatocytes isolated from rats treated *in vivo* with LPS. Rats were treated with LPS (4 mg/kg) 2 hours prior to hepatocyte isolation. The indicated concentrations of allyl alcohol were added to the culture medium, and cytotoxicity was measured 90 minutes later. For reference, allyl alcohol cytotoxicity in hepatocytes from naïve rats is presented. Data are expressed as mean $\pm$ S.E.M. a, significantly different from control not exposed to allyl alcohol. N = 5.


Naive Rats
LPS-treated rats
a
a
ALT Released (% of Total)
100
80
60
40
20
0
0
10
50
100
200
Allyl Alcohol (μM)

contribute to the enhancement of hepatotoxicity of allyl alcohol because allyl alcohol-induced cytotoxicity in isolated hepatocytes was not altered by pretreatment of cells with LPS (Figure 4.1). Thus, these results support the hypothesis that factors other than LPS alone are responsible for the enhancement of hepatotoxicity seen *in vivo*. This hypothesis is consistent with results of studies in which inhibition of Kupffer cell function prevented enhancement of allyl alcohol hepatotoxicity by LPS (Sneed *et al.,* 1997). One of the inflammatory mediators produced by LPS-activated Kupffer cells is the proinflammatory cytokine, TNF, which plays a critical role in liver injury from large doses of LPS (Beutler *et al.,* 1985; Tracey *et al.,* 1987; Hewett *et al.,* 1993). Accordingly, we examined the role of TNF in LPS potentiation of allyl alcohol hepatotoxicity.

The methylxanthine, PTX, inhibits the synthesis of TNF (Zabel *et al.,* 1989; Han *et al.,* 1990; Doherty *et al.,* 1991; Noel *et al.,* 1990; Dezube *et al.,* 1993; Semmler *et al.,* 1993; Zabel *et al.,* 1993), and results presented here (Table 4.1) confirm this. Furthermore, administration of PTX prior to LPS treatment protected animals from the enhanced hepatotoxicity of allyl alcohol. These data suggested that TNF may be involved in the mechanism by which LPS augments the hepatotoxicity of allyl alcohol. PTX has multiple pharmacological effects, however; therefore a more specific approach, neutralization of TNF with an anti-TNF serum, was used to test further whether inhibition of TNF afforded protection. The anti-TNF

serum did not diminish LPS enhancement of allyl alcohol hepatotoxicity despite complete neutralization of circulating TNF activity. Similar protection by PTX and lack of protection by anti-serum-induced neutralization of TNF have been observed in a rat model of intestinal injury induced by nonsteroidal anti-inflammatory drugs (Reuter and Wallace, 1999) and in a model of bacteria-induced lung injury in rabbits (Miyazaki *et al.*, 1999).

One explanation for the disparate results observed with PTX and anti-TNF serum in these studies is that since PTX inhibits synthesis of TNF, it affords a more complete blockade of TNF action in the liver, whereas TNF is still produced by Kupffer cells after treatment with anti-TNF serum and can act locally before neutralization by the anti-serum. An alternative explanation is that TNF is not involved in the mechanism by which LPS enhances the hepatotoxicity of allyl alcohol. This explanation is supported by results obtained from *in vitro* experiments presented here. Exposure of isolated hepatocytes to TNF did not alter the cytotoxic response to allyl alcohol (Figure 4.4), indicating that direct effects of TNF on hepatocytes are not sufficient to increase sensitivity to allyl alcohol. Others have also shown that TNF alone is not cytotoxic to isolated hepatocytes, but that cell damage requires the addition of other cytokines or induction of oxidative stress in the cells (Adamson and Billings, 1992; Sieg and Billings, 1997).

Experiments were performed using hepatocytes isolated from rats treated two hours earlier with LPS. Since TNF activity in plasma reaches a peak 90 minutes after administration of LPS, these hepatocytes were exposed to TNF *in vivo*. Despite this exposure to TNF and other mediators evoked by treatment with LPS, allyl alcohol was neither more potent nor more toxic in these cells. Maximal cytotoxicity was observed at the same concentration of allyl alcohol (100 μM) in both cell populations, and the concentration of allyl alcohol required to achieve half-maximal cytotoxicity was greater, not smaller, in hepatocytes from LPS-treated rats compared to those from naïve rats. One possible explanation for these results is that the procedure used to isolate hepatocytes could select cells that are resistant to the effects of LPS and the mediators elicited by LPS. Alternatively, these results suggest that exposure *in vivo* to LPS-induced mediators for up to two hours is not sufficient to increase sensitivity of hepatocytes toward allyl alcohol. It is possible that interactions among hepatocytes and nonparenchymal cells in the intact liver may be needed at times beyond 2 hours, or augmentation of toxicity may only be observed under conditions of continuous exposure to LPS-induced soluble mediators. Another possibility is that the presence of a mediator that becomes involved at times beyond 2 hours after exposure to LPS may be required for enhanced hepatotoxicity. For example, thrombin is necessary for liver injury from larger doses of LPS, and protection is afforded when

the action of thrombin is blocked up to 2 hours after administration of LPS (Moulin *et al.,* 1996).

If TNF is not involved in the mechanism by which LPS enhances allyl alcohol hepatotoxicity, then the protective effect produced by PTX is due to one or more of the other pharmacological properties of this drug. The phosphodiesterase properties of PTX cause increases in intracellular levels of cyclic adenosine monophosphate (cAMP), and this increase in cAMP may inhibit macrophage function (Taffet *et al.,* 1989). PTX also improves blood flow in tissues (Ward and Clissold, 1987). This effect of PTX has been demonstrated to be protective in one model of sepsis in which high mortality was associated with hemodynamic shock (Yang *et al.,* 1999). In addition PTX can reduce the levels of toxic free radicals. PTX attenuates the expression of inducible nitric oxide synthase (Wu *et al.,* 1999) and decreases the respiratory burst of neutrophils (Kowalski *et al.,* 1999). A combination of the above factors may be involved in the ability of PTX to protect animals from LPS-enhanced allyl alcohol hepatotoxicity.

In summary, inflammatory mediators may participate in the ability of LPS to enhance the hepatotoxicity of certain xenobiotics. In LPS-induced enhancement of allyl alcohol hepatotoxicity however, circulating TNF does not appear to play a major role, although the possibility of autocrine or paracrine hepatic effects of TNF cannot be completely ruled out. The drug

PTX protects animals from this enhancement and may do so by affecting the responses of Kupffer cells to the presence of LPS.

Chapter 5

SUMMARIES AND CONCLUSIONS

5. A. Characterization of the Model

Very small, nontoxic doses of exogenous LPS can significantly potentiate the liver injury observed in rats exposed to allyl alcohol. Furthermore, this potentiation occurs at doses of allyl alcohol which are nontoxic or only mildly so. Histologically, the lesions produced are much more allyl alcohol-like than LPS-like in appearance, suggesting that the hepatotoxicity of allyl alcohol is potentiated, not that of LPS.

Based upon the observations presented in Chapter 2, LPS accelerates the development of liver damage from allyl alcohol. Pretreatment of rats with LPS reduces the time for significant liver injury to occur. Normally allyl alcohol produces hepatic lesions at 18 to 24 hours. In the presence of LPS, lesions are apparent as early as 2 hours. LPS also makes the lesions more severe: animals cotreated with LPS and 30 mg/kg of allyl alcohol have hepatic lesions that resemble those of animals treated with 40 mg/kg of allyl alcohol.

Allyl alcohol must be bioactivated to the aldehyde acrolein for liver damage to occur. This is also true of LPS-induced potentiation of allyl alcohol. However, LPS has no effects on the actual metabolism of allyl alcohol. LPS does not affect the hepatocellular concentration of reduced glutathione.

5. B. The Role of the Innate Immune System

LPS is a potent stimulant for the innate immune system. Under normal conditions the stimulation provided by LPS is just adequate to elicit an immediate response from the innate immune system towards invading bacteria. However, sometimes, the components of the innate immune system will overrespond to the stimulus provided by LPS and, in turn, damage the body that they are defending.

The two primary divisions of the innate immune system, macrophages and neutrophils, have prominent roles in high dose models of LPS hepatotoxicity. Disabling either cell population will protect a host from the toxic effects of endotoxemia. Recent research has also suggested that these cell populations participate in the toxic mechanisms of certain xenobiotics. Depletion of circulating neutrophils protects animals from the liver injury produced by ANIT, while inactivation of Kupffer cells protects rats from the hepatotoxicity of ethyl alcohol (Dahm *et al.*, 1991; Adachi *et al.*, 1994).

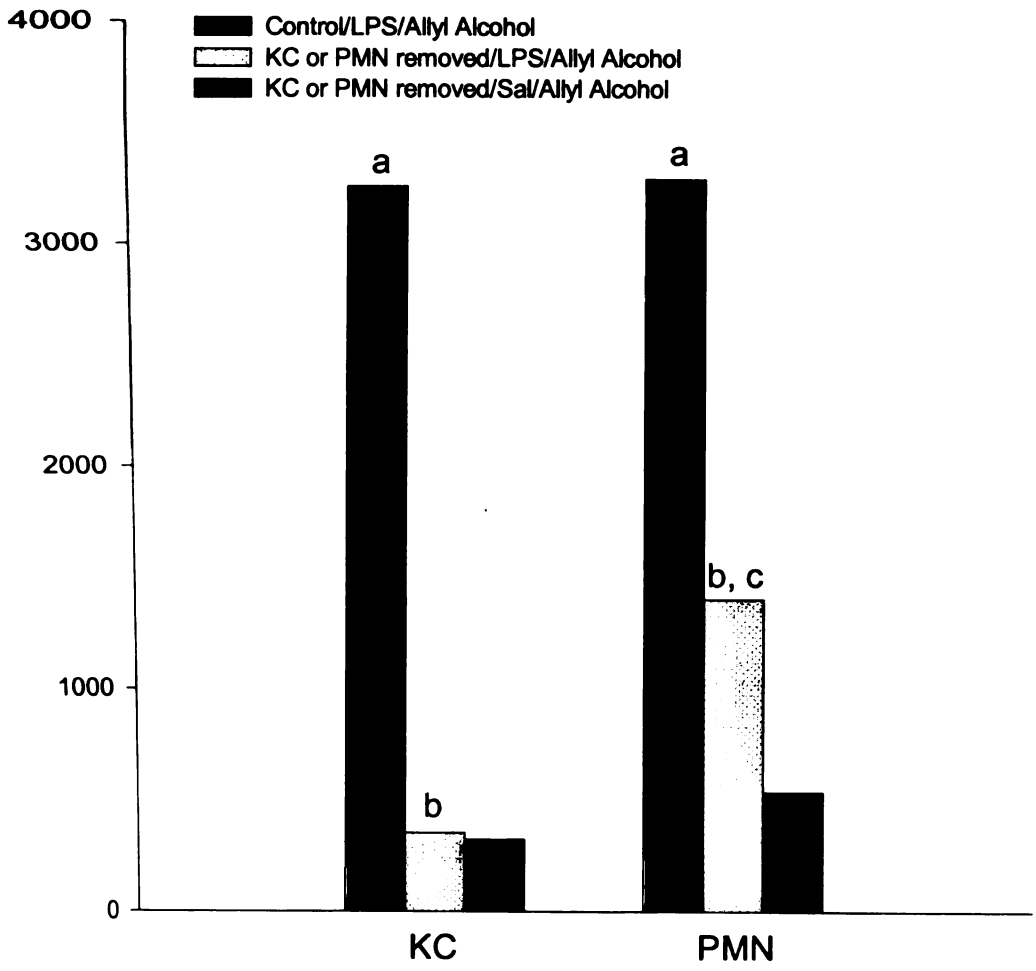
Based on the data presented in Chapter 3, the current state of one major population of innate immune system cells, Kupffer cells, was crucial to the ability of allyl alcohol to produce significant hepatotoxicity. Stimulation of these cells with LPS prior to exposure to allyl alcohol resulted in a significant increase in liver injury compared to no prior

stimulation. Disabling Kupffer cells protected experimental animals from enhanced hepatotoxicity of allyl alcohol even if they were pretreated with LPS. These data fulfilled two of Koch's postulates concerning the involvement of agents in a disease process.

