MEDIATORS OF INFLAMMATION AND α-NAPHTHYLISOTHIOCYANATE HEPATOTOXICITY

by

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ABSTRACT

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α-Naphthylisothiocyanate (ANIT) is an experimental cholangiolitic hepatotoxicant that causes cholestasis, periportal edema, and biliary epithelial cell and hepatic parenchymal cell necrosis in rats. Since neutrophils and glutathione are requisite for the expression of ANIT-induced liver injury, the role of leukotrienes (LTs) in this model was assessed. Rats were treated with LT biosynthesis inhibitors (A63162, Zileuton, MK-886) or the gamma-glutamyltranferase inhibitor AT-125, and ANIT-induced liver injury was evaluated. Treatment with A63162 or AT-125 afforded partial protection from ANIT hepatotoxicity while treatment with Zileuton or MK-886 did not attenuate hepatotoxicity. Since Zileuton and MK-886 inhibited LT biosynthesis without affording protection, the weight of the evidence suggests that leukotrienes are not requisite in ANIT hepatotoxicity. Since platelets can contribute to tissue injury, the role of platelets or platelet-derived prostanoids was assessed in ANIT toxicity. Treatment of rats with antiplatelet serum effectively reduced circulating platelet numbers and attenuated ANIT-induced increases in serum bilirubin concentration and ALT activity, while bile flow and serum GGT activity remained unaffected. Inhibitors of prostaglandin biosynthesis did not ameliorate ANIT hepatotoxicity. Platelet-activating factor (PAF) is a mediator that contributes

to inflammatory injury. The role of PAF in ANIT-induced liver injury was determined by treating rats with a PAF receptor antagonist, alone or in combination with Zileuton. Neither of these treatment paradigms altered ANIT hepatotoxicity. Since macrophages may contribute to hepatotoxicity, their role in ANIT toxicity was determined. Enhancement of hepatic macrophage number or function with zymosan or vitamin A attenuated ANIT hepatotoxicity, while inhibition of macrophage function had no effect. Vitamin A treatment decreased the concentration of ANIT in bile and may afford protection through this action. Zymosan protection may relate to decreased circulating neutrophil numbers or function. These results suggest that: 1) LTs are not required in ANIT injury; 2) platelets contribute partially to ANIT hepatotoxicity, but not through prostanoid production; 3) PAF, alone or with LTs, does not contribute to ANIT hepatotoxicity; 4) macrophages are not required for ANIT hepatotoxicity; 5) zymosan or vitamin A attenuates the effects of ANIT. These findings delineate further the mechanism(s) of ANIT-induced liver injury.

In memory of my mother

Elaine Taub Bailie

and for my wife, son and father

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
ALT	Alanine Aminotransferase
ANIT	Alpha-Naphthylisothiocyanate
APAP	Acetaminophen
ASA	Aspirin
BNIT	Beta-Naphthylisothiocyanate
CAb	Control Serum
CCl_4	Carbon Tetrachloride
CO	Corn Oil
CO_2	Carbon Dioxide
DMSO	Dimethylsulfoxide
EIA	Enzyme Immunoassay
FMLP	N-Formyl-Methionyl-Leucyl-Phenylalanine
$GdCl_3$	Gadolinium Chloride
GGT	Gamma-Glutamyltransferase
GSH	Glutathione
GS-ANIT	Glutathione-α-Naphthylisothiocyanate Conjugate
5-HPETE	5-Hydroperoxyeicosatetraenoic Acid
12-HPETE	12-Hydroperoxyeicosatetraenoic Acid
HPLC	High-Performance Liquid Chromatography
IB	Ibuprofen
LTs	Leukotrienes
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
MPS	Mononuclear-Phagocyte System
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
NADPH	Reduced form of Nicotinamide-Adenine Dinucleotide Phosphate
PAb	Anti-Platelet Serum
PAF	Platelet-Activating Factor
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGF_2	Prostaglandin F_2
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2

List of Abbreviations (cont.)

PGI_2	Prostaglandin I_2
PLA_2	Phospholipase A_2
PMA	Phorbol-Myristate-Acetate
PMN	Polymorphonuclear Leukocyte
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
SAL	Saline
TNF-α	Tumor Necrosis Factor-Alpha
TxA_2	Thromboxane A_2
VEH	Vehicle
ZIL	Zileuton

CHAPTER I

INTRODUCTION

REACTION OF LIVER TO HEPATOTOXICANTS

Liver injury is a consequence of exposure to a variety of agents including chemicals, drugs, bacteria and bacterial products. The hepatic insult caused by xenobiotics is dependent on inherent properties of the agent and the species used to evaluate the response. The hepatic response to toxic compounds is often categorized by the region of liver affected (ie, centrilobular, midzonal or periportal), or as "drug induced" versus "toxic hepatitis". Whereas the former is a description of the histologic distribution of the lesion within the hepatic architecture, the latter relates to specific characteristics of the expression of liver injury. Generally, the description of "toxic hepatitis" refers to agents which cause an hepatic lesion with distinct morphologic distribution within the hepatic parenchyma. Additionally, this type of liver injury has an identifiable latent period, is dose related, occurs in all individuals exposed, and is reproducible in animal models (Klatskin, 1969).

In contrast, agents which produce "drug induced hepatitis" tend to lack an exact zonal pattern of lesions, a fixed dose related effect or specific latent period. In addition, not all exposed individuals express liver injury and the toxicity is, in general, not reproducible in animal models (Klatskin, 1969). These characteristics make agents which cause "drug-induced hepatitis" difficult to study.

Compounds which cause "drug-induced" liver injury are often further subdivided by the type of hepatic insult expressed after exposure to a given agent. Halothane

is an inhalation anesthetic which causes cytotoxic hepatotoxicity. This injury is characterized by a marked hepatic parenchymal necrosis with limited involvement of biliary epithelium or cholestasis. Evidence suggests that the severe form of this liver injury may be immunologic in origin (Vergani et al., 1980; Davis et al., 1981).

In contrast, erythromycin estolate and chlorpromazine are compounds which cause a cholangiolitic hepatitis. This form of "drug-induced" hepatitis is characterized by biliary epithelial insult, cessation of bile flow (ie cholestasis), and limited hepatic parenchymal necrosis (Zimmerman, 1978). Whereas hepatic parenchymal necrosis predominates in halothane-induced liver injury, agents which cause cholangiolitic hepatitis primarily produce biliary epithelial cell insult. The mechanism(s) of toxicity for agents which cause cholangiolitic "drug-induced" hepatitis are poorly understood.

α-Naphthylisothiocyanate (ANIT) is an experimental hepatotoxicant that causes a dose-dependent hepatotoxicity with a predictable latent period and which is reproducible in numerous animal models (Indacochea-Redmond and Plaa, 1971; Capizzo and Roberts, 1971). Histologically, the hepatic insult caused by ANIT is reminiscent of a cholangiolitic "drug-induced hepatitis". Because of this similarity, ANIT has gained interest as a model for agents which cause "drug-induced" hepatotoxicities characterized by cholestasis, biliary epithelial injury and mild hepatic parenchymal insult. Furthermore, a number of developmental compounds from various pharmaceutical companies have demonstrated hepatotoxic effects not

unlike those seen after ANIT administration (personal communications). Thus, a renewed interest in ANIT and its mechanism(s) of toxicity has occurred. As a model hepatotoxicant, ANIT may prove to be a useful compound for determining mechanisms of toxicity of other cholangiolitic compounds.

INFLAMMATION AS A CONTRIBUTOR TO TISSUE INJURY

The process of inflammation is complex and relies on the interaction of a myriad of cellular and soluble components. It is defined as the "reaction of vascularized living tissue to local injury" (Cotran et al., 1989). This response includes the local accumulation of fluid, soluble substances and cellular constituents into an area of tissue insult which contributes to the resolution of injury. Until the last decade or so, inflammation was believed to be entirely beneficial to the organism. The inflammatory response is associated with tissue insult and is paramount in limiting the spread of injury. In addition, inflammation is requisite for the cleanup of cellular debris and for the promotion of tissue repair (Cotran et al., 1989).

A growing body of evidence suggests that inflammation may, in many circumstances, contribute to tissue injury. If the inflammatory response is overly vigorous or inappropriate, loss of cell viability can occur. Many pathologic conditions, including arthritis, hypersensitivity reactions, certain forms of fatal renal disease and hepatitis are due to an inappropriate inflammatory response (Cotran et al., 1989). Inflammation can cause or enhance localized tissue insult such as pulmonary vascular injury after cutaneous thermal insult, immune complex injury

to the kidney and ischemia/reperfusion injury of myocardium or liver (Till et al., 1983; Till et al., 1982; Johnson and Ward, 1982; Romson et al., 1983). Further, inflammation and the associated release of cytokines and autacoids contributes to the pathophysiology of bacterial sepsis. Although incomplete, this list of pathophysiologic changes associated with inflammation demonstrates the diverse pathologic reactions mediated by this response.

The inflammatory process is dynamic, and the cells and soluble mediators which contribute to inflammation vary depending on the duration of the inflammatory response. Circulating cells most often associated with acute inflammation include neutrophils, monocytes and platelets. Certain tissue-fixed cells such as macrophages and mast cells may also contribute to the inflammatory process. Cells contribute to inflammation by both their physical interaction at the site of injury and through the release of soluble mediators such as cytokines and autacoids. The surrounding tissue parenchyma can modify the inflammatory response by the release and/or processing of soluble mediators. Thus, the process of inflammation is dependent on the tissue in which it occurs and the interaction of blood-derived cells with tissue-fixed inflammatory cells and soluble mediators.