The role of neutrophils was not as clear. Early in the development of this model, large numbers of neutrophils were present in the necrotic areas of the hepatic sinsoids. Their contribution to the injury was unclear: were they active participants or were they there in response to tissue necrosis. Depletion of circulating neutrophils provided significant protection from the ability of LPS to potentiate the hepatotoxicity of allyl alcohol; however, unlike in the case of $GdCl_3$, removal of neutrophils did not totally eliminate the increase in liver injury seen in LPS-potentiated allyl alcohol hepatotoxicity. A significant difference in plasma ALT activities still existed between neutrophil-depleted animals from the LPS/allyl alcohol group and animals from the saline/allyl alcohol group. These data suggested that neutrophils did not act independently of Kupffer cells but most likely acted in concert with them to potentiate the hepatotoxicity seen when animals were given both LPS and allyl alcohol (Figure 5.1).

While the data from Chapter 3 demonstrate the important role of both Kupffer cells and neutrophils in LPS-enhanced hepatotoxicity of allyl alcohol, they do not indicate the relative contribution of each cell population to the experimental model. Inhibition of either leg of the innate

Figure 5.1. Comparison of the protective effects of depletion of functional Kupffer cells versus circulating neutrophils. Rats were depleted of either functional Kupffer cells or circulating neutrophils prior to treatment with LPS (100 μg/kg) or saline control. Two hours later, animals were treated with allyl alcohol (30 mg/kg) or saline control. LPS potentiated the hepatotoxicity of allyl alcohol in control animals in both studies while depletion of functional Kupffer cells or circulating neutrophils provided significant protection from liver injury. Exclusive depletion of neutrophils, however, did not provide the same degree of protection as did depletion of Kupffer cells. The level of hepatotoxicity in neutrophil-depleted, cotreated animals was significantly higher than in the same group in the absence of LPS. a, significantly different from value in the absence of LPS. b, significantly different from value in the presence of functional Kupffer cells or circulating neutrophils. c, significantly different value in absence of LPS.


4000
3000
2000
1000
0
Control/LPS/Allyl Alcohol
KC or PMN removed/LPS/Allyl Alcohol
KC or PMN removed/Sal/Allyl Alcohol
a
a
b
b, c
KC
PMN

immune system is equally protective even though inactivation of Kupffer cells provided more absolute protection than did depletion of neutrophils. The latter observation may indicate that neutrophils work in concert with Kupffer cells to produce the liver injury associated with sequential administration of LPS and allyl alcohol. Such an observation may serve as a starting point for other research questions such as how do Kupffer cells and neutrophils interact to produce the LPS-induced potentiation of allyl alcohol hepatotoxicity.

5. C. The Role of TNF

LPS does not directly make hepatocytes more sensitive to the toxic effects of allyl alcohol. Simply exposing cultured hepatocytes to LPS has none of the effects seen in intact animals. As has already been established, participation of Kupffer cells and neutrophils is crucial to the ability of LPS to potentiate the hepatotoxicity of allyl alcohol. A logical supposition would be that these cells produce a factor or factors that make hepatocytes more sensitive to the toxic effects of allyl alcohol.

As described in the Chapter 1, both Kupffer cells and neutrophils produce a number of biochemical mediators in response to exposure to LPS and some of these mediators play an important role in the liver injury

seen in endotoxemia. It is quite possible that a secretory product of these cells has a deleterious effect on adjacent hepatocytes.

One of these mediators is the cytokine TNF. Inhibition of TNF activity in intact animals protects them from the organ damage and death associated with endotoxemia and bacteremia. In the current model of LPS-induced enhancement of allyl alcohol toxicity, pretreatment of rats with PTX, a known inhibitor of TNF synthesis, is protective. However, inhibition of TNF activity with an anti-TNF serum is not. These data may indicate that TNF is not involved and they may indicate that PTX provides protection via another mechanism such as affecting cyclic adenosine monophosphate levels in Kupffer cells or inhibiting the respiratory burst in neutrophils.

Adding TNF directly to cultured hepatocytes does not affect their sensitivity to allyl alcohol. Similarly, coculturing them with LPS-stimulated peritoneal macrophages or RAW cells has no effect. Based on the results described above, the complex cellular interactions found in the intact animal are missing from cell culture situations.

5. D. A New Hypothesis

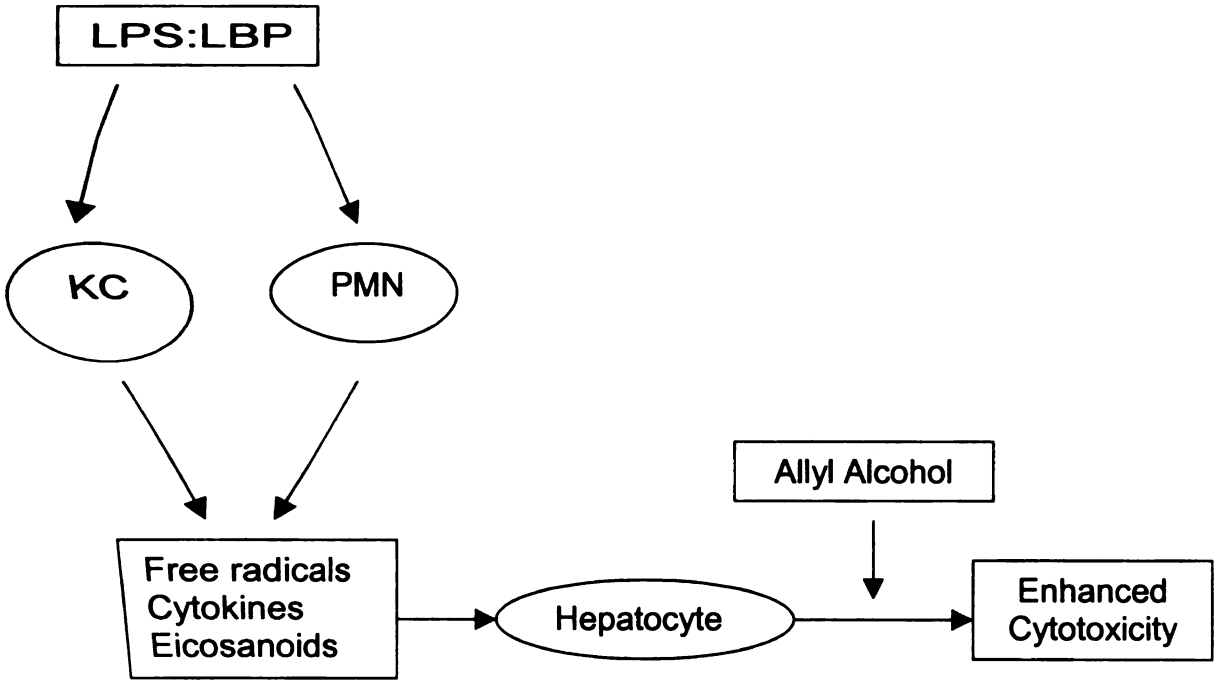
In the work presented in this dissertation, we have established that very small doses of LPS can potentiate the hepatotoxicity of allyl alcohol. We

have also proven our original hypothesis that cells of the innate immune system are crucial elements in this model of liver injury. Our experimental data correlate quite well with a growing body of research linking exposure to LPS to the augmentation of the hepatotoxicity of other biologically-based toxicants or synthetic xenobiotic agents. Even though these compounds vary in their mechanism of hepatotoxicity, coexposure to LPS is the common factor.

The data generated for this dissertation has answered the original hypothesis, but they have in turn generated a new hypothesis and a new set of questions to be solved. A new hypothesis might be the following: After exposure to LPS, Kupffer cells and neutrophils release biochemical mediators that make hepatocytes more sensitive to the toxic properties of allyl alcohol (Figure 5.2). The identity of the mediator needs to discovered as well as how it exerts its effect on hepatocytes to make them more sensitive.

The data from this dissertation has also generated a number a new questions. One interesting aspect of this model is the inability to reproduce it in *in vitro* culture systems. The simple presence of LPS does not make hepatocytes more sensitive to allyl alcohol. Questions arising from this finding might be: What are the cell types crucial to the mechanism? Is there something in the environment of the sinusoid that is missing from the *in vitro* system? Finding the answer to these and related

Figure 5. 2. A hypothetical model. Kupffer cells and neutrophils release inflammatory mediators in the presence of LPS. When these mediators reach hepatocytes, they alter the physiology of the parenchymal cells such that the hepatocytes are more susceptible to injury by allyl alcohol.


LPS:LBP
KC
PMN
Allyl Alcohol
Free radicals
Cytokines
Eicosanoids
Hepatocyte
Enhanced
Cytotoxicity

questions will provide valuable insight into the workings of the ability of LPS to potentiate allyl alcohol hepatotoxicity.

The knowledge gained from solving the mechanism behind this model may prove useful in understanding other models in which the interaction of LPS and a second agent results in enhanced liver injury. This knowledge may also provide insight into some of the variable and idiosyncratic responses of individual humans to various toxicants. The current state of their innate immune system may be a very important factor in how that person will respond to a given toxicant.

BIBLIOGRAPHY

Bibliography

Adachi, Y., Bradford, B. U., Gao, W., Bojes, H. K., Thurman, R. G. (1994). Inactivation of Kupffer cells prevents early alcohol-induced liver injury. *Hepatology*. **20**, 435-460.

Adamson, G. M., and Billings, R. E. (1992). Tumor necrosis factor-induced oxidative stress in isolated mouse hepatocytes. *Arch. Biochem. Biophys*. **294**, 223–229.

Aida, Y., and Pabst, M. J. (1990). Priming of neutrophils by lipopolysaccharide for enhanced release of superoxide. Requirement for plasma but not for tumor necrosis factor. *J. Immunol*. **145**, 3017-3025.

Albina, J. E., and Reichner, J. S. (1998). Role of nitric oxide in mediation of macrophage cytotoxicity and apoptosis. *Cancer Metast. Rev*. **17,** 39-53.

Albrecht, C., Meijer, D. K., Lebbe, C., Sagesser, H., Melgert, B. N., Poelstra, K., and Reichen, J. (1997). Targeting naproxen coupled to human serum albumin to nonparenchymal cells reduces endotoxin-induced mortality in rats with biliary cirrhosis. *Hepatology*. **26,** 1553-1559.

Armbrust, T., and Ramadori, G. (1996). Functional characterization of two different Kupffer cell populations of normal rat liver. *J. Hepatol*. **25**, 518-528.

Anderson, D. C., Schmalsteig, F. C., Shearer, W., Becker-Freeman, K., Kohl, S., Smith, C. W., Tosi, M. F., and Springer, T. (1985). Leukocyte LFA-1, OKM1, p150,95 deficiency syndrome: functional and biosynthetic studies of three kindreds. *Fed. Proc*. **44**, 2671-2677.

Anderson, M. S., Bulawa, C. E., and Raetz, C. R. H. (1985). The biosynthesis of gram-negative endotoxin. *J. Biol. Chem*. **260**, 15536-15541.

Aono, K., Isobe, K., Nakashima, I., Kondo, S., Miyachi, M., and Nimura, Y. (1994). Kupffer cells cytotoxicity against hepatoma cells is related to nitric oxide. *Biochem. Biophys. Res. Commun*. **201**, 1175-1181.

Arthur, M. J. P., Bently, I. S., Tanner, A. R., Saunders, P. K., Millward-Sadler, G. H., and Wright, R. (1985). Oxygen-derived free radicals promote hepatic injury in the rat. *Gastroenterology* **89**, 1114-1122.

Arthur, M. J. P., Kowalski-Saunders P., and Wright, R. (1986). Corynebacterium parvum-elicited hepatic macrophages demostrated enhanced respiratory burst activity compared with resident Kupffer cells in the rat. *Gastroenterology.* **91**, 174-181.

Arthur, M. J. P., Kowalski-Saunders, P., Wright, R. (1988). Effect of endotoxin on release of reactive oxygen intermediates by rat hepatic macrophages. *Gastroenterology.* **95**, 1588-1594.

Badr, M. Z., Belinsky, S. A., Kauffman, F. C., and Thurman, R. G. (1986). Mechanism of hepatotoxicity to periportal regions of the liver lobule due to allyl alcohol: role of oxygen and lipid peroxidation. *J. Pharmacol. Exper. Ther.* **238**, 1138-1142.

Balis, J. U., Patterson, J. F., Shelley, S. A., Larson, C. H., Fareed, J., and, Gerber, L. I. (1979). Glucocorticoid and antibiotic effects on hepatic microcirculation and associated host responses in lethal Gram-negative bacteremia. *Lab. Invest.* **40**, 55-65.

Ball, H. A., Cook, J. A., Wisse, W. C., and Halushka, P. V. (1986). Role of thromboxane, prostaglandins and leukotrienes in endotoxic and septic shock. *Intensive Care Med.* **12**, 116-126.

Baue, A. E. The multiple organ or system failure syndrome. (1993). In: *Pathophysiology of Shock, Sepsis, and Organ Failure.* Schlag, G. and Redl, H., eds. 1004 -1018.

Bautista, A. P., Mezaros, K., Bojto, J., and Spitzer J. J. (1990). Superoxide anion generation in the liver during the early stage of endotoxemia in rats. *J. Leukoc. Biol.* **48**, 123-128.

Bazil, V., Baudys, M., Hilgert, I., Stefanova, I., Low, M.G., Zbrozek, J., and Horejsi, V. (1989). Structural relationship between the soluble and membrane bound forms of human monocyte surface glycoprotein CD14. *Mol. Immunol.* **26**, 657-662.

Beauchamp, Jr., R. O., Andjelkovich, D. A., Kligerman, A. D., Morgan, K. T., and Heck, H., d'A. (1985). A critical review of the literature on acrolein toxicity. *Crit. Rev. Toxicol.* **14**, 309-378.

Belinsky, S. A., Matsumura, T., Kauffman, and Thurman, R. G. (1984a). Rates of allyl alcohol metabolism in periportal and pericentral regions of the liver lobule. *Mol. Pharmacol.* **25**, 158-164.

Belinsky, S. A., Popp, J. A., Kauffman, F. C., and Thurman R. G. (1984b). Trypan blue uptake as a new method to investigate hepatotoxicity in periportal and pericentral regions of the liver lobule: studies with allyl alcohol in the perfused liver. *J. Pharmacol. Exper. Ther.* **230**, 755-760.

Bemelmans, M. H. A., van Tits, L. J. H., and Buurman, W. A. (1996). Tumor necrosis factor: function, release and clearance. *Crit Rev. Immunol.* **16**, 1-11.

Beutler, B., Milsark I. W., and Cerami, A. C. (1985). Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science.* **229**, 869-871.

Beutler, B., and Cerami, A. (1987). Cachectin: more than a tumor necrosis factor. *New Eng. J. Med.* **316**, 379-385.

Bhagwandeen, S. B., Apte, M., Manwarring, L., and Dickeson, J. (1987). Endotoxin-induced necrosis in rats on an alcoholic diet. *J. Pathol.* **151**, 47-53.

Billiar, T. R., Maddaus, M. A., West, M. A., Curran, R. D., Wells, C. A., and Simmons, R. L. (1988). Intestinal bacterial overgrowth *in vivo* augments the *in vitro* response of Kupffer cells to endotoxin. *Ann. Surg.* **208**, 532-540.

Billiar, T. R., Curran, R. D., West, M. A., Hofmann, K., and Simmons, R. L. (1989a). Kupffer cell cytotoxicity to hepatocytes in coculture requires L-arginine. *Arch. Surg.* **124**, 1416-1421.

Billiar, T. R., Curran, R. D., Stuehr, D. J., West, M. A., Bentz, B. G., and Simmons, R. L. (1989b). An L-arginine-dependent mechanism of hepatocyte protein synthesis *in vitro*. *J. Exper. Med.* **169**, 1467-1472.

Blyuger, A. F., Maiore, A. Y., Bolotova, M. A., Strozhe, T. Ya., and Zaltsmane, V. K. (1974). Dynamics of functional and ultrastructural changes in the liver cells during the development of allyl alcohol necrosis. *Bull. Exper. Biol. Med.* **77**, 328-331.

Bowers, G. J., MacVittie, T. J., Hirsch, E. F., Conklin, J. C., Nelson, R. D., Roethel R. J., and Fink, M. P. (1985). Prostanoid production by lipopolysaccharide-stimulated Kupffer cells. *J. Surg. Res.* **38**, 501-508.

Bouwens, L., and Wisse, E. (1992). The origin of Kupffer cells and their anatomic relationship to hepatocytes. In *Hepatocytes and Kupffer Cell Interactions.* (T. Billiar and R. D. Curran, Eds.) pp. 4-19. CRC Press, Boca Raton.

Brandtzaeg, P., Waage, A., Mollnes, T. E., Oktedalen, O., and Kierulf, P. (1990). Severe human septic shock involves more than tumor necrosis factor. In *Progress in Clinical and Biological Research*, (A. Sturk, J. H. van Deventer, J. Wouter ten Cate, L. G. Thijis, and J. Levin, Eds.), Vol. 367, pp. 25-42. Wiley-Liss, Inc., New York.

Brouwer, A., Barelds, R. J., de Leeuw, A. M., Blauw, E., Plas, A., Yap, S. H., van den Broek, A. M., and Knook, D. L. (1988). Isolation and culture of Kupffer cells from human liver. Ultrastructure, endocytosis and prostaglandin synthesis. *J. Hepatol.* **6**, 36-49.

Brouwer, A., Parker, S. G., Hendriks, H. F. J., Gibbons, L., and Horan, M. A. (1995). Production of eicosanoids and cytokines by Kupffer cells from young and old rats stimulated by endotoxin. *Clin. Sci.* **88**, 211-217.

Brouckaert, P., Libert, C., Everaerdt, B., Takahashi, N., and Cauwels, A., and Fiers, W. (1993). Tumor necrosis factor, its receptors and the connection with interleukin 1 and interleukin 6. *Immunobiol.* **187**, 317-329.

Brown, A. P., Harkema, J. R., Schultze, A. E., Roth, R. A., and Ganey, P. E. (1997). Gadolinium chloride pretreatment protects against hepatic injury but predisposes the lungs to alveolitis following lipopolysaccharide administration. *Shock.* **7**, 186-192.

Bufler, P., Stiegler, G., Schuchmann, M., Hess, S., Kruger, C., Stelter, F., Eckerskorn, C., Schutt, C., and Englelmann, H. (1995). Soluble lipoploysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants. *Eur. J. Immunol.* **25**, 604-610.

Butterworth, K. R., Carpanini, F. M. B., Dunnington, D., Grasso, P., and Pelling, D. (1978). The production of periportal necrosis by allyl alcohol in the rat. *Br. J. Pharmacol.* **63**, 353P-354P.

Calcamuggi, G., Lanzio, M., Dughera, L., Babini, G., and Emanuelli, G. (1992). Endotoxin tolerance and polymyxin B modify liver damage and cholestasis induced by a single dose of alpha-naphthylisothiocyanate in the rat. *Arch. Toxicol.* **66**, 126-30.

Callery, M. P., Mangino, M. J., Kamei, T., and Flye, M. W. (1990). Interleukin-6 production by endotoxin-stimulated Kupffer cells is regulated by prostaglandin E_2. *J. Surg. Res.* **48**, 523-527.

Callery, M. P., Kamei, T., and Flye, M. W. (1992). Endotoxin stimulates interleukin-6 production by human Kupffer cells. *Cir. Shock.* **37**, 185-188.

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. USA.* **72**, 3666-3670.

Casteleijn, E., Kuiper, J., van Rooij, H. C. J., Koster, J. F., and van Berkel, T. J. C. (1988a). Conditioned media of Kupffer and endothelial liver cells influence protein phosphorylation in parenchymal liver cells. Involvement of prostaglandins. *Biochem. J.* **252**, 601-605.

Casteleijn, E., Kuiper, J., van Rooij, H. C. J., Kamps, J. A. A. M., Koster, J. F., and van Berkel, T. J. C. (1988b). Hormonal control of glycogenolysis in parenchymal liver cells by Kupffer and endothelial liver cells. *J. Biol. Chem.* **263**, 2699-2703.