CELLULAR MEDIATORS OF INFLAMMATION

The Neutrophil:

The neutrophil is a short-lived, circulating leukocyte with a prominent role in the acute phase of inflammation. Neutrophils (polymorphonuclear leukocytes, PMNs) act to phagocytize and degrade bacteria, cellular debris, immune complexes and other particulate matter. Neutrophils are a source of soluble mediators including cytokines, platelet activating factor, leukotrienes and prostaglandins. Although the actions of neutrophils are paramount in resolving tissue injury, the potential role of the neutrophil as a mediator of organ damage cannot be overlooked.

Neutrophils destroy particulates through two distinct mechanisms. Certain stimuli (particularly bacteria) cause the activation of a membrane-bound NADPH oxidase, which leads to the production of superoxide anion (0_2^-). Superoxide anion can undergo conversion to hydrogen peroxide, either spontaneously or via the action of the enzyme, superoxide dismutase. Further, these two oxidizing agents can react to form a more potent oxidant, the hydroxyl radical ($OH^\cdot$). Lastly, myeloperoxidase, an enzyme located in and released from the neutrophil granules, catalyzes the reaction of hydrogen peroxide with chloride ions to form cytotoxic hypochlorous acid. The ability to generate highly reactive oxidant species provides the neutrophil with a means of destroying bacteria and other pathologic agents. Reactive oxidants, however, can also destroy viable host tissue. This is well documented in myocardial ischemia/reperfusion injury (Jolly et al., 1984; Romson

et al., 1983).

The neutrophil contains granules, the contents of which contribute to the capacity of the neutrophil to destroy pathogens. Two distinct granular types are evident histologically in the neutrophil. The azurophilic granules contain primarily myeloperoxidase but also accommodate many lysosomal enzymes including neutral proteases, lysozyme, acid hydrolases and β-glucuronidase. The secondary granules of the neutrophil are characterized by the presence of lysozyme, neutral proteases and lactoferrin but lack myeloperoxidase (Barrett, 1983; Cotran et al., 1989). Upon phagocytosis, the granules present in the neutrophil coalesce with the phagosome and degrade the engulfed particulates. It should be noted that the contents of these granules, if released into the extracellular environment, may degrade cellular constituents. Thus, the neutrophil is capable of both confining and extending tissue insult.

The role of the neutrophil in inflammation is not limited to phagocytosis and destruction of particulates. Neutrophils are a rich source of soluble mediators of inflammation including, but not limited to, leukotrienes, prostaglandins and platelet activating factor (acetyl glycerol ether phosphocholine, PAF) (Table 1). The leukotrienes and prostaglandins are metabolic products of arachidonic acid catalyzed by the 5-lipoxygenase and cyclooxygenase enzymes, respectively. Certain prostanoids, and the leukotrienes in particular, are potent inflammatory agents produced when neutrophils and other cells are stimulated. PAF is a lipid-

derived, inflammatory mediator produced by neutrophils and a variety of other cells. PAF, leukotrienes and prostanoids derived from neutrophils can affect other inflammatory cells, local microvasculature, tissue parenchyma and the formation and release of other soluble mediators. In addition, many soluble mediators of inflammation not only affect cells other than neutrophils but also possess autocrine effects.

The Monocyte/Macrophage:

The monocyte is another blood-borne granulocyte that plays a pivotal role in the inflammatory response. The monocyte, responding to chemotactic factors, emigrates to an area of injury and upon entering the tissue differentiates into a macrophage. Once in the injured area the macrophage carries out many functions similar to those of the neutrophil. The macrophage is a highly phagocytic cell, engulfing particulates, bacteria and cellular debris. Further, the activated macrophage produces a myriad of compounds which may contribute to the expansion of the inflammatory response (Table 1). Reactive oxygen species, chemotactic factors, proteases, arachidonic acid metabolites and cytokines are just a few of the agents produced by macrophages upon activation. In contrast to the neutrophil (circulating half-life of approximately 6 hr), the monocyte/macrophage is a relatively long-lived cell both in circulation (circulating half-life approximately 17.5 hr) and at a site of tissue injury (Van Furth, 1992). Macrophage infiltration is often associated with inflammation somewhat later than the neutrophil. Since the macrophage has the capacity to produce many cytotoxic agents it, like the

neutrophil, may contribute to tissue insult rather than resolution of injury.

Interestingly, macrophages not only emigrate from the circulation into a region of inflammation, but they may also be present in tissues as resident phagocytic cells. This category of tissue-fixed phagocytic cell is exemplified by the Kupffer cells of the liver and the alveolar macrophages in lungs. These cells share the same capacity to engulf particulates and release soluble mediators as the monocyte-derived emigratory macrophage, however they are present in tissue as part of the normal parenchymal cellular constituent. Tissue-fixed macrophages comprise the primary phagocytic cells of the mononuclear-phagocyte system or MPS (previously known as the reticuloendothelial system). These cells are associated with the parenchymal vasculature and act as sentinels against introduction of foreign material into host tissue by clearing particulates from the circulation. In response to tissue injury, macrophages release soluble inflammatory mediators which stimulate chemotaxis and emigration of circulating granulocytes, alterations in vascular permeability and vasoreactivity, and subsequent inflammation. Recently, the macrophage has gained attention as a potential mediator of tissue injury, particularly with regard to chemically-induced hepatotoxicity.

The Mast cell:

Mast cells, which are only fixed in tissues, are widely distributed throughout connective tissue and are often found in perivascular locales (Cotran et al., 1989; Barrett, 1983). Mast cells are abundant in many organs including heart, spleen,

kidney, lung and liver. An outstanding feature of the mast cell is the heavily granulated cytoplasm. These granules contain a complex of zinc, heparin and histamine (Barrett, 1983). In addition, chemotactic factors for eosinophils and neutrophils are present in mast cell granules.

As with the other granulocytes discussed, the mast cell is capable of producing membrane-derived, soluble mediators of inflammation including leukotrienes (particularly LTC4,D4 and E4), prostaglandins and PAF. Mast cell degranulation occurs in response to a variety of specific immunologic and chemical stimuli and leads to the release of soluble mediators of inflammation. Pathophysiologic processes such as anaphylaxis have been attributed to mast cell degranulation. Thus, the mast cell is yet another cell with the potential to contribute vigorously to local inflammation and tissue injury.

The Platelet:

Thrombocytes (platelets), although derived from precursor cells that are of myeloid origin, are not granulocytic leukocytes. Platelets are cellular fragments derived from megakaryocytes in bone marrow that function primarily to maintain vascular hemostasis. Once released into the circulation, platelets remain viable for 2-4 days.

The fundamental role of thrombocytes is interaction with the vascular endothelium, subendothelium and soluble factors to maintain vascular continuity, particularly

after a vessel is breached. Normally, the platelet in circulation is quiescent and therefore does not adhere to surfaces or release agents which promote thrombosis. Upon encountering an area of vascular injury, the platelet 1) adheres to the components underlying the endothelium and 2) undergoes a morphologic change associated with adherence of other platelets and blood components.

Concurrent with these events, platelets secrete, from cytoplasmic granules, a variety of proaggregatory compounds. In addition, the platelet can synthesize de novo other proaggregatory agents, such as TxA_2 (Table 1). The production and release of these soluble mediators leads to a cascade of events including recruitment of other platelets, continued stimulation of platelet aggregation, vasoconstriction, and generation of an environment that promotes the formation of fibrin. The sum of the platelet aggregatory response is the generation of a thrombus at the site of vascular compromise (Cotran et al., 1989).

As previously mentioned, platelets store a variety of compounds in their cytoplasmic granules. Of particular importance with regard to inflammation are the components of the dense granules which include the inflammatory mediators, histamine and 5-hydroxytryptamine. Upon stimulation, thrombocytes produce proinflammatory eicosanoids, including 12-HPETE and TxA_2 (Weiss, 1982). Evidence in vitro suggests that platelets can interact with neutrophils and, by transcellular synthesis, produce the potent inflammatory agent LTC_4 (Maclouf et al., 1990). Whereas neither the neutrophil nor platelet can produce cysteinyl LTs

independently, interactions between these cells extends their capacity to contribute to inflammation. Thus, the platelet may contribute to tissue insult through the release of soluble mediators of inflammation, alterations in local tissue blood flow or modulation of cellular function.

LIPID MEDIATORS OF INFLAMMATION

The list of potentially important soluble mediators of inflammation is extensive and growing rapidly. Because of the overwhelming number of these agents, the following discussion will be limited to those that are addressed in the experimental sections. In particular this section will address the roles of leukotrienes, prostaglandins (specifically TxA_2, PGE_2 and PGI_2) and PAF in the inflammatory response. These agents have the capacity to contribute to inflammation and produce pathophysiologic alterations consistent with those seen after ANIT administration.

Leukotrienes (LTs):

Stimulation of arachidonic acid metabolism can lead to the production of a variety of soluble substances. When arachidonate undergoes metabolism by 5-lipoxygenase it leads to the initial formation of 5-HPETE which is then dehydrated to form LTA_4. LTA_4 can then undergo at least two distinct metabolic conversions. Addition of water to LTA_4 via a hydrolase leads to the formation of the proinflammatory agent LTB_4, whereas conjugation of LTA_4 to glutathione by a glutathione-S-transferase leads to the formation of LTC_4 (Maycock et al.1989).