Casteleijn, E., Kuiper, J., van Rooij, H. C., Kamps, J. A., Koster, J. F., and Van Berkel T. J. (1988c). Endotoxin stimulates glycogenolysis in the liver by means of intercellular communication. *J. Biol. Chem.* **263**, 6953-6955.

Chamulitrat, W., Jordan, S. J., and Mason, R. P. (1994). Nitric oxide production during endotoxic shock in carbon tetrachloride-treated rats. *Mol. Pharmacol.* **46**, 391-397.

Chang, H. R., Vesin, C., Grau, G. E., Pointaire, P., Arsenijevic, D., Strath, M., Pechere, J. C. and Piguet, P. F. (1993). Respective role of polymorphonuclear leukocytes and their integrins (CD-11/18) in the local or systemic toxicity of lipopolysaccharide. *J. Leukoc. Biol.* **53**, 636- 639.

Creasey, A. A., Stevens, P., Kenny, J., Allison, A. C., Warren, K., Carlett, R., Hinshaw, L., and Taylor Jr., F. B. (1991). Endotoxin and cytokine profile in plasma of baboons challenged with lethal and sublethal Escherichia coli. *Circ .Shock.* **33**, 84-91.

Cybulsky, M. I., Colditz, I. G., and Movat, H. Z. (1986). The role of interleukin-1 in neutrophil leukocyte emigration induced by endotoxin. *Am. J. Pathol.* **124**, 367-372.

Czaja, M. J., Xu, J., Ju, Y., Alt, E., Schmiedeberg, P. (1994). Lipopolysaccharide-neutralizing antibody reduces hepatocyte injury from acute hepatotoxin administration. *Hepatology.* **19**, 1282-1289.

Dahm, L. J., Schultze, A. E., and Roth, R. A. (1991). An antibody to neutrophils attenuates a-naphthylisothiocyanate-induced liver injury. *J. Pharmacol. Exper. Ther.* **256**, 412-420.

Deaciuc, I. V., Bagby, G. J., Niesman, M. R., Skrepnik, N., and Spitzer, J. J. (1993). Modulation of hepatic sinusoidal endothelial cell function by Kupffer cells: An example of intercellular communication in the liver. *Hepatology.* **19**, 464-470.

Decker, K. (1990). Biologically active products of stimulated liver macrophages (Kupffer cells). *Eur. J. Biochem.* **192**, 245-261.

Dezube, B. J., Sherman, M. L., Fridovich-Keil, J. L., Allen-Ryan, J., and Pardee, A.. B. (1993). Down-regulation of tumor necrosis factor expression by pentoxifylline in cancer patients: a pilot study. *Cancer Immunol. Immunother.* **36**, 57-60.

Dinarello, C. A. (1983). Molecular mechanisms in endotoxin fever. *Agents Actions.* **13**, 470-486.

Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino Jr., M. A., and O'Conner, J. V. (1986). Tumor necrosis factor (cachetin) is an endogenous pyrogen and induces production of interleukin 1. *J. Exp. Med.* **163**, 1433-1450.

Dinarello, C.A. (1988). Biology of interleukin 1. *FASEB J.* **2**, 108-15.

Dofferhoff, A. S. M., Bom, V. J. J., van Ingen, J., de Vries-Hospers, H. G., Hazenberg, B. P. C., vd Meer J., Mulder P. O. M., and Weits, J. (1991). Patterns of cytokines, plasma endotoxin, and acute phase protein during the treatment of severe sepsis in humans. In *Progress in Clinical and Biological Research* Vol 367. (A. Sturk, J. H. van Deventer, J. Wouter ten Cate, H. R. Buller, L. G. Thijis and J. Levin, Eds.) pp. 43-54. Wiley-Liss.

Dogterom, P., Nagelkerke, J. F., van Steveninck, J., and Mulder, G. J. Inhibition of lipid peroxidation by disulfiram and diethyldithiocarbamate does not prevent hepatotoxin-induced cell death in isolated rat hepatocytes. A study with allyl alcohol, tert-butyl hydroperoxide, diethyl maleate, bromoisovalerylurea and carbon tetrachloride. (1988) *Chem.-Biol. Interactions.* **66**, 251–265.

Dogterom P., Kroese, E. D., Mulder, G. J., and Nagelkerke, J. F. (1989a). Extracellular calcium alleviates cell toxicity due to hepatotoxins that induce lipid peroxidation, but has no effect on toxins that do not cause lipid peroxidation. A study in isolated rat hepatocytes. *Biochem. Pharmacol.* **38**, 4225-4230.

Dogterom P., Mulder G. J., and Nagelkerke, J. M. (1989b). Lipid peroxidation-dependent and -independent protein thiol modifications in isolated rat hepatocytes: differential effects of vitamin E and disulfiram. *Chem.-Biol. Interactions.* **71**, 291-306.

Dogterom P. and Mulder G. J. (1993). Differential effects of extracellular calcium on lipid peroxidation dependent (ethacrynic acid and allyl alcohol) and lipid peroxidation independent (disulfiram)-induced cytotoxicity in normal and vitamin E-deficient rat hepatocytes. *Chem.-Biol. Interactions.* **86**, 171-181.

Doherty, G.M., Jensen, J.C., Alexander, H.R., Buresh, C.M., and Norton, J.A. (1991). Pentoxifylline suppression of tumor necrosis factor gene transcription. *Surgery.* **110**, 192-198.

Doi, F., Goya, T., and Torisu, M. (1993). Potential role of hepatic macrophages in neutrophil-mediated liver injury in rats with sepsis. *Hepatology.* **17**, 1086-1094.

Durham, S. K., Brouwer, A., Barelds, R. J., Horan, M. A., and Knook D. L. (1990). Comparative endotoxin-induced hepatic injury in young and aged rats. *J. Pathol.* **162**, 341-349.

Durum, S. K., and Oppenheim, J. J. (1989). Macrophage-derived mediators: interleukin 1, tumor necrosis factor, interleukin 6, and related cytokines. *Fundamental Immunology*, 2nd edition. (Paul. W. E., ed.) 639-661.

Edwards, M. J., Keller, B. J., Kauffman, F. C., and Thurman, R. G. (1993). The involvement of Kupffer cells in carbon tetrachloride toxicity. *Toxicol. Appl. Pharmacol.* **119**, 275-279.

ElSisi, A. E. D., Earnest, D. L., and Sipes, I. G. (1993a). Vitamin A potentiation of carbon tetrachloride hepatotoxicity: role of liver macrophages and active oxygen species. *Toxicol. Appl. Pharmacol.* **119**, 295-301.

ElSisi, A. E. D., Hall, P., Sim, W-L. W., Earnest, D. L., and Sipes, I. G. (1993b). Characterization of vitamin A potentiation of carbon tetrachloride-induced liver injury. *Toxicol. Appl. Pharmacol.* **119**, 280-288.

ElSisi, A. E. D., Earnest, D. L., and Sipes, I. G. (1993c). Vitamin A potentiation of carbon tetrachloride hepatotoxicity: enhanced lipid peroxidation without enhanced biotransformation. *Toxicol. Appl. Pharmacol.* **119**, 289-294.

Eskandari, M. K., Nguyen, D. T., Kunkel, S. L., and Remick, D. G. (1990). WEHI 164 subclone 13 assay for TNF: sensitivity, specificity, and reliability. *Immunol. Invest.* **19**, 69-79.

Espevik, T. and Nissen-Myer, J. (1986). A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods.* **95**, 99-105.

Evans, R., Fong, M., Fuller, J., Kamdar, S., Meyerhardt, J., and Strassmann, G. (1992). Tumor cell IL-6 gene expression is regulated by IL-1 alpha/beta and TNF alpha: proposed feedback mechanisms induced by the interaction of tumor cells and macrophages. *J. Leukoc. Biol.* **52**, 436- 468.

Fariss, M. W., and Reed, D. J., (1987). High-performance liquid chromatography of thiols and disulfides: Dinitrophenol derivatives. *Methods Enzymol.* **143**, 101-114.

Feingold, K. R., Staprans, I., Memon, R. A., Moser, A. H., Shigenaga, J. K., Doerrler, W., Dinarello, C. A., and Grunfeld, C. (1992). Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses stimulate hepatic triglyceride production while high doses inhibit clearance. *J. Lipid Res.* **33**, 1765- 1776.

Ferrali, M., Ciccoli, L., and Comporti, M. (1989). Allyl alcohol-induced hemolysis and its relation to iron release and lipid peroxidation. *Biochem. Pharmacol.* **38**, 1819-1825.

Fawcett D W. (1986). The liver and gallbladder. In *A Textbook of Histology*. (D. W. Fawcett, ed.) pp. 679-715. W. B. Saunders Company.

Fong, Y., Tracey, K. J., Moldawer, L. L., Hesse, D. G., Manogue, K. B., Kenney, J. S., Lee, A. T., Kuo, G. C., Allison, A.C., and Lowry, S. F. (1989). Antibodies to cachectin/tumor necrosis factor reduce interleukin 1 beta and interleukin 6 appearance during lethal bacteremia. *J. Exp. Med.* **170**, 1627-1633.

Fox, E. S., Thomas, P., and Broitman, S. A. (1989). Clearance of gut-derived endotoxins by the liver. Release and modification of ^{3}H, ^{14}C-lipopolysaccharide by isolated rat Kupffer cells. *Gastroenterology*. **96**, 456 -461.

Fujita, J., Yoshida, O., Tsurusawa, M., Izumi, H., and Mori, K. J. (1983). Effects of reticuloendothelial system blockade on haemopoietic responses to irradiation of LPS non-responsive C3H/HeJ mice. *Cell Tissue Kinet.* **16**, 107-114.

Fukumura, D., Yonei, Y., Kurose, I., Saito, H., Ohishi, T., Higuchi, H., Miura, S., Kato, S., Kimura, H., Ebinuma, H., and Ishi, H. (1996). Role in nitric oxide in Kupffer cell-mediated hepatoma cell cytotoxicity in vitro and ex vivo. *Hepatology*. **24**, 141-149.

Gaillard, T., Mulsch, A., Busse, R., Klein, H., and Decker, K. (1991). Regulation of nitric oxide production by stimulated rat Kupffer cells. *Pathobiology*. **59**, 280-283.

Gallin, J. I. (1989). Inflammation. *Fundamental Immunology*, 2nd edition. (Paul, W. E., ed.) 721-733.

Galanos, C., Freudenberg, M, A., and Reutter, W. (1979). Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc. Natl. Acad. Sci., USA*. **76**, 5939-5943.

Ganey, P. E., Bailie, M. B., VanCise, S., Colligan, M. E., Madhukar, B. V., Robinson J. P., and Roth, R. A. (1994). Activated neutrophils from rat injured isolated hepatocytes. *Lab. Invest.* **70**, 53 - 60.

Ganey, P. E. , and Schultze, A. E. (1995). Depletion of neutrophils and modulation of Kupffer cell function in allyl alcohol-induced hepatotoxicity. *Toxicology*. **99**, 99-106.

Giger, U., Boxer, L. A., Simpson, P. J., Lucchesi, B. R., and Todd, R. F. 3d. (1987). Deficiency of leukocyte surface glycoproteins Mo1, LFA-1, and Leu M5 in a dog with recurrent bacterial infections: an animal model. *Blood*. **69**, 1622-1230.

Goldblum, S. E., Brann, T. W., Ding, X., Pugin, J., and Tobias, P. S. (1994). Lipopolysaccharide (LPS)-binding protein and soluble CD14 function as accessory molecules for LPS-induced changes in endothelial barrier function, *in vitro*. *J. Clin. Invest.* **93**, 692-702.

Golenbock, D. T., Bach, R. R., Lichenstein, H., Juan, T. S., Tadavarthy, A., and Moldow, C. F. (1995). Soluble CD14 promotes LPS activation of CD14-deficient PNH monocytes and endothelial cells. *J. Lab. Clin. Med.* **125**, 662-671.

Grewe, M., Gausling, R., Gyufko, K., Hoffmann, R., and Decker, K. (1994). Regulation of the mRNA expression for tumor necrosis factor-alpha in rat liver macrophages. *J. Hepatol.* **20**, 811-818.

Gumucio, J. J., Balabaud, C., Miller, D. L., DeMason, L. J., Appelman, H. D., Stoecker, T. J., and Franzblau, D. R. (1978). Bile secretion and liver cell heterogeneity in the rat. *J. Lab. Clin. Med.* **91**, 350-362.

Guzman, J. J., Marks, J. F., and Caren, L. D. (1991). *In vivo* and *in vitro* studies on the effects of vitamin A on the chemiluminescent response of murine peritoneal exudate cells. *Toxicol. Lett.* **57**, 125-137.

Haenen, G. R. M. M., Vermeulen, N. P. E., Tai Tin Tsoi, J. N. L. (1988). Activation of the microsomal glutathione-S-transferase and reduction of the glutathione dependent protection against lipid peroxidation by acrolein. *Biochem. Pharmacol.* **37**, 1933-1938.

Hagmann, W., Denzlinger, C., and Keppler, D. (1985). Production of peptide leukotrienes in endotoxic shock. *FEBS*. **180**, 309-313.

Hailman, E., Lichenstein, H. S., Wurfel, M. M., Miller, D. S., Johnson, D. A., Kelley, M., Busse, L. A., Zukowski, M. M., and Wright, S. D. (1994).

Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J. Exp. Med.* **179**, 269-77.

Han, J., Thompson, P., and Beutler, B. (1990). Dexamethasone and pentoxifylline inhibit endotoxin-induced cachectin/tumor necrosis factor synthesis at separate points in the signaling pathway. *J. Exper. Med.* **172**, 391-394.

Hansen, J., Cherwitz, D. L., and Allen, J. I. (1994). The role of tumor necrosis factor-alpha hepatotoxicity in ethanol-fed rats. *Hepatology.* **20**, 461-474.

Harbrecht, B. G., McClure, E. A., Simmons, R. L., and Billiar, T. R. (1995). Prostanoids inhibit Kupffer cell nitric oxide synthesis. *J. Surg. Res.* **58**, 625-629.

Haziot, A., Chen, S., Ferrero, E., Low, M. G., Silber, R., and Goyert, S. M. (1988). The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J. Immunol.* **141**, 547-552.

Haziot, A., Rong, G. W., Silver, J., and Goyert, S. M. (1993a). Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. *J. Immunol.* **151**, 1500-1507.

Haziot, A., Tsuberi, B. Z., and Goyert, S. M. (1993b). Neutrophil CD14: biochemical properties and role in the secretion of tumor necrosis factor-alpha in response to lipopolysaccharide. *J. Immunol.* **150**, 5556-5565.

Helle, M., Boeije, L., Pascual-Salcedo, D., and Aarden, L. (1991). Differential induction of interleukin-6 production by moncytes, endothelial cells and smooth muscle cells. (A. Sturk, J. H. van Deventer, J. Wouter ten Cate, H. R. Buller, L. G. Thijis and J. Levin, Eds.) pp. 43-54.

Hesse, D. G., Tracey, K. J., Fong, Y., Manogue, K. R., Palladino, M. A. Jr., Cerami, A., Shires, G. T., and Lowry, S. F. (1988). Cytokine appearance in human endotoxemia and primate bacteremia. *Surg. Gynecol. Obstet.* **166**, 147-153.

Heumann D., Gallay P., Barras C., Zaech P., Ulevitch R.J., Tobias P.S., Glauser M-P., and Baumgartner J. D. (1992). Control of lipopolysaccharide (LPS) binding and LPS-induced tumor necrosis factor

secretion in human peripheral blood monocytes. *J. Immunol.* **148**, 3505-3512.

Hewett, J. A., Schultze, VanCise, S., and Roth, R. A. (1992). Neutrophil depletion protects against liver injury from bacterial endotoxin. *Lab. Invest.* **66**, 347-361.

Hewett, J. A., Jean, P. J., Kunkel, S. L., and Roth, R. A. (1993). Relationship between tumor necrosis factor-a and neutrophils in endotoxin-induced liver injury. *Am. J. Physiol.* **265**, G1011-G1015.

Ho, J. S., Buchweitz, J. P., Roth, R. A., and Ganey, P. E. (1996). Identification of factors from rat neutrophils responsible for cytotoxicity to isolated hepatocytes. *J. Leukoc. Biol.* **59**, 716-724.

Hormann, V. A., Moore, D. R., and Rikans, L. E. (1989). Relative contributions of protein sulfhydryl loss and lipid peroxidation to allyl alcohol-induced cytotoxicity in Isolated rat hepatocytes. *Toxicol. Appl. Pharmacol.* **98**, 375-384.

Hirata, K., Kaneko, A., Ogawa, K., Hayasaka, H., and Onoe, T. (1980). Effect of endotoxin on rat liver. Analysis of acid phosphatase isozymes in the liver of normal and endotoxin-treated rats. *Lab. Invest.* **43**, 165-171.

Husztik, E., Lazar, G., Parducz, A. (1980). Electron microscopic study of Kupffer-cell phagocytosis blockade induced by gadolinium chloride. *Br. J. Exp. Pathol.* **61**, 624-630.

Iimuro, Y., Yamamoto, M., Kohno, H., Itakura, J., Fujii, K., and Matsumoto, Y. (1994). Blockade of liver macrophages by gadolinium chloride reduces lethality in endotoxemic rats -- analysis of mechanisms of lethality in endotoxemia. *J. Leukoc. Biol.* **55**, 723-728.

Jack, R. S., Grunwald, U., Stelter, F., Workalemhu, G., and Schutt, C. (1995). Both membrane-bound and soluble forms of CD14 bind to gram-negative bacteria. *Eur. J. Immunol.* **25**, 1436-1441.

Jaeschke, H., Kleinwaechter, C., and Wendel, A. (1987). The role of acrolein in allyl alcohol-induced lipid peroxidation and liver cell damage in mice. *Biochem. Pharmacol.* **36**, 51 -57.

Jaeschke, H., Farhood, A., and Smith C.W. (1991). Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. *Am. J. Physiol.* **261**, G1051-G1056.

Jaeschke, H., Kleinwaechter, C., and Wendel, A. (1992). NADH-dependent reductive stress and ferritin-bound iron in allyl alcohol-induced lipid peroxidation in vivo: the protective effect of vitamin E. *Chem.-Biol. Interactions*. **81**, 57-68.

Jean, P. E., Bailie, M. B., and Roth, R. A. (1995). 1-Naphthylisothiocyanate-induced elevation of biliary glutathione. *Biochem. Pharmocol.* **49**, 197-202.

Jirik, F. R., Podor, T. J., Hirano, T., Kishimoto, T., Loskutoff, D. J., Carson, D. A., and Lotz, M. (1989). Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. *J. Immunol.* **142**, 144-147.

Jones, E. A., and Summerfield, J. A. (1989). Kupffer cells. In: *The Liver Biology and Pathophysiology*, 2nd ed. (I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, and D. A. Shafritz, Eds), pp. 683-704. Raven Press, New York.

Joseph, C. K., Byun, H. S., Bittman, R., and Kolesnick, R. N. (1993). Substrate recognition by ceramide-activated protein kinase. *J. Biol. Chem.* **268**, 20002-20006.

Joseph, C. K., Wright S. D., Bornmann, W. G., Randolph, J. T., Kumar, E. R., Bittman, R., Liu, and Kolesnick, R. N. (1994). Bacterial lipopolysaccharide has structural similarity to ceramide and stimulates ceramide-activated protein kinase in myloid cells. *J. Biol. Chem.* **269**, 17606-17610.

Karck, U., Peters, T., and Decker, K. (1988). The release of tumor necrosis factor from endotoxin-stimulated rat Kupffer cells is regulated by prostaglandin E_2 and dexamethasone. *J. Hepatol.* **7**, 352-361.

Kawada, N., Ueda, N., Mizoguchi, Y., Kobayashi, K., Monna, T., Morisawa, S., Ishimura, K., Suzuki, T., and Yamamoto, S. (1992). Increased 5-lipoxygenase activity in massive hepatic cell necrosis in the rat correlates with neutrophil infiltration. *Hepatology*. **16**, 462-468.

Kaye, C. (1973). Biosynthesis of mercapturic acids from allyl alcohol, allyl esters and acrolein. *Biochem. J.* **134**, 1093-1101.

Keppler, D., Hagmann, W., and Rapp, S. (1987). Role of leukotrienes in endotoxin action *in vivo*. *Rev. Infect. Dis.* Suppl. **9**, S580-S584.

Kirkland, T. N., Finley, F., Leturcq, D., Moriarty, A., Lee, J. D., Ulevitch, R. J., and Tobias, P. S. (1993). Analysis of lipolysaccharide binding by CD14. *J. Biol. Chem.* **268**, 24818-24823.

Kispert, P. H. (1992). The role of Kupffer cells in control of acute pphase protein synthesis. In *Hepatocytes and Kupffer Cell Interactions.* (T. Billiar and R. D. Curran, Eds.) pp. 4-19. CRC Press, Boca Raton.

Klaunig, J. E., Goldblatt, P. J., Hinton, D. E., Lipsky, M. M., Chacko, J., and Trump, B. F. (1981). Mouse liver cell culture I. Hepatocyte isolation. *In Vitro.* **17**, 913-925.

Kolesnick, R. N., and Hemer, M. R. (1991). Characterization of a ceramide kinase activity from human leukemia (HL-60) cells. Separation from diacylglycerol kinase activity. *J. Biol. Chem.* **265**, 18803-18808.