LTC_4 undergoes sequential metabolism by gamma-glutamyltransferase and dipeptidase to form two other biologically active, soluble compounds LTD_4 and LTE_4, respectively (Moncada et al., 1985).

Collectively, LTC_4,D_4, and E_4 are referred to as either cysteinyl, sulfidopeptidyl or thiolether LTs. Thus, activation of the 5-lipoxygenase enzyme leads to the formation of two distinct types of proinflammatory LTs with different pathophysiologic actions. Whether LTB_4 or the cysteinyl LTs are formed upon release of arachidonic acid depends on the enzymatic constituents of the cell type involved, the local cellular environment and the activating stimulus.

Leukotriene Sources and Actions:

Cellular formation of LTs requires the presence of 5-lipoxygenase. Further, generation of the cysteinyl LTs requires glutathione-S-transferase (LTC_4 synthase) and glutathione. Many inflammatory cells have the enzymatic constituents to produce LTB_4. Although neutrophils are a prominent source of this mediator, they lack LTC_4 synthase and therefore the capacity to produce cysteinyl LTs independently. However, neutrophils acting in concert with platelets or endothelium can produce physiologically relevant quantities of LTC_4 (Maclouf et al., 1990; Feinmark et al., 1989). Thus, neutrophils may potentially contribute both LTB_4 and cysteinyl LTs to a biologic system.

Like the neutrophil, macrophages and mast cells are capable of producing LTB_4.

Moreover, these cells are independently capable of producing significant quantities of the cysteinyl LTs (Table 1). Mast cells produce a profile of LTs that favors the formation of the cysteinyl LTs, whereas macrophages produce somewhat more LTB_4 (Hagmann and Keppler, 1988).

Interestingly, there is contrasting experimental evidence concerning the ability of Kupffer cells (tissue-fixed macrophages) to produce significant quantities of LTs (Decker, 1990a). Kupffer cells, when stimulated with calcium ionophore, phorbol esters, lipopolysaccharides, tumor necrosis factor and some viruses generate large quantities of prostaglandins. In addition, Sakagami et al. (1988, 1989) reported that isolated rat Kupffer cells stimulated with the calcium ionophore A23187 produced nanogram quantities of both LTB_4 and cysteinyl LTs. However, only a minuscule formation of 5-lipoxygenase products and no significant 5-lipoxygenase activity from Kupffer cell extracts could be documented by Decker and colleagues (1990a). Nonetheless, it is evident that many inflammatory cells have the enzymic machinery to generate LTs. Whether the most important cellular source of LTs is mast cells or Kupffer cells remains to be identified. However, the liver, when appropriately stimulated, is a rich source of cysteinyl LTs (Hagmann et al., 1991).

Stimuli for release of LTs are varied and include, but are not limited to, the calcium ionophore A23187, bacterial lipopolysaccharide, viral proteins, PAF, phagocytosis and chemotactic peptides such as zymosan or N-formyl-methionyl-leucyl-phenylalanine (FMLP) (Hagmann and Keppler, 1988). Almost any stimulus that,

in the appropriate cell, releases arachidonate or elevates intracellular calcium will generate eicosanoids. Whether this arachidonic acid results in generation of prostanoids, LTs or a mixture of both is cell and stimulus dependent.

Although many inflammatory cells produce both LTB_4 and the cysteinyl LTs, the actions of these agents are markedly different (Table 2). The action of LTB_4 is primarily directed at neutrophils, eosinophils, macrophages and monocytes and includes stimulation of chemotaxis, chemokinesis, adhesion, superoxide generation and lysosomal enzyme release (Hagmann and Keppler, 1988). It should be noted that the action of LTB_4 on these cells, and on the neutrophil in particular, is species specific.

In the rat, LTB_4 produces enhanced PMN adhesion and aggregation (Hughes et al., 1992; Suematsu and Tsuchiya, 1991). The capacity of LTB_4 to stimulate other cellular actions such as chemotaxis is debatable. In vitro, LTB_4 does not appear to stimulate chemotaxis or chemokinesis in a Boyden chamber (Thieroff et al., 1988). In vivo, however, Karasawa et al. (1991) reported that LTB_4 receptor blockade attenuated the increase of myeloperoxidase activity (a marker of PMN accumulation) in intestinal tissue and enhanced survival following splanchnic artery occlusion/reperfusion in the rat. LTB_4 has also been implicated as requisite for PMN accumulation in a model of experimentally-induced inflammation of tooth pulp in the rat (Okiji et al., 1991). Furthermore, LTB_4 may mediate the pulmonary accumulation of neutrophils following ischemia/reperfusion of the hind limbs of rats

(Goldman et al., 1991). These data, taken together, suggest that in vivo LTB_4 is capable of causing PMN chemotaxis and activation in the rat, either directly or through the release of secondary mediators of inflammation.

Although LTB_4 is capable of inducing inflammatory cell accumulation and activation, an event which is paramount in tissue injury caused by inflammation, LTB_4 cannot produce many of the other pathophysiologic responses associated with inflammation. Cysteinyl LTs alter vascular tone, tissue perfusion, and vascular permeability with subsequent edema formation and are associated with inflammation in a variety of organs and pathologic conditions (Piper, 1984; Bach, 1983). Moreover, cysteinyl LTs have been implicated as playing a role in the pathophysiology of various models of inflammatory liver injury including galactosamine-potentiated endotoxin hepatitis in mice and frog 3-induced viral hepatitis in rats (Wendel and Tiegs, 1987; Tiegs and Wendel, 1988; Hagmann et al., 1987). Further, under hypoxic conditions, LTC_4 causes hepatic parenchymal cell lysis (Hagmann and Keppler, 1988) suggesting a potential role for this mediator in ischemic liver injury.

LTs are produced by most inflammatory cells and although the specific array of LTs elicited is dependent on a variety of factors, an inflammatory nidus contains the cellular constituency to generate both LTB_4 and the cysteinyl LTs. Together, these LTs are capable of initiating and propagating all of the pathophysiologic changes associated with inflammation (Table 2). Thus, the LTs are one potentially

important group of mediators of tissue injury associated with inflammation.

Platelet Activating Factor (PAF):

PAF is a lipid mediator produced by most inflammatory cells. It is enzymically synthesized by either remodeling of released lipid or via a de novo pathway from a glycerol phosphate. When synthesized by "remodeling", phospholipase A2 catalyzes the hydrolysis of membrane bound sn-2 fatty acyl residues to produce the biologically inactive PAF precursor lyso-PAF and a free fatty acid such as arachidonate. Lyso-PAF is then converted to PAF by the action of a distinct acetyl-coenzyme A known as lyso-PAF acetyltransferase (Yue et al., 1991). This cascade not only leads to the production of PAF, but also the release of arachidonate and the potential for generation of eicosanoids.

When produced de novo, PAF is generated from the precursor 1-O-alkyl-2-lyso-sn-glycerol-3-phosphate which undergoes acetylation via acetyl-CoA-transferase followed by phosphorylation to PAF by a specific CDP-choline phosphotransferase (Snyder, 1990; Yue et al., 1991). Although de novo synthesis of PAF does not occur in all cells that produce this mediator, this synthetic pathway is constitutive in some cells and has been hypothesized as having a role in the regulation of basal physiology (Yue et al., 1991). Thus, PAF synthesis occurs through two distinct pathways, one of which produces only PAF, the other potentially producing PAF as well as other lipid mediators.

PAF sources and actions:

When appropriately stimulated, most inflammatory cells as well as endothelium produce biologically significant quantities of PAF (Table 1). Relevant to the scope of this discussion are neutrophils, monocytes/macrophages, Kupffer cells, platelets and endothelium. All of these cell types, when stimulated with the calcium ionophore, A23187, produce PAF via PLA2 activation. A multitude of other stimuli, both physiologic (cytokines, antigens, complement components, LTs and thrombin) and experimental (PMA, FMLP, zymosan) also activate PAF formation.

After production, PAF often undergoes extracellular secretion, although in endothelium most newly-synthesized PAF remains cell-associated. Depending on the stimulus, PAF generation in neutrophils and macrophages frequently leads to intracellular accumulation of this mediator in both the membranous fraction and, to some extent, in specific granules (Yue et al., 1991). Since the production of PAF is not always associated with its release, this mediator may serve as a potential intracellular regulatory molecule in certain cells.

In addition to the calcium ionophore A23187, thrombin stimulates PAF generation in platelets. On a cell basis, the quantities of PAF produced by thrombocytes is small (2-5 fmol/10^6 cells) relative to other cellular sources of this mediator (10-170 pmol/10^6 cells) (Bratton and Henson, 1989). However, platelets can accumulate in large numbers at sites of injury and may thus contribute significant PAF to a locale.

Whether PAF produced by thrombocytes is released remains to be elucidated. It has been suggested that platelets may release lyso-PAF for processing to PAF by other cells via transcellular biosynthesis (Bratton and Henson, 1989). Although platelets are a potential source of PAF or lyso-PAF, the roles of these platelet-derived mediators in inflammation are questionable.