Kolesnick, R., and Golde, D. W. (1994). The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell* **77**, 325-328.

te Koppele, J. M., Keller, B. J., Caldwell-Kenkel, J. C., Lemasters, J. J., and Thurman, R. G. (1991). Effect of hepatotoxic chemicals and hypoxia on hepatic nonparenchymal cells: impairment of phagocytosis by Kupffer cells and disruption of the endothelium in rats livers perfused with colloidal carbon. *Toxicol. Appl. Toxicol.* **110**, 20-30.

Kowalski, J., Kosmider, M., Pasnik, J., Zeman, K., Baj, Z., Janiszewska-Drobinska, B., and Czekalska, R. (1999). Pentoxifylline decreases neutrophil respiratory bursts in patients with stable angina. *Fundam. Clin. Pharmacol.* **13**, 237-242.

Krebs, H. A., Freeland, R. A., Hems, R., and Stubbs, M. (1969). Inhibition of hepatic gluconeogenesis by ethanol. *Biochem. J.* **112**, 117-124.

Kuiper, J., Zijlstra, F. J., Kamps, J. A., and van Berkel, T. J. (1988). Identification of prostaglandin D_2 as the major eicosanoid from liver endothelial and Kupffer cells. *Biochim. Biophys. Acta.* **959**, 143-52.

Kunkel, S. L., Chensue, S. W., and Phan, S. H. (1986). Prostaglandin as endogenous mediators od interleukin 1 production. *J. Immunol.* **136**, 186-192.

Kyle M. E., Serroni A., and Farber J. L. (1989). The inhibition of lipid peroxidation by disulfiram prevents the killing of cultured hepatocytes by allyl alcohol, tert-butyl hydroperoxide, hydrogen peroxide and diethyl maleate. *Chem.-Biol. Interactions.* **72**, 269-275.

Laskin, D. L., Heck, D. E., Gardner, C. R., Feder, L. S., and Laskin, J. D. (1994). Distinct patterns of nitric oxide production in hepatic macrophages and endothelial cells following acute exposure of rats to endotoxin. *J. Leukoc. Biol.* **56**, 751-758.

Lavnialkova, N., Burdelya, L., Lakhotia, A., Patel, N., Prokhorova, S., and Laskin, D. L. (1997). Macrophage and interleukin-1 induced nitric oxide production and cytostasis in hamster tumor cells varying in malignant potential. *J. Leukoc. Biol.* **61**, 452-458.

Liew, F.Y., Li, Y., and Millott, S. (1990a). Tumour necrosis factor (TNF-alpha) in leishmaniasis. II. TNF-alpha-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. *Immunology.* **71**, 556-559.

Liew, F. Y., Li, Y., and Millott, S. (1990b). Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide. *J. Immunol.* **145**, 4306-4310.

Lin, J. Y., and Chadee, K. (1992). Macrophage cytotoxicity against Entamoeba histolytica trophozoites is mediated by nitric oxide from L-arginine. *J. Immunol.* **148**, 3999-4005.

Liu, J., Mathias, S., Yang, Z., and Kolesnick, R. N. (1994). Renaturation and tumor necrosis factor-alpha stimulation of a 97-kDa ceramide-activated protein kinase. *J. Biol. Chem.* **269**, 3047-3052.

Levy, E., Path, F. C., and Ruebner, B. H. (1967). Hepatic changes produced by a single dose of endotoxin in the mouse. Light microscopy and histochemistry. *Am. J. Pathol.* **52**, 269-285.

Lind, R. C., Gandolfi, A. J., Sipes, G., and Brown, Jr. B. R. (1985). The involvement of endotoxin in halothane-associated liver injury. *Anesthesiology*. **61**, 544-550.

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* **15**, 323-350.

McCuskey, R. S., Urbaschek, R., McCuskey, P. A., and Urbaschek, B. (1982). *In vivo* microscopic studies of the responses of the liver to endotoxin. *Klin. Woehenschr.* **60**, 749-751.

McIntyre, K. W., Stepan, G. J., Kolinsky, K. D., Benjamin, W. R., Plocinski, J. M., Kaffka, K. L., Campen, C. A., Chizzonite, R. A., and Kilian, P. L. (1991). Inhibition of interleukin 1 (IL-1) binding and bioactivity *in vitro* and modulation of acute inflammation *in vivo* by IL-1 receptor antagonist and anti-IL-1 receptor monoclonal antibody. *J. Exp. Med.* **174**, 931-939.

Maellaro, E., Casini, A. F., Del Bello, B., and Comporti, M. (1990). Lipid peroxidation and antioxidant systems in the liver injury produced by glutathione depleting agents. *Biochem. Pharmacol.* **39**, 1513-1521.

Maellaro, E., Del Bello, B., Sugherini, L., Pompella, A., Casini, A. F., and Comporti, M. (1994). Protection by ascorbic acid against oxidative injury of isolated hepatocytes. *Xenobiotica.* **24**, 281-289.

Marshall, J. C., Lee, C., Meakins, J. L., Michel, R. P., and Christou, N. V. (1987) Kupffer cell modulation of the systemic immune response. *Arch. Surg.* **122**, 191-196.

Mathias, S., Dressler, K. A., and Kolesnick, R. N. (1991). Characterization of a ceramide-activated protein kinase: stimulation by tumor necrosis factor alpha. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10009-10013.

Mathias, S., Younes, A., Kan, C. C., Orlow, I., Joseph, C., and Kolesnick, R. N. (1993). Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1 beta. *Science.* **259**, 519-522.

Mathison, J. C., Tobias, P. S., Wolfson, E., and Ulevitch, R. J. (1992). Plasma lipopolysaccharide (LPS)-binding protein. A key component in macrophage recognition of gram-negative LPS. *J. Immunol.* **149**, 200-206.

Matuschak, G. M., Pinsky, M. R., Klein, E. C., van Thiel, D. H., and Rinaldo, J. E. (1990). Effects of D-galactosamine-induced acute liver injury on mortality and pulmonary responses to Escherichia coli lipopolysaccharide. Modulation by arachidonic acid metabolites. (1990) *Am. Rev. Resp. Dis.* **141**, 1296-1306.

Mayer, A. M. S. and Spitzer, J. A. (1991). Continuous infusion of *Escherichia coli* endotoxin *in vivo* primes *in vitro* superoxide anion release in rat polymorphonuclear leukocytes and Kupffer cells in a time-dependent manner. *Infec. Immun.* **59**, 4590-4598.

van der Meer, J. W. M., and Vogels, M. T. E. (1991). Interleukin-1 as a therapeutic agent in serious infections. In *Progress in Clinical and Biological Research*, (A. Sturk, J. H. van Deventer, J. Wouter ten Cate, L. G. Thijis, and J. Levin, Eds.), Vol. 367, pp. 25-42. Wiley-Liss, Inc., New York.

Miccadei, S., Nakae, D., Kyle M., Gilfor, D., and Farber, J. L. (1988) Oxidative cell injury in the killing of cultured hepatocytes by allyl alcohol. *Arch. Biochem. Biophys.* **265**, 302-310.

Michie, H. R., Manogue, K. R., Spriggs, D. R., Revhaug, A., O'Dwyer, S., Dinarello, C. A., Cerami, A., Wolff, S. M., and Wilmore, D. W. (1988). Detection of circulating tumor necrosis factor after endotoxin administration. *Science.* **318**, 1481-1486.

Miyazaki, H., Broaddus, V. C., Wiener-Kronish, J. P., Sawa, T., Pittet, J. F., Kravchenko, V., Mathison, J. C., Nishizawa, H., Hattori, S., Yamakawa, T., Yamada, H., and Kudoh, I. (1999). The effects of two antiinflammatory pretreatments on bacterial-induced lung injury. *Anesthsiology.* **90**, 1650-1662.

Moore, R. N., Oppenheim, J. J., Farrar, J. J., Carter Jr., C. S., Waheed, A., and Shadduck, R. K. (1980). Production of lymphocyte-activating factor (interleukin-1) by macrophages activated with colony-stimulating factors. *J. Immunol.* **125**, 1302-1305.

Moriguchi, S., Werner, L., and Watson, R. R. (1985). High dietary vitamin A (retinyl palmitate) and cellular immune functions in mice. *Immunology* **56**, 169-177.

Morrison, D. C., and Ulevitch, R. J. (1978). The effects of bacterial endotoxins on host mediation systems. *Am. J. Pathol.* **93**, 526-617.

Moulin, F., Pearson, J. M., Schultze, A. E., Scott, M. A., Schwartz, K. A., Davis, J. M., Ganey, P. E., and Roth, R. A. (1996). Thrombin is a distal mediator of lipoplysaccharide-induced liver injury in the rat. *J. Surg. Res.* **65**, 149-158.

Movat, H. Z., Cybulsky, M. I., Colditz, I. G., Chan, M. K., and Dinarello, C. A. (1987). Acute inflammation in gram-negative infection: endotoxin, interleukin 1, tumor necrosis factor, and neutrophils. *Fed. Proc.* **46**, 97-104.

Nagai, H., Shimazawa, T., Yakuo, I., Aoki, M., Koda, A., and Kasahara, M. (1989). Role of peptide-leukotrienes in liver injury in mice. *Inflammation* **13**, 673-680.

Nakao, A., Taki, S., Yasui, M., Kimura, Y., Nonami, T., Harada, A., and Takagi, H. (1994). The fate of intravenously injected endotoxin in normal rats and in rats with liver failure. *Hepatology.* **19**, 1251-1256.

Neta, R., Douches, S., and Oppenheim, J. J. (1986a). Interleukin 1 is a radioprotector. *J. Immunol.* **136**, 2483-2485.

Neta, R., Vogel, S. N., Oppenheim, J. J., and Douches, S. D. (1986b). Cytokines in radioprotection. Comparison of the radioprotective effects of IL-1 to IL-2, GM-CSF and IFN gamma. *Lymphokine Res.* **Suppl 1**, S105-110.

Noel, P., Nelson, S., Bokulic, R., Bagby, G., Lippton, H., Lipscomb, G., and Summer, W. (1990). Pentoxifylline inhibits lipopolysaccharide-induced serum tumor necrosis factor and mortality. *Life Sci.* **47**, 1023-1029.

Nolan, J. P. The role of endotoxin in liver injury. (1975). *Gastroenterology.* **69**, 1346-1356.

Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N., and Levitzki, A. (1994). Prevention of lipopolysaccharide-induced toxicity by tyrosine kinase inhibitors. *Science* **264**, 1319-1322.

O'Brien, A. D., Rosenstreich, D. L., Scher, I., Campbell, G. H., MacDermott, R. P., and Formal, S. B. (1980). Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. *J. Immunol.* **124**, 20-24.

Ohno, Y., Ormstad, K., Ross, D., and Orrenius, S. (1985). Mechanism of allyl alcohol toxicity and protective effects of low-molecular weight thiols studied with rat hepatocytes. *Toxicol. Appl. Pharmacol.* **78**, 169 -179.

Okumura, Y., Ishibashi, H., Shirahama, M., Kurokawa, S., Kudo, J., Okubo, H., and Niho, Y. (1987). Kupffer cells modulate natural killer cell activity *in vitro* by producing prostaglandins. *Cell. Immunol.* **107**, 89-98.

Patarroyo M., and Makgoba, M.W. (1989). Leucocyte adhesion to cells in immune and inflammatory responses. *Lancet* **2**, 1139-1142.

Patel, J. M., Wood, J. C., and Leibman, K. C. (1980). The biotransformation of allyl alcohol and acrolein in rat liver and lung preparations. *Drug Metabol. Dispos.* **8**, 305-308.

Paul, N. L. and Ruddle NH. (1988). Lymphotoxin. *Annu. Rev. Immunol.* **6**, 407-438.

Pearson, J. M., Brown, A. P., Ganey, P. E. and Roth, R. A. (1996). Gadolinium chloride treatment attenuates hepatic platelet accumulation after lipopolysaccharide administration. *Shock* **5**, 408-415.

Pearson, J. M., Bailie, M. B., Fink, G. D., and Roth, R. A. (1997). Neither platelet activating factor nor leukotrienes are critical mediators of liver injury after lipopolysaccharide administration. *Toxicology.* **121**, 181-189.

Pearson, J. M., Schultze, A.E., Jean, P.A., and Roth, R.A. (1995). Platelet participation in liver injury from gram-negative bacterial lipopolysaccharide in the rat. *Shock* **4**, 178-186.

Penttilä, K. (1987). Allyl alcohol cytotoxicity and glutathione depletion in isolated periportal and perivenous rat hepatocytes. *Chem. Biol. Interactions.* **65**, 107-121.

Peters, T., Karck, U., and Decker, K. (1990). Interdependence of tumor necrosis factor, prostaglandin E_2, and protein synthesis in lipopolysaccharide-exposed rat Kupffer cells. *Eur. J. Biochem.* **191**, 583-589.

Pompella, A., Romani, A., Fulceri, R., Benedetti, A., and Comporti, M. (1988). 4-Hydroxynonenal and other lipid peroxidation products are formed in mouse liver following intoxication with allyl alcohol. *Biochim. Biophys. Acta* **961**, 293-298.

Pompella, A., Romani, A., Benedetti, A., and Comporti, M. (1991). Loss of membrane protein thiols and lipid peroxidation in allyl alcohol hepatotoxicity. *Biochem. Pharmacol.* **41**, 1255-1259.

Portoles, M. T., Arahuetes, R. M., and Pagani, R. (1994). Intracellular calcium alterations and free radical formation evaluated by flow cytometry in endotoxin-treated rat liver Kupffer and endothelial cells. *Eur. J. Cell Biol.* **65**, 200-205.

Pugin, J., Schurer-Maly, C. C., Leturcq, D., Moriarty, A., Ulevitch, R. J., and Tobias, P. S. (1993). Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2744-2748.

Przybocki, J. M., Reuhl, K. R., Thurman, R. G., and Kauffman, F. C. (1992). Involvement of nonparenchymal cells in oxygen-dependent hepatic injury by allyl alcohol. *Toxicol. Appl. Pharmacol.* **5**, 57-63.

Raetz, C. R. H., Brozek, K. A., Clementz, T., Coleman, J. D., Galloway, S. M., Golenbock, D. T., and Hampton, R. Y. (1988). Gram-negative endotoxin: a biologically active lipid. *Cold Spring Harbor Symposia on Quantitative Biology*. **53**, 973-982.

Raetz, C. R. H., Ulevitch, R. J., Wright, S. D., Sibley, C. H., Ding, A., and Nathan, C. F. (1991). Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* **5**, 2652-2660.

Raines, M. A., Kolesnick, R. N., and Golde, D. W. (1993). Sphingomyelinase and ceramide activate mitogen-activated protein kinase in myeloid HL-60 cells. *J. Biol. Chem.* **268**, 14572-14575.

Ramadori, G., Meyer zum Buschenfelde, K. H., Tobias, P. S., Mathison, J. C., and Ulevitch, R. J. (1990). Biosynthesis of lipopolysaccharide-binding protein in rabbit hepatocytes. *Pathobiology*. **58**, 89-94.

Reid, W. D. (1972). Mechanism of allyl alcohol-induced hepatic necrosis. *Experimentia*. **28**, 1058-1061.

Reuter, B. K. and Wallace, J. L. (1999). Phosphodiesterase inhibitors prevent NSAID enteropathy independently of effects on TNF-alpha release. *Am. J. Physiol.* **277**, G847-G854.

Rietschel, E. T., Kirkae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zahringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994). Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* **8**, 217-225.

Rikans, L. E. (1987). The oxidation of acrolein by rat liver aldehyde dehydrogenases. Relation to allyl alcohol hepatotoxicity. *Drug Metab. Dispos.* **15**, 356-362.

Rikans, L. E., and Kosanke, S. D. (1984). The effect of glutathione levels and hepatocellular injury from carbon tetrachloride, allyl alcohol, or galactosamine. *Drug Chem. Toxicol.* **7**, 595-604.

Rikans, L. E., and Hornbrock, K. R. (1986). Isolated hepatocytes as a model for aging effects on hepatotoxicity: studies with allyl alcohol. *Toxicol. Appl. Pharmacol.* **84**, 634-639.

Rikans, L. E., and Moore, D. R. (1987). Effect of age and sex on allyl alcohol hepatotoxicity in rats: role of liver alcohol and aldehyde dehydrogenase activities. *J. Pharmacol. Exper. Ther.* **243**, 20-26.

Rikans, L. E., and Cai, Y. (1994). Dithiothreitol reversal of allyl alcohol cytotoxicity in isolated rat hepatocytes. *Toxicology.* **86**, 147-161.

Rippe, D. F., and Berry, L. J. (1972). Study of inhibition of induction of phosphoenolpyruvate carboxylase by endotoxin with radial immunodiffusion. *Infect. Immun.* **6**, 766-772.

Rodriguez de Turco, E. B. and Spitzer, J. A. (1990). Eicosanoid production of nonparenchymal liver cells isolated from rats infused with *E. coli* endotoxin. *J. Leukoc. Biol.* **48**, 488-494.

Roland, C. R., Mangino, M. J., Duffy, B. F., and Flye, M. W. (1993). Lymphocyte suppression by Kupffer cells prevents portal venous tolerance induction: a study of macrophage function after intravenous gadolinium. *Transplantation.* **55**, 1151-1158.

Rosengren, R. J., Sauer, J.-M., Hooser, S. B. and Sipes, I, G. (1995) The interactions between retinol and five different hepatotoxicants in the Swiss Webster mouse. *Fundam. Appl. Toxicol.* **25**, 281-292.

Saito, H., Kurose, I., Ebinuma, H., Fukumura, D., Higuchi, H., Atsukawa, K., Tada, S., Kimura, H., Yonei, Y., Masuda, T., Miura, S.,

and Ishii, H. (1996). Kupffer cell-mediated cytotoxicity against hepatoma cells occurs through production of nitric oxide and adhesion via ICAM-1/CD18. *Int. Immunol.* **8**, 1165-1172.

Sasse, D. and Maly I. P. (1991). Studies on the periportal hepatotoxicity of allyl alcohol. *Prog. Histochem. Cytochem.* **23**, 146-149.

Sato, T., Shinzawa, H., Abe, Y., Takahashi, T., Arai, S., and Sendo, F. (1993). Inhibition of *Corynebacterium parvum*-primed and lipopolysaccharide-induced hepatic necrosis in rats by selective depletion of neutrophils using a monoclonal antibody. *J. Leukoc. Biol.* **53**, 144-150.

Sato, T., Yamamoto, H., Sasaki, C., and Wake, K. (1998). Maturation of rat dendritic cells during intrahepatic translocation evaluated using monoclonal antibodies and electron microscopy. *Cell Tissue Res.* **294**, 503-514.

Sauer, J. M., Waalkes, M. P., Hooser, S. B., Kuester, R. K., McQueen CA, and Sipes IG (1997). Suppression of Kupffer cell function prevents cadmium induced hepatocellular necrosis in the male Sprague-Dawley rat. *Toxicology* **121**, 155-164.

Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch, R. J. (1990). Structure and function of lipopolysaccharide binding protein. *Science.* **249**, 1429-1431.

Seglen, P. O. (1973). Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. *Exper. Cell Res.* **82**, 391-398.

Semmler, J., Gebert, U., Eisenhut, T., Moeller, J., Schonharting, M. M., Allera, A., and Endres, S. (1993). Xanthine derivatives: comparison between suppression of tumour necrosis factor-a production and inhibition of cAMP phosphodiesterase activity. *Immunology.* **78**, 520-525.

Serafini-Cessi, F. (1972). Conversion of allyl alcohol to acrolein by rat liver. *Biochem. J.* **128**, 1103-1107.

Sieg, D. J. and Billings, R. E. (1997). Lead/cytokine-mediated oxidative DNA damage in cultured mouse hepatocytes. *Toxicol. Appl. Pharmacol.* **142**, 106-115.

Siegel, J. H., Goodzari, S., Guadelupi, P., Coleman, W. P., Malcolm, D., Blevins, S., Frankenfield, D., Badellino, M. C., Boetker, T., and Duek, S. D.

(1993). The host defense to trauma and sepsis: multiple organ failure as a manifestation of host defense failure disease. In: Pathophysiology of Shock, Sepsis and Organ Failure. (H. Schlag and H. Redl, Eds) pp. 626-664.

Simmons, D. L., Tan, S., Tenen, D. G., Nicholson-Weller, A., and Seed, B. (1989). Monocyte antigen CD14 is a phospholipid anchored membrane protein. *Blood.* **73**, 284-289.

Shibayama, Y. (1987). Sinusoidal circulatory disturbance by microthrombosis as a cause of endotoxin-induced hepatic injury. *J. Pathol.* **151**, 315-321.

Shiratori, Y., Kawase, T., Shiina, S., Okano, K., Sugimoto, T., Teraoka, H., Matano, S., Matsumoto, K., and Kamii, K. (1988). Modulation of hepatotoxicity by macrophages in the liver. *Hepatology.* **8**, 815-821.

Silva, J. M., and O'Brien, P. J. (1989). Allyl alcohol- and acrolein-induced toxicity in isolated rat hepatocytes. *Arch. Biochem. Biophys.* **275**, 551-588.

Sleyster, E. C., and Knook D. L. (1982). Relation between localization and function of rat liver Kupffer cells. *Lab. Invest.* **47**, 484-90

Sneed, R. A., Grimes, S. D., Schultze, A. E., Brown, A. P., and Ganey, P. E. (1997). Bacterial endotoxin enhances the hepatotoxicity of allyl alcohol. *Toxicol. Appl. Pharmacol.* **144**, 77-87.