The actions of PAF are varied and cell specific. PAF most likely initiates cellular responses by interaction with a surface receptor (Decker, 1990b; Yue et al., 1991; Koltai et al., 1991). However, this receptor has only been characterized in crude systems and, as yet, has not been purified. The actions of PAF on neutrophils are proinflammatory and may contribute to the recruitment and activation of this cell in injured tissue. In the neutrophil, PAF causes chemotaxis, aggregation, granular enzyme release, and superoxide anion production (Hosford et al., 1993; Koltai et al., 1991; Yue et al., 1991). Further, it primes neutrophils for activation by other soluble mediators (cytokines) and enhances PMN production of LTB_4 (Braquet et al., 1989; Yue et al., 1991). The syntheses of PAF and LTB_4 are closely associated and may reflect activation of PLA2 and subsequent arachidonate release.

The effects of PAF in macrophages are, as in neutrophils, proinflammatory. Macrophages produce PAF, and when exposed to this mediator they generate superoxide anion and the inflammatory cytokines, IL-1 and TNF-α (Yue et al., 1991; Snyder, 1990). PAF also primes monocytes for the production of IL-1 and TNF,

thereby enhancing the generation of these cytokines in the presence of other stimuli such as endotoxin (Braquet et al., 1989). Additionally, macrophage synthesis of PGI_2 and TxA_2 is enhanced by PAF.

PAF production and release by macrophages is stimulus dependent. FMLP, endotoxin and A23187 all cause increased generation of PAF, whereas only endotoxin and A23187 appear to cause significant liberation of PAF (Braquet et al., 1989). The function of cell-associated PAF has not been completely explored. However, PAF may serve an autocrine function or as a signal for cell adhesion (Bratton and Henson, 1989).

PAF has been implicated in hepatic cirrhosis and pathophysiologic alterations associated with obstructive jaundice in the rat (Zhou et al., 1992). After bile duct ligation, PAF content of the liver increases. Further, PAF antagonism affords protection in this model of obstructive jaundice (Zhou et al., 1992). Although Kupffer cells, hepatic endothelial cells and hepatocytes all produce PAF in control or bile-duct ligated rats, only Kupffer cells release detectable quantities of this mediator. The release of PAF by Kupffer cells is elevated four-fold by bile duct ligation. These results suggest that Kupffer cell-derived PAF may be an important mediator of the pathophysiologic events associated with obstructive jaundice. In addition, evidence suggests that endotoxin may be a primary stimulus for this Kupffer cell-mediated PAF release and subsequent liver injury (Zhou et al., 1992).

Although PAF derives its name from its actions on platelets, the actual effect of this mediator on thrombocytes is limited and species-specific. PAF causes morphologic changes, aggregation and degranulation of platelets (Snyder, 1990). In the rat, platelet aggregation in vitro does not occur after PAF administration (Personal observation; Klee et al., 1991). However, at least one report suggests that PAF may cause platelet aggregation in vivo in the rat (Klee et al., 1991). This response is likely due to PAF-induced activation of other cells and subsequent release of secondary mediators causing thrombocyte activation. The fact that PAF can stimulate platelet aggregation in vivo underscores the importance of interactions among cellular and soluble mediators.

Prostaglandins (PGs):

This class of soluble mediators includes a broad array of compounds with a plethora of biologic effects. Due to their actions on platelet activity, vascular tone and vascular permeability, this discussion will be limited primarily to three specific prostanoids, thromboxane A_2 (TxA_2), prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2).

The prostaglandins are another group of lipid mediators under the broad classification of eicosanoids. They are short-lived autacoids which act through cell surface receptors and are of primary importance in maintaining organ homeostasis. In contrast to the LTs, which are produced by the action of 5-lipoxygenase, PGs are generated by the action of the cyclooxygenase enzyme.

Cyclooxygenase acts on arachidonate to form the unstable intermediate, PGG_2, which undergoes enzymic conversion to PGH_2. This precursor then undergoes enzymic and nonenzymic isomerization to form the biologically active prostanoids. TxA_2 and prostacyclin are formed from PGH_2 by the actions of thromboxane synthetase or prostacyclin synthetase, respectively. PGE_2 is an isomer of PGH_2 which is generated either through spontaneous isomerization or via catalysis by an isomerase (Moncada et al., 1985).

The cells mentioned in the preceding sections have the capacity to produce TxA_2, PGE_2 and/or prostacyclin (Table 1). The quantities and relative proportions of the mediators produced are cell-specific and stimulus-dependent. In general, prostacyclin is the major prostanoid generated by endothelium, where it plays a primary role in preventing platelet adhesion to the normal vascular intima. Platelet metabolism of arachidonate favors the formation of the proaggregatory mediator, TxA_2. Thus, under normal homeostasis, there is balanced production of PGI_2 and TxA_2, allowing for maintenance of vascular integrity and blood flow (Moncada et al., 1985). However, under conditions of vascular insult, endothelium may produce TxA_2 and enhance platelet activation and aggregation (McDonald et al., 1983; Ingerman et al., 1980).

TxA_2 may contribute to pathophysiologic states in certain organs. One such example is phorbol myristate acetate (PMA)-induced injury to isolated rat lungs (Carpenter and Roth, 1987; Carpenter-Deyo and Roth, 1989). In this model,

isolated rat lungs were exposed to a nontoxic concentration of PMA in the presence of PMNs which resulted in the production of pulmonary edema. Inhibition of TxA_2 synthesis in either the isolated lungs or the PMNs was sufficient to ameliorate PMA-induced pulmonary edema (Carpenter et al.1987; Carpenter and Roth, 1987; Carpenter-Deyo and Roth, 1989). Platelets, when stimulated with PMA, cause a TxA_2-dependent injury to isolated lungs (Wang et al., 1991). These findings demonstrate that TxA_2 may play a role in inflammatory-mediated tissue injury and suggests that PMNs and platelets are, at least in one model of tissue injury, an important source of this mediator.

Kupffer cells are another potential source of prostanoids. When appropriately stimulated, these cells generate PGD_2, PGE_2 and TxA_2 (Decker, 1990a). The role of PGD_2 in the physiology or pathophysiology of the rat is unclear (Giles and Leff, 1988). PGD_2 synthesis and receptors are widespread in rat tissues, but the physiologic and pathologic actions of this mediator are incompletely understood. PGE_2 and TxA_2 have numerous effects on both Kupffer cells and hepatic parenchymal cells. In the Kupffer cell, PGE_2 inhibits lipopolysaccharide-induced synthesis of additional PGE_2 and TNF-α, suggesting a regulatory role for this prostanoid (Decker, 1990a). Conversely, PGE_2 stimulates other activities of the Kupffer cell including collagenase synthesis (Decker, 1990a). Further, PGE_2, and to an even greater extent, TxA_2, increase hepatic parenchymal cell glycogenolytic activity (Decker, 1990a). Thus, the local production of these autacoids appears to modulate the function of hepatic parenchymal cells and resident hepatic

macrophages.

In addition to modulating cell function, PGE_2, PGI_2 and TxA_2 are important regulators of vascular tone and permeability (Table 2). TxA_2 is a potent vasoconstrictor whereas prostacyclin and PGE_2 are vasodilatory (Cotran et al., 1989). Although these prostanoids are only modestly vasodilatory by themselves, PGE_2 and prostacyclin markedly enhance the vascular permeability and chemotactic effects of other mediators (Davies and MacIntyre, 1992; Cotran et al., 1989). Thus, the actions of these lipid mediators may contribute to the sequelae of inflammation through their interaction with other mediators.

The effects of these prostanoids on vascular tone may contribute to their role in various models of endotoxin shock and certain forms of liver injury. Inhibition of prostanoid synthesis or specific antagonism of TxA_2 attenuates many of the pathophysiologic changes associated with endotoxin shock (Feuerstein and Hallenbeck, 1987; Ball et al.1986). Alternately, administration of the vasodilatory prostanoids, PGI_2, PGE_2 or the synthetic analog 16,16-dimethyl PGE_2 appears to ameliorate pathophysiologic changes after endotoxin administration (Feuerstein and Hallenbeck, 1987). Further, 16,16-dimethyl PGE_2 has been shown to attenuate liver injury caused by 4-pentanoic acid, carbon tetrachloride or ANIT in rats and murine hepatitis virus 3 in mice (Hidaka et al., 1991; Rush et al., 1986; Ruwart et al., 1984; Sinclair et al., 1990). The mechanism of PGE_2 protection is unknown. Since PGE_2 inhibits PMN function in vitro and reduces inflammation in

vivo, it may act to reduce the inflammatory component in these models of injury (Lehmeyer and Johnston, 1978; Fantone et al., 1982). It is apparent that whether a particular prostanoid is protective or detrimental depends on the intrinsic activity and local concentration of the PG, the variety of other mediators present, and the tissue involved. These examples serve to emphasize the equilibrium among mediators and the importance of this relationship in pathophysiologic states.

INFLAMMATION AND LIVER INJURY

Only recently has inflammation as a mechanism of tissue injury garnered serious investigation. Although there are numerous instances in which inflammation plays a key role in extra-hepatic tissue injury, limited research has been carried out on inflammatory-mediated liver insult, especially on the role of inflammation in chemically induced liver damage. This section will address the role of mediators of inflammation in liver injury, with emphasis on models of chemically induced hepatic insult.

One of the first reports suggesting that inflammatory cells might contribute to chemically induced liver injury was presented by Laskin et al. (Laskin and Pilaro, 1986; Laskin et al., 1986). These investigators demonstrated the accumulation of macrophages in hepatic parenchyma following oral administration of acetaminophen (APAP) to rats. Hepatic macrophages isolated from APAP-treated rats exhibited morphologic alterations consistent with activation. They also displayed enhanced chemotaxis, chemokinesis, and phagocytic activity, and

depending on the stimulus, either enhanced or depressed generation of superoxide anion (Laskin and Pilaro, 1986). Furthermore, hepatocytes treated in vitro with APAP released a heat and trypsin labile chemotactic factor for macrophages/monocytes (Laskin et al., 1986).

The accumulation of macrophages in livers of rats treated with APAP is well documented (Laskin et al., 1984; Laskin et al., 1986; Bailie, unpublished observation). Although the presence of macrophages in livers from APAP-treated rats has been demonstrated, a role for these cells in APAP-induced liver injury has not been confirmed. The rat is a species with marked resistance to the hepatotoxic effects of APAP. APAP administration does cause the collection of macrophages in the livers of these animals, however, significant hepatotoxicity, as evidenced by a rise in serum enzyme markers of hepatic parenchymal cell lysis, does not occur. Further, no clear relationship between macrophage accumulation and hepatic insult has been revealed in animals that are sensitive to APAP intoxication.

The presence of inflammatory cells in a chemically induced liver lesion is not sufficient to implicate the cells as causal in that insult. This is clearly illustrated by methylene dianiline hepatotoxicity. This agent causes a marked hepatic accumulation of neutrophils, which precedes the frank onset of hepatic parenchymal insult (Bailie et al., 1993). Selective depletion of circulating neutrophils or induction of panleukopenia with cyclophosphamide does not

attenuate methylene dianiline hepatotoxicity, suggesting that this influx of neutrophils is an epiphenomenon and not requisite for the toxicity (Roth et al., 1993). The accumulation of macrophages in the liver following APAP administration may represent a similar phenomenon. Until the role of macrophages in APAP hepatotoxicity is adequately addressed, the implication that these cells are causal in toxicity (Laskin et al., 1986) remains speculative.

Although the role of macrophages in APAP-induced hepatotoxicity has not been completely explored, evidence suggests that prostanoids contribute to the liver injury caused by APAP administration. In mice, enhancement of prostaglandin synthesis with hyperosmotic NaCl or xylitol magnifies APAP-induced liver injury (Ben-Zvi et al., 1990). Further, pretreatment with indomethacin or aspirin attenuates hepatic insult. This effect was apparently not due to modulation of APAP pharmacokinetics or bioactivation since neither aspirin nor indomethacin altered APAP-induced depletion of hepatic glutathione content, and indomethacin did not change the plasma concentration, half-life or clearance of APAP (Ben-Zvi et al., 1990). Although these results do not enhance our understanding of the macrophage in APAP hepatotoxicity, they do serve to demonstrate the importance of soluble mediators in potentiating tissue injury.

The potential role of macrophages in other hepatotoxicities has also been explored. Rats given a single, oral administration of carbon tetrachloride (CCl_4) exhibit massive centrilobular hepatic necrosis. However, if pretreated with

gadolinium chloride, an agent which inhibits Kupffer cell function, the injury is ameliorated (Edwards et al., 1993). Further, enhancement of Kupffer cell function by pretreatment with vitamin A for 7 days potentiates a minimally toxic dose of CCl_4 (ElSisi et al., 1993). Moreover, methyl palmitate, an agent which inhibits Kupffer cell function, administered after the 7 day regimen of vitamin A, abolishes the potentiation (ElSisi et al., 1993b). The enhanced toxicity is apparently due to Kupffer cell-derived reactive oxygen products, since treatment with superoxide dismutase or catalase attenuates vitamin A potentiation of CCl_4 hepatotoxicity (ElSisi et al., 1993b).

Interestingly, vitamin A amplifies an array of chemically induced hepatotoxicities, including those of endotoxin, APAP and allyl alcohol (ElSisi et al., 1993). This suggests that either Kupffer cells are important in several models of chemically induced liver injury or that vitamin A pretreatment sensitizes hepatic parenchyma to the deleterious effects of xenobiotic agents.

Although vitamin A pretreatment potentiates allyl alcohol hepatotoxicity, the role of Kupffer cells in the hepatotoxic effect of this agent is still a matter of debate. Przybocki and colleagues (1992) reported that inhibition of Kupffer cell function with gadolinium chloride ameliorated allyl alcohol hepatotoxicity in vivo. In contrast, these investigators found that gadolinium chloride provided no protection against allyl alcohol hepatotoxicity in the isolated liver. They suggest that expression of allyl alcohol hepatotoxicity in vivo is dependent on Kupffer cells and other

circulating mediators of inflammation (Przybocki et al., 1992). In contrast to these findings, Ganey and coworkers could not demonstrate protection against allyl alcohol hepatotoxicity in vivo using an identical treatment paradigm (Ganey et al., 1993a). Further, cyclophosphamide-induced leukopenia did not attenuate liver injury caused by allyl alcohol, suggesting that circulating leukocytes do not participate in this toxicity (Ganey, personal communication).

One explanation for this controversy may be the quality of animals used. While Ganey and colleagues used specific, pathogen-free animals; Przybocki and coworkers used standard Sprague-Dawley rats. It is conceivable that Przybocki and associates were studying the hepatotoxic effects of allyl alcohol in the presence of a viral or bacterial contaminant. Thus, macrophages may not contribute to allyl alcohol toxicity per se, but may play a role in augmenting toxicity in the presence of other compromising factors. Clearly, further evaluation is needed before a definitive role for macrophages in allyl alcohol-induced hepatotoxicity can be confirmed.

The capacity of inflammation to contribute to the injury of ischemia/reperfusion in various organs has garnered much attention. A common finding in most models of ischemia/reperfusion-induced tissue insult is a large accumulation of PMNs (Hughes et al., 1992). In the liver, damage due to ischemia/reperfusion is a biphasic event. Modest functional changes occur after brief ischemia (45 minutes) which progresses to fulminant necrosis by 15 hrs of reperfusion (Hughes et al.,

1992). Although modest, initial insult occurs shortly after reinitiation of perfusion and most likely represents Kupffer cell-mediated oxidant stress (Jaeschke and Farhood, 1991). The severity of liver injury increases markedly as PMNs accumulate in the hepatic parenchyma and reperfusion proceeds. Since depletion of circulating PMNs attenuates the extension of injury by continued reperfusion, the severe progression of injury is presumably neutrophil-mediated (Jaeschke et al., 1990).

Although PMNs are requisite for this latter phase of reperfusion injury, the signal for neutrophil accumulation is unknown. LTB_4 does not appear to be a chemotactic factor for PMN influx in this model because inhibition of LT biosynthesis does not alter PMN infiltration or ischemia/reperfusion induced injury (Hughes et al., 1992). The mechanism(s) of neutrophil accumulation in the reperfusing liver as well as the specific pathogenic role that neutrophils play in hepatic parenchymal insult remains to be elucidated.

In culture, PMA-stimulated human neutrophils or PMA or FMLP-stimulated rat PMNs are capable of killing rat hepatocytes (Guigui et al., 1988; Ganey et al., 1993b). In cocultures, the generation of oxidant species does not appear to contribute to the cytotoxic action of PMNs since administration of superoxide dismutase and/or catalase does not affect PMN-mediated hepatocyte killing. Evidence suggests that neutrophil-derived proteases may be the primary cytotoxic agents released by PMNs stimulated with either PMA, FMLP or zymosan (Guigui

et al., 1988; Ganey et al., 1993b; Mavier et al., 1988). These studies suggest that PMNs have the capacity to destroy liver parenchyma directly. Their action may be mediated by lysosomal constituents rather than through the generation of reactive oxidants.

Circulating inflammatory cells also contribute to liver injury from bacterial endotoxin. In the rat, intravenous administration of E. coli lipopolysaccharide causes fulminant hepatitis characterized by inflammatory infiltrates, platelet thrombi and marked hepatocellular necrosis (Hewett et al., 1992) In this model, either depletion of circulating neutrophils or platelets attenuates the hepatotoxicity (Hewett et al., 1992; Pearson et al., 1994). As is the case in galactosamine sensitized mice treated with endotoxin, TNF-α may be the terminal mediator of tissue injury since agents which block TNF-α formation or action (pentoxifylline or anti-TNF antibody, respectively) ameliorate endotoxin-induced hepatitis in the rat (Hewett et al., 1993).

Certainly, various models of endotoxin-induced systemic and hepatic insult have generated enormous interest with regard to inflammatory-mediated tissue injury and hepatic insult. Galactosamine sensitization of mice followed by endotoxin administration causes fulminant hepatitis. Pharmacologic interventions which prevent the formation of cysteinyl LTs or antagonize LTD_4 receptors attenuate the hepatic insult in this model (Tiegs and Wendel, 1988). Further, LTD_4, but not LTE_4, when administered to galactosamine-sensitized mice produces hepatic injury reminiscent of that produced by endotoxin (Wendel and Tiegs, 1987; Tiegs and

Wendel, 1988). The effect of LTD_4 in this model of liver injury may be due to its vasoconstrictive properties, suggesting an ischemia/reperfusion related hepatic insult (Wendel et al., 1987). It should be noted that although LTD_4 is causal in the galactosamine-sensitized mouse model of endotoxin hepatitis, the terminal mediator of this injury is probably TNF-α (Tiegs et al., 1989).