Spolarics, Z., Bautista, A.P., and Spitzer, J.J. (1993). Primed pentose activity supports production and elimination of superoxide anion in Kupffer cells from rats treated with endotoxin *in vivo*. *Biochim. Biophys. Acta* **1179**, 134-140.

*Strubelt, O., Younes, M., and Pentz, R. (1986). Influence of extracellular calcium on allyl alcohol-induced hepatotoxicity. *Acta Pharmacol. et Toxicol.* **59**, 47-52.

Sveinbjornsson, B., Olsen, R., Seternes, O. M., and Seljelid, R. (1996). Macrophage cytotoxicity against murine meth A sarcoma involves nitric oxide-mediated apoptosis. *Biochem. Biophys. Res. Commun.* **223**, 643-649.

Tachibana, K., Sone, S., Tsubura, E., and Kishino, Y. (1984). Stimulatory effect of vitamin A on tumoricidal activity of rat alveolar macrophages. *Br. J. Cancer.* **49**, 343-348.

Taffet, S. M., Singhel, K. J., Overholtzer, J. F., and Shurtleff, S. A. (1989). Regulation of tumor necrosis factor expression in a macrophage-like cell line by lipopolysaccharide and cyclic AMP. *Cell. Immunol.* **120**, 291-300.

Tai, J. H., and Pestka, J. J. (1988). Synergistic interaction between the trichothecece T-2 toxin and *Salmonella typhimurium* lipopolysaccharide in C3H/HeN and C3H/Hej mice. *Toxicol Lett.* **44**, 191-200.

Thorgeirsson, S. S., Mitchell, J. R., Sasame, H. A., and Potter, W. Z. (1976). Biochemical changes after hepatic injury by allyl alcohol and *n*-hydroxy-2-acetylaminofluorene. *Chem.-Biol. Interactions.* **15**, 139-147.

Tiegs, G. and Wendel, A. (1988). Leukotriene-mediated liver injury. *Biochem. Pharmacol.* **37**, 2569-2573.

Tobias, P. S., Soldau, K., and Ulevitch, R. J. (1986). Isolation of a lipopolysaccharide-binding acute phase reactant from rabbit serum. *J. Exp. Med.* **164**, 777-793.

Tobias, P. S., Mathison, J. C., and Ulevitch, R. J. (1988). A family of lipopolysaccharide binding proteins involved in responses to gram negative sepsis. *J. Biol. Chem.* **263**, 13479-13481.

Tobias, P. S., Soldau, K., and Ulevitch, R. J. (1989). Identification of lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. *J. Biol. Chem.* **264**, 10867-10871.

Tobias, P. S., Soldua, K., Gegner, J. A., Mintz, D., and Ulevitch, R. J. (1995). Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. *J. Biol. Chem.* **270**, 10482-10488.

Toth, C. A., and Thomas, P. (1992). Liver endocytosis and Kupffer cells. *Hepatology.* **16**, 255-266.

Tracey, K. J., Fong, Y., Hesse, D. G., Manogue, K. R., Lee, A. T., Kuo, G. C., Lowry, S. F., and Cerami, A. (1987). Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature.* **330**, 662 -664.

Tracey, K. J., Wei, H., Manogue, K. R., Fong, Y., Hess D. G., Nguyen, H. T., Kuo, G. C., Beutler, B., Cotran, R. S., and Creami, A. (1988). Cachectin/tumor necrosis factor induces cachexia, anemia, and inflammation. *J. Exp. Med.* **167**, 1211-1127.

Tracey, K. J. and Cerami, A. (1993). Tumor necrosis factor, other cytokines and disease. *Annu. Rev. Cell Biol.* **9**, 317-343.

Tracey, K. J. and Cerami, A. (1994). Tumor necrosis factor: a pleiotropic cytokine and therapuetic target. *Annu. Rev. Med.* **45**, 491-503.

Unanue, E. R. (1989). Macrophages, antigen-presenting cells, and the phenomena of antigen handling and presentation. *Fundamental Immunology*, 2nd edition. (Paul. W. E., Ed.) 95-115.

Utili, R., Abernathy, C. O., and Zimmerman, H. J. (1977). Endotoxin and the liver. *Life Sci.* **20**, 553-566.

Van Bossuyt, H., Desmaretz, C., Rombaut, B., and Wisse, E. (1988). Response of cultured rat Kupffer cells to lipopolysaccharide. *Arch. Toxicol.* **62**, 316-324.

Van Bossuyt, H., and Wisse, E. (1988). Structural changes produced in Kupffer cells in the rat liver by injection of lipopolysaccharide. *Cell Tissue Res.* **251**, 205-214.

Vogel, S. N., and Hogan, M. M. (1990). Role of cytokines in endotoxin-mediated host responses. In *Immunophysiology*. (Oppenheim, J. J., and Shevach, E. M., Eds) pp. 238-258.

Vouldoukis, I., Becherel, P. A., Riveros-Moreno, V., Arock, M., da Silva, O., Debre, P., Mazier, D., and Mossalayi, M. D. (1997). Interleukin-10 and interleukin-4 inhibit intracellular killing of *Leishmania infantum* and *Leishmania major* by human macrophages by decreasing nitric oxide generation. *Eur. J. Immunol.* **27**, 860-865.

Wang, J. H., Redmond, H. P., Watson, R. W., and Bouchier-Hayes, D. (1995). Role of lipopolysaccharide and tumor necrosis factor-alpha in induction of hepatocyte necrosis. *Am. J. Physiol.* **269**, G297-304.

Ward, A. and Clissold, S. P. (1987). Pentoxifylline. A review of its pharmocodynamic and pharmacokinetic properties and its therapeutic efficacy. *Drugs.* **34**, 50-97.

Watson, R. W. G., Redmond, H .P., and Bouchier-Hayes, D. (1994). Role of endotoxin in mononuclear phagocyte inflammatory responses. *J. Leukoc. Biol.* **56**, 95-103.

Weinstein, S. L., Gold, M. R., and DeFranco, A. L. (1991). Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. *Proc. Natl. Acad. Sci. USA.* **88**, 4148-4152.

Weinstein, S. L., Sanghera, J. S., Lemke, K., DeFranco, A. L., and Pelech, S. L. (1992). Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J.Biol. Chem.* **267**, 14955-14962.

West, M. A., Keller, G. A., Hyland, B. J., Cerra, F. B., and Simmons, R. L. (1985). Hepatocyte function in sepsis: Kupffer cell mediate a biphasic protein synthesis response in hepatocytes after exposure to endotoxin or killed *Escherichia coli. Surgery.* **98**, 388-395.

West, M. A., Keller, G. A., Hyland, B. J., Cerra, F. B., and Simmons, R. L. (1986). Further characterization of Kupffer cell/macrophage-mediated alterations in hepatocyte protein synthesis. *Surgery.* **100**, 416-422.

West, M. A., Billiar, T. R., Hyland, B. J., Jordan, M. L., Simmons, R. L. (1987). Regulation of Kupffer cell-mediated alterations in hepatocyte protein synthesis *in vitro. Curr. Surgery.* **44**, 467-469.

West, M. A., Billiar, T. R., Mazuski, J. E., Curran, R. J., Cerra, F. B., and Simmons, R. L. (1988.) Endotoxin modulation of hepatocyte secretory and cellular protein synthesis is mediated by Kupffer cells. *Arch. Surg.* **123**, 1400-1405.

Wilson, R. A., and Hart, J. R. (1981). *In vivo* drug metabolism and liver lobule heterogeneity in the rat. *Gastroenterology.* **81**, 563-569.

Wisse, E., Braet, F., Luo, D., de Zanger, R., Jans, D., Crabbe, E., and Vermoesen, A. (1996). Structure and function of sinusoidal lining cells in the liver. *Toxicol. Pathol.* **24**, 100-111.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science.* **249**, 1431-1433.

Wright, S. D., and Kolesnick, R. N. (1995). Does endotoxin stimulate cells by mimicking ceramide? *Immunology Today*. **16**, 297-302.

Wu, C. C., Liao, M. H., Chen, S. J., and Yen, M. H. (1999). Pentoxifylline improves circulatory failure and survival in murine models of endotoxaemia. *Eur. J. Pharmacol.* **373**, 41-49.

Xu, H., Gonzalo, J. A., St. Pierre, Y., Williams, I. R., Kupper, T. S., Cotran, R. S., Springer, T. A., and Gutierrez-Ramos, J. C. (1994). Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. *J. Exp. Med.* **180**, 95-109.

Yamate, J., Kumagai, D., Tsujino, K., Nakatsuji, S., Kuwamura, M., Kotani, T., Sakuma, S., and LaMarre, J. (1999). Macrophage populations and apoptotic cells in the liver before spontaneous hepatitis in Long-Evans cinnamon (LEC) rats. *J. Comp. Pathol.* **120**, 333-346.

Yamauchi, M., Potter, J. J., and, Mezey, E. (1988). Lobular distribution of alcohol dehydrogenase in the rat liver. *Hepatology*. **8**, 243-247.

Yang, Z., Costanzo, M., Golde, D. W., and Kolesnick, R. N. (1993). Tumor necrosis factor activation of the sphingomyelin pathway signals nuclear factor kappa B translocation in intact HL-60 cells. *J. Biol. Chem.* **268**, 20520-20523.

Yang, S., Zhou, M., Koo, D. J., Chaudry, I. H., and Wang, P. (1999). Pentoxifylline prevents the transition from the hyperdynamic to hypodynamic response during sepsis. *Am. J. Physiol.* **277**, H1036-H1044.

Zabel, P., Wolter, D. T., Schonharting, M. M., and Schade, U. F. (1989). Oxpentifylline in endotoxaemia. *Lancet.* **2**, 1474- 1477.

Zabel, P., Schonhartig, M. M., Schade, U. F., and Schlaak, M. (1991). Effects of pentoxifylline in endotoxiemia in human volunteers. In *Progress in Clinical and Biological Research* (A. Sturk, J. H. van Deventer, J. Wouter ten Cate, H. R. Buller, L. G. Thijis, and J. Levin, Eds.), Vol. 367, pp. 207-213. Wiley-Liss, New York.

Zabel, P., Schade, F. U., and Schlaak, M. (1993). Inhibition of endogenous TNF formation by pentoxifylline. *Immunobiology*. **187**, 447-463.

Zentella, A., Manogue, K., and Cerami, A. (1991). The role of cachectin/TNF and other cytokines in sepsis. In *Progress in Clinical and Biological Research* Vol 367. (A. Sturk, J. H. van Deventer, J. Wouter ten Cate, H. R. Buller, L. G. Thijis and J. Levin, Eds.) pp. 43-54.

Zieve, L., Anderson, W. R., Lyftogt, C., and Draves, K. (1986). Hepatic regenerative enzyme activity after pericentral and periportal lobular toxic injury. *Toxicol. Appl. Pharmacol.* **86**, 147-158.