In contrast to the finding that LTD_4 has a primary role in the galactosamine/endotoxin model of hepatitis in mice, LTs do not apparently contribute to the hepatic insult caused by endotoxin administration to rats. Inhibition of LT biosynthesis does not afford protection from endotoxin-induced hepatitis in the rat (Bailie and Hewett, unpublished observation). Further, Yoshikawa and Goto observed that LT receptor antagonism does not reduce lipopolysaccharide-provoked hepatitis in rats (Yoshikawa and Goto, 1992). However, PAF receptor antagonism attenuates endotoxin liver injury in their model (Yoshikawa and Goto, 1992). Interestingly, in the galactosamine/endotoxin model of liver injury in mice, just the opposite is true. PAF receptor antagonism fails to attenuate liver injury, while abolishing the effects of LTs effectively reduces the hepatic insult (Tiegs and Wendel, 1988). Although there are distinct differences in these models, they serve to demonstrate the importance of both cellular and soluble mediators in the expression of endotoxin-induced liver injury and confirm the significance of inflammation in certain forms of hepatic insult.

INFLAMMATION AND ANIT HEPATOTOXICITY

The histologic lesions associated with ANIT-induced liver injury have been well characterized in the rat. Twenty four hours after a single, oral administration, ANIT causes portal edema, periportal inflammation comprising primarily neutrophils, mild hepatic parenchymal cell insult and biliary epithelial cell necrosis (McLean and Rees, 1958; Dahm et al., 1991b). Microscopic alterations in liver are detectable as early as 6 hr after ANIT adminstration and consist of mild portal edema and inflammation. At 12 hr, the inflammatory infiltrate is marked and biliary epithelial cell swelling is detectable. By 24 hr, frank biliary epithelial cell necrosis and scattered hepatic parenchymal cell necrosis is evident (McLean and Rees, 1958; Dahm et al., 1991b; Ungar et al., 1962; Goldfarb et al., 1962). The biliary epithelial injury is distributed throughout the portal regions but not all bile ducts are affected. The extent of biliary epithelial necrosis is, when present in a given bile duct, severe and results in loss of biliary epithelial integrity and sloughing of epithelial cells into the biliary space.

The loss of biliary epithelium and consequent plugging of bile ducts with cellular debris was, until recently, considered to be the mechanism of cholestasis associated with ANIT intoxication. Evidence now suggests that alterations in bile formation rather than physical obstruction of the biliary canaliculi may be the initial cause of bile flow cessation (Kossor et al., 1992) Sloughing of the biliary epithelium with subsequent physical obstruction of bile ducts may act to enhance and prolong the cholestasis (Kossor et al., 1992).

ANIT-induced injury to hepatic parenchyma is less prominent than the injury to biliary epithelium. The loss of parenchymal cells is mild and confined primarily to the periportal regions that demonstrate associated biliary epithelial injury and edema. Although the extent of hepatic parenchymal involvement is modest, biochemical and histologic evidence suggests a significant and reproducible loss of parenchymal cells. Interestingly, the hepatic parenchymal insult, portal edema and biliary epithelial injury occur in concert, and clear dissociation of these events has not been demonstrated, suggesting that expression of hepatic parenchymal cell necrosis is dependent on ANIT-induced biliary epithelial cell injury.

Six to 24 hours after ANIT administration, neutrophils are associated with the biliary tree and infiltrate the surrounding periportal parenchyma (McLean and Rees, 1958; Ungar et al., 1962; Goldfarb et al., 1962; Dahm et al., 1991). The prominence of this inflammatory infiltrate suggests that inflammation may play a role in the pathogenesis of ANIT intoxication. Confirmation of the role of neutrophils in ANIT hepatotoxicity was recently provided by Dahm and colleagues (Dahm et al., 1991). They observed that specific depletion of circulating neutrophils with anti-neutrophil serum attenuated ANIT-induced liver injury whereas depletion of circulating lymphocytes did not. Further, ANIT has the capacity to stimulate and/or prime neutrophils to release superoxide anion and β-glucuronidase in vitro, while the nonhepatotoxic congener BNIT does not share this capability (Roth and Hewett, 1990).

Although ANIT stimulates PMNs to release reactive oxidants in vitro, the toxic effect of ANIT in vivo is not apparently mediated through the release of reactive oxygen species. Treatment of rats with polyethylene glycol-coupled superoxide dismutase and catalase does not reduce the hepatic insult caused by ANIT administration (Dahm et al., 1991). This is compatible with evidence in vitro suggesting that isolated neutrophils do not injure cultured hepatotocytes by production of oxidants but rather through the release of proteases (Guigui et al., 1988; Ganey et al., 1993b).

Although ANIT hepatotoxicity has been well characterized histologically, the pathophysiologic mechanism(s) of ANIT-induced liver injury are not well delineated. For example, the role of metabolism and bioactivation in ANIT hepatotoxicity has been incompletely addressed. Capizzo and Roberts (1970) demonstrated that $^{14}CO_2$ was detected in expired air when ^{14}C-ANIT labeled at the isothiocyanate moiety was administered to rats. Demonstration of radiolabeled inorganic sulfate in the urine of rats given ^{35}S-ANIT confirmed the finding that the isothiocyanate moiety of ANIT undergoes metabolism (Traiger et al., 1984). In addition, using thin layer chromotography, Capizzo and Roberts showed the presence of several ^{14}C-labeled metabolites in the urine of various species after ANIT administration. The rabbit, a species that does not manifest ANIT hepatotoxicity, did not excrete a particular ^{14}C metabolite in urine as did the susceptible species. This finding suggests that this metabolite might be important in the expression of ANIT toxicity.

Although there is evidence that ANIT undergoes metabolism, identification of specific metabolites is lacking. The only confirmed metabolite of ANIT to be conclusively identified is α-naphthylamine (Mennicke et al., 1978) and its function in ANIT hepatotoxicity is unknown . Although the role of bioactivation in the expression of ANIT toxicity is unclear, evidence from studies using inhibitors and inducers of hepatic monooxygenase activity suggest that bioactivation might be required for expression of liver injury. Phenobarbital and 3-methylcholanthrene pretreatment enhance hepatic monooxygenase activity and potentiate ANIT hepatotoxicity (Roberts and Plaa, 1965; El-Hawari and Plaa, 1977). Conversly, piperonyl butoxide, disulfiram, diethyldithiocarbamate and cobaltous chloride inhibit hepatic monooxygenase activity and attenuate ANIT-induced hepatic insult (Roberts and Plaa, 1965; El-Hawari and Plaa, 1977; Traiger et al., 1984). SKF-525A demonstrates a biphasic effect on ANIT toxicity consistant with the ability of this compound to increase or decrease monooxygenase activity.

While inducers and inhibitors of hepatic monooxygenase activity increase or decrease ANIT injury respectively, recent evidence provided by Dahm and coworkers suggests a possible alternative mechanism for the protection or potentiation afforded by these compounds (Dahm and Roth, 1991). In addition to modulating hepatic monooxygenase activity, the agents mentioned above can influence hepatic glutathione content or the activity of glutathione S-transferase (Dahm and Roth, 1991). Since glutathione appears to be required for the expression of ANIT injury, the protection afforded by inhibitors of monooxygenase

activity and conversly the enhancement of ANIT hepatotoxicity caused by inducers of monooxygenase activity, is consistent with their actions on hepatic glutathione status (Dahm and Roth, 1991). Clearly, the metabolic fate of ANIT and the potential contribution of an ANIT metabolite to the expression of hepatotoxicity requires further investigation.

The function of glutathione (GSH) in ANIT-induced liver injury is somewhat unusual (Dahm and Roth, 1991). In many models of chemical hepatotoxicity, GSH provides a protective mechanism for hepatocytes. The concentration of GSH in hepatic parenchymal cells is high (5-7mM), and conjugation of various xenobiotic agents to this tripeptide occurs readily. Generally, this conjugation leads to biologic inactivation of the chemical and subsequent elimination. GSH is also a requisite cofactor for glutathione peroxidase and in this capacity contributes to cellular defenses against oxidant stress (Kehrer, 1993).

In many models of liver injury, depletion of cellular GSH augments the chemical insult. In contrast, ANIT hepatotoxicity is abolished by decreased hepatic non-protein sulfhydryl content (a marker of GSH status), suggesting that GSH is required for the expression of liver injury (Dahm and Roth, 1991). One explanation for these results might relate to the formation of cysteinyl LTs.

GSH is requisite for, and rate limiting in, the generation of cysteinyl LTs (Austen and Soberman, 1988). It is plausible that ANIT administration causes 5-

lipoxygenase activation and subsequent formation of these compounds. If cysteinyl LTs are causal in ANIT hepatotoxicity, GSH depletion could afford protection by preventing the formation of these mediators. As was previously presented, the cysteinyl LTs are requisite for expression of toxicity in at least one other model of liver injury, and have the capacity to produce pathophysiologic changes consistent with those seen after ANIT administration. Inflammation is a critical component in the expression of ANIT hepatotoxicity. The observations that PGE_2 administration, GSH depletion and reduction in circulating neutrophils all attenuate ANIT-induced liver injury suggest a role for inflammatory mediators in this model. The capacity of other cellular and soluble mediators to contribute to ANIT hepatotoxicity remains to be addressed. Further, the signals for hepatic accumulation of inflammatory cells have not been elucidated.

Hypothesis: Cellular and soluble mediators of inflammation are requisite for the expression of ANIT hepatotoxicity.

The overall aim of this project was to evaluate the contribution of selected cellular and soluble inflammatory mediators to ANIT hepatotoxicity. Since GSH is requisite for the formation of cysteinyl LTs and for the expression of ANIT injury, the potential role of LTs in ANIT-induced liver injury was addressed. LTs can produce many of the pathophysiologic changes associated with ANIT intoxication including edema formation, neutrophil aggregation, altered vascular tone and hepatic parenchymal cell lysis. PAF can mediate many of the pathophysiologic changes

associated with inflammation, and has been shown to contribute to organ injury in several models of inflammatory insult. Further, PAF appears to have redundant effects with LTs in the systemic effects of endotoxin. Therefore, the role of PAF, alone or in combination with LTs, in ANIT-induced injury was evaluated.

Since platelets can contribute to tissue injury both directly and through the release of soluble mediators, experiments were performed to determine if platelets and prostanoids are requisite for the expression of ANIT hepatotoxicity. Finally, since Kupffer cells and macrophages release soluble mediators and cytotoxic species which contribute to a variety of chemically induced liver injuries, the role of these cells in ANIT hepatotoxicity was addressed.

TABLE 1. MAJOR SOURCES OF SELECTED SOLUBLE INFLAMMATORY MEDIATORS IN THE RAT.

	PAF	LTB_4	Cyst. LTs	TxA_2	PGE_2	PGI_2
Neutrophil	+++[a]	+++[h]	-[d]	++[j]	+++[p]	+[p]
Macrophage or Kupffer cells	+++[ac]	+++[e]	++[f]	+++[l]	+++[l]	+/-[l]
Mast cell	++[b]	+++[g]	+++[g]	N.F.	N.F.	N.F.
Platelet	+/-[a]	+/-[i]	+/-[i]	++[k]	-[m]	-[m]
Endothelium	++[c]	+/-[i]	+/-[i]	+/-[n,o]	+++[m]	+++[m]

Cyst. LTs = Cysteinyl leukotrienes
N.F. = Not Found

a) Salari et al., 1990
b) Hogaboam et al., 1992
c) Zhou et al., 1992
d) Piper, 1984
e) Sakagami et al., 1989
f) Sakagami et al., 1988
g) Heavey et al., 1988
h) Bailie dissertation
i) Maclouf et al., 1990 (possible via transcellular biosynthesis)
j) Carpenter-Deyo and Roth, 1989 (8ng $TxA_2/10^6$ cells)
k) Ganey and Roth, 1987 (2ng $TxA_2/10^6$ cells)
l) Decker, 1990
m) Davies and MacIntyre, 1992
n) Smith, 1986
o) McDonald et al., 1983 (ovine)
p) Dray et al., 1980

TABLE 2. EFFECT OF SOLUBLE INFLAMMATORY MEDIATORS IN THE RAT.

MEDIATOR	ACTION
PAF	-PMN chemotaxis/activation/priming -Macrophage activation/priming -Platelet aggregation (not in vitro in the rat)
LTB_4	-PMN adhesion/activation -Modulates cytokine production in certain cells -PMN chemotaxis in vitro does not occur in the rat -Similar action in eosinophils and macrophages
CYSTEINYL LTs	-Modulates vascular tone and permeability -Alters tissue perfusion -Can cause hepatic parenchymal cell lysis under appropriate conditions
TxA_2	-Platelet aggregation -Vasoconstriction -Modifies hepatic parenchymal metabolism
PGE_2	-Modulates Kupffer cell functions -Vasodilatory (modest effect alone, enhances effect of other agents) -Exogenous administration ameliorates a variety of pathophysiologic states

See text for references

CHAPTER II

LEUKOTRIENES AND ALPHA-NAPHTHYLISOTHIOCYANATE INDUCED LIVER INJURY

Summary

α-Naphthylisothiocyanate (ANIT) administration to rats results in periportal hepatic inflammation and injury. Glutathione (GSH) appears to be necessary for the liver injury to occur. The leukotrienes (LTs) are metabolites of arachidonic acid and potent mediators of inflammation that have been implicated in certain liver injury models. Inasmuch as GSH is a requisite cofactor for the synthesis of cysteinyl LTs and since inflammation is a prominent component of ANIT injury, we hypothesized that LTs are involved in producing the hepatic insult that results from ANIT administration. To test this hypothesis, rats were treated with one of several inhibitors of LT biosynthesis (A63162, Zileuton or MK-886). Each of these agents prevented the formation of LTB_4 in Ca^{++} ionophore-stimulated whole blood from rats treated with the inhibitors. A63162 attenuated the hepatic parenchymal injury caused by ANIT and resulted in a modest decrease in ANIT-induced cholestasis. In contrast, neither Zileuton nor MK-886 attenuated liver injury. AT-125 inhibits γ-glutamyl transferase (GGT), the enzyme that catalyzes the formation of LTD_4 from LTC_4. AT-125 pretreatment did not prevent ANIT-induced hepatic parenchymal insult. It did, however, ameliorate the cholestasis caused by ANIT. In conclusion, the partial protection afforded by A63162 and AT-125 likely results from effects unrelated to the formation of LTs, since Zileuton and MK-886 inhibited LT synthesis without affording protection. The lack of protection by Zileuton and MK-886 in the face of LT synthesis inhibition suggests that LTs are not necessary for the expression of injury after ANIT administration.

Introduction

Acute administration of α-naphthylisothiocyanate (ANIT) to rats causes a cholangiolitic hepatopathy with a periportal distribution (Goldfarb et al., 1962; McLean and Rees, 1958; Ungar et al., 1962). The hepatic lesion is characterized by periportal edema, biliary epithelial and hepatic parenchymal cell injury and neutrophil infiltration. Although the mechanisms of ANIT toxicity are incompletely understood, neutrophil (PMN) depletion prior to ANIT administration prevents the hepatotoxicity, suggesting that ANIT-induced hepatic insult depends upon PMNs (Dahm et al., 1991b). Thus, the inflammatory response seems to be critical to the development of hepatic lesions in this model.

Lipid mediators, particularly the leukotrienes (LTs), produce a myriad of effects that contribute to inflammation and tissue injury (Cotran et al., 1989). The LTs have been implicated in the pathophysiology of asthma, the adult respiratory distress syndrome, arthritis and inflammatory bowel disease (Cotran et al., 1989). In addition, they are important mediators in the liver injuries caused by endotoxin/galactosamine in mice and by frog 3 virus in rats (Tiegs and Wendel, 1988; Wendel and Tiegs, 1987; Hagmann et al., 1987). LTs are produced in inflammatory cells by the action of 5-lipoxygenase on arachidonic acid. Mast cells, macrophages and neutrophils are among the cells which produce LTs, and many of their functions are modified by the LTs (Cotran et al., 1989).

The observation that GSH appears to be required for the expression of ANIT-

induced inflammatory liver injury (Dahm and Roth, 1991) raised further interest in the role of LTs in this model. Glutathione (GSH) is a requisite cofactor in the biosynthesis of the cysteinyl LTs (ie, LTC_4, LTD_4 and LTE_4). These are formed by S-conjugation of GSH to LTA_4 followed by the sequential metabolism of the glutathione sidechain by γ-glutamyl transferase and a dipeptidase. The cysteinyl LTs can contribute to inflammation by inducing cell chemotaxis and tissue edema. In addition, LTs modulate vasoactivity, and, under low oxygen conditions, LTC_4 is directly cytotoxic to hepatocytes in vitro (Keppler et al.,1985).

Since PMNs and GSH appear to mediate ANIT-induced liver injury and since both are important in the synthesis and actions of LTs, the role of LTs in ANIT-induced hepatotoxicity was evaluated using inhibitors of leukotriene biosynthesis.

Materials and Methods

Materials.

ANIT, A23187, AT-125, L-γ-glutamyl-p-nitroanilide, Kit 605-D for bilirubin determination, and Kit 59-20 for alanine aminotransferase activity were purchased from Sigma Chemical Co. (St. Louis, MO). A63162 and Zileuton were gifts from Abbott Laboratories (Abbott Park, IL). MK-886 was obtained from the Merck Frosst Co. (Montreal, Canada). Enzyme immunoassay reagents for measurement of LTB_4 were obtained from Caymen Chemical Co. (Ann Arbor, MI). Polyethylene tubing was purchased from Clay Adams (Parsippany, NJ). All other reagents were of the highest quality commercially available.

Animals.

Male, Sprague-Dawley rats (VAF/plus, CF:CD(SD)BR, Charles River, Portage MI) weighing 150-275g were housed under conditions of controlled temperature (18-21° C) and humidity (55 ± 5%) and were maintained on a 12 hr light/dark cycle. They were bedded on hardwood chips and allowed food (Wayne Lab Blox, Allied Mills, Chicago, IL) and water ad libitum. Animals were fasted for 24 hr prior to ANIT administration and throughout the duration of experiments. All procedures on animals were carried out according to the humane guidelines of the American Association of Laboratory Animal Sciences.

Methods.

Effect of inhibitors of leukotriene biosynthesis on ANIT-induced liver injury. Zileuton (40 mg/kg in 0.2% methylcellulose, 4 ml/kg) or vehicle was administered to rats po 1 hr prior to ANIT (35 mg/kg in corn oil, 2 ml/kg, ip). They received Zileuton or its vehicle every 6 hr thereafter. A63162 (100 mg/kg in 0.2% methylcellulose, 2 ml/kg, po) or vehicle was administered to rats 1 hr prior to ANIT (35 mg/kg, 2 ml/kg, ip) or corn oil vehicle and every 8 hr thereafter. MK-886 (5 mg/kg in dimethysulfoxide, 2 ml/kg, ip) or vehicle was given 1 hr prior to oral administration of ANIT or its vehicle and every 8 hr thereafter. Inhibition of leukotriene biosynthesis was verified as described below.

Twenty-four hours after ANIT administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and placed on a heating pad to maintain body temperature. A midline laparotomy was performed for measurement of bile flow as previously described (Dahm and Roth, 1991). Rats were euthanized via exsanguination from the descending aorta, and collected blood was used for measurement of markers of liver injury. Serum total bilirubin content was measured spectrophotometrically as previously described (Dahm and Roth, 1991) and used as an indirect measure of cholestasis. Serum GGT activity was assessed by a modification of the method of Szaz and was used as an index of biliary epithelial integrity (Szaz, 1969). Serum ALT activity was measured spectrophotometrically based on the method of Bergmeyer et al. (1978) using Sigma Kit 59-20, and was assumed to reflect loss of hepatic parenchymal cell

integrity. Liver sections were collected and fixed in 10% phosphate-buffered formalin for histologic analysis.

Verification of inhibition of leukotriene biosynthesis.

Ex-vivo stimulation of LT synthesis in whole blood with Ca^{++} ionophore was performed in a manner similar to that described by McMillan et al. (1986) and Gresele et al. (1986). Briefly, heparin-treated whole blood was collected, and 700 μl were placed immediately in a 1.5 ml Eppendorf tube containing 7 μl of either calcium ionophore (A23187, 30 μg/ml, final concentration) or dimethylsulfoxide vehicle. The tubes were gently mixed then placed in a water bath at 37°C for 30 min. The reaction was terminated by placing the tubes on ice for 5 min followed by centrifugation at 15,000 X g for 2 min. Plasma was separated from the cell pellet, and 100 μl were placed in 400 μl of ice-cold 100% methanol. This was stored at -20°C until it was assayed for LTB_4 immunoreactive substances. Measurement of LTB_4 was performed by the University of Michigan Ligand Core laboratory of the Diabetes Research and Training Center using an LTB_4 enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI).

Effect of AT-125 (Acivicin) on ANIT-induced hepatic insult.

AT-125 (10 mg/kg, 2 ml/kg, ip), an inhibitor of GGT, or sterile water vehicle was administered 1 hr prior to ANIT (35 mg/kg, 2 ml/kg, po). Twenty-four hours later, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and processed as described above. Serum GGT activity was measured 24 hr after ANIT

administration to verify inhibition by AT-125.

Statistical analysis.

Results are expressed as the mean ± SE. Homogeneity of variance was tested with the F-max test. Prior to further analysis, a log-transformation was performed on nonhomogeneous data. Data were analyzed with a completely random, two-way analysis of variance (ANOVA). Comparisons between treatment means were carried out using Tukey's ω test or the least significant difference test as appropriate (Steel and Torrie, 1980). The criterion for significance was $p \leq 0.05$.

Results

Efficacy of leukotriene biosynthesis inhibitors.

To confirm efficacy of treatment regimens using inhibitors of LT biosynthesis, LTB_4 concentration was examined in blood from drug-treated animals which was stimulated with Ca^{++} ionophore. LTB_4 concentrations were small (approximately 0.75 ng/ml) but detectable in methanol-extracted plasma from unstimulated blood (Fig 1A,B). Stimulation of blood with A23187 caused a 20-fold increase in plasma LTB_4 concentration (Fig 1A,B). This increase was inhibited 85%-95% by pretreatment with either A63162 or Zileuton (Fig 1A,B). Similarly, MK-886 inhibited the production of LTB_4 by ionophore-stimulated whole blood by greater than 95% (dimethylsulfoxide treated= 18.9 ng/ml LTB_4, MK-886 treated= 0.7 ng/ml LTB_4: n=2).

Leukotriene synthesis inhibition and ANIT-induced liver injury.

ANIT administration caused hepatic parenchymal and biliary epithelial injury as demonstrated by increases in serum ALT and GGT activities (Figs. 2,3,4). Injury to these cells was confirmed by histologic analysis of livers from ANIT-treated rats. Additionally, cholestasis was reflected by increased serum total bilirubin concentration and decreased bile flow. These changes are consistent with previous reports (Dahm et al., 1991a,b; Dahm and Roth, 1991).

Pretreatment with A63162 attenuated the changes in serum ALT activity and bilirubin concentration caused by ANIT (Fig. 2A,B) and returned bile flow toward

control values (Fig 2C). The protection afforded was modest: a 43% decrease in ALT and a 30% decrease in serum bilirubin concentration. A63162 did not alter the increase in serum GGT activity caused by ANIT administration. A larger dose of A63162 (100mg/kg) did not provide additional protection against ANIT-induced hepatopathy (data not shown).

In contrast to A63162, neither Zileuton nor MK-886 ameliorated ANIT-induced liver injury. This was reflected in the lack of attenuation of any of the markers of liver injury evaluated (Fig 3A-C, 4A-D).

Effect of AT-125 on ANIT-induced liver injury.

LTD_4 helps to mediate liver injury caused by gram-negative bacterial endotoxin in galactosamine-sensitized mice (Wendel and Tiegs, 1987; Tiegs and Wendel, 1988). This inflammatory LT is produced by the action of GGT on LTC_4. Accordingly, the effect of GGT inhibition on ANIT-induced liver injury by AT-125 was examined. AT-125 treatment maintained GGT inhibition throughout the duration of the experiment as demonstrated by the lack of significant GGT activity 24 hr after AT-125 treatment (Fig 5D). In addition, GGT activity was not detectable in AT-125/corn oil treated animals (Fig 5D). Histologic evaluation of livers from ANIT/AT-125 treated animals demonstrated biliary epithelial insult similar to that seen in those from ANIT/VEH treated rats (data not shown). This finding confirmed that the decrease in GGT activity seen 24 hr after ANIT administration was due to AT-125 inhibition of GGT activity rather than protection from biliary epithelial insult. AT-125 did not alter the

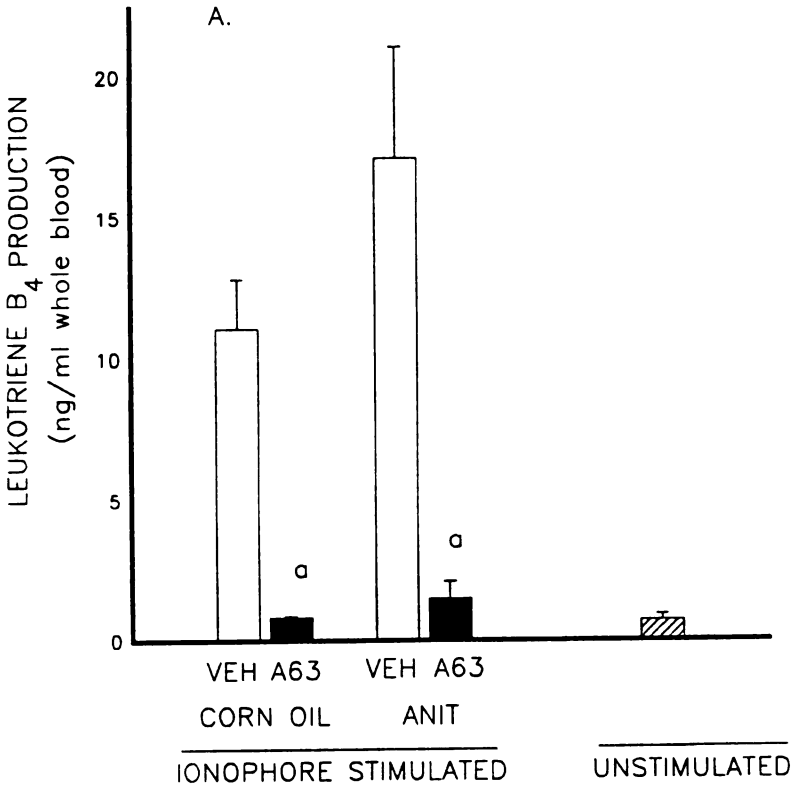
hepatic parenchymal insult caused by ANIT as reflected by elevated serum ALT activity (Fig 5A). However, GGT inhibition did provide a modest decrease in ANIT-induced cholestasis as reflected by both bile flow (Fig 5B) and serum total bilirubin concentration (Fig 5C).

Figure 1. Effect of A63162 and Zileuton on LTB4 production in Ca^{++} ionophore-stimulated whole blood.

A) A63162 effect on LTB_4 production. Rats were pretreated with either A63162 (50 mg/kg, po, every 8 hr) or its vehicle and with ANIT (35 mg/kg, ip) or corn oil vehicle.

(B) Zileuton effect on LTB_4 production. Rats were pretreated with either Zileuton (40 mg/kg, po, every 6 hr) or its vehicle and with ANIT (35 mg/kg, ip) or corn oil vehicle.

Twenty-four hours after ANIT administration, anticoagulated blood was treated with Ca^{++} ionophore (A23187,30 μg/ml) or DMSO vehicle (7 μl/vial) as described in Methods. LTB_4 concentration was measured using an enzyme immunoassay (EIA). Bars represent means ± SE. N = 3-6. *Significantly different from respective vehicle-treated group.


A.
LEUKOTRIENE B_4 PRODUCTION
(ng/ml whole blood)
20
15
10
5
0
a
a
VEH A63
CORN OIL
VEH A63
ANIT
IONOPHORE STIMULATED
UNSTIMULATED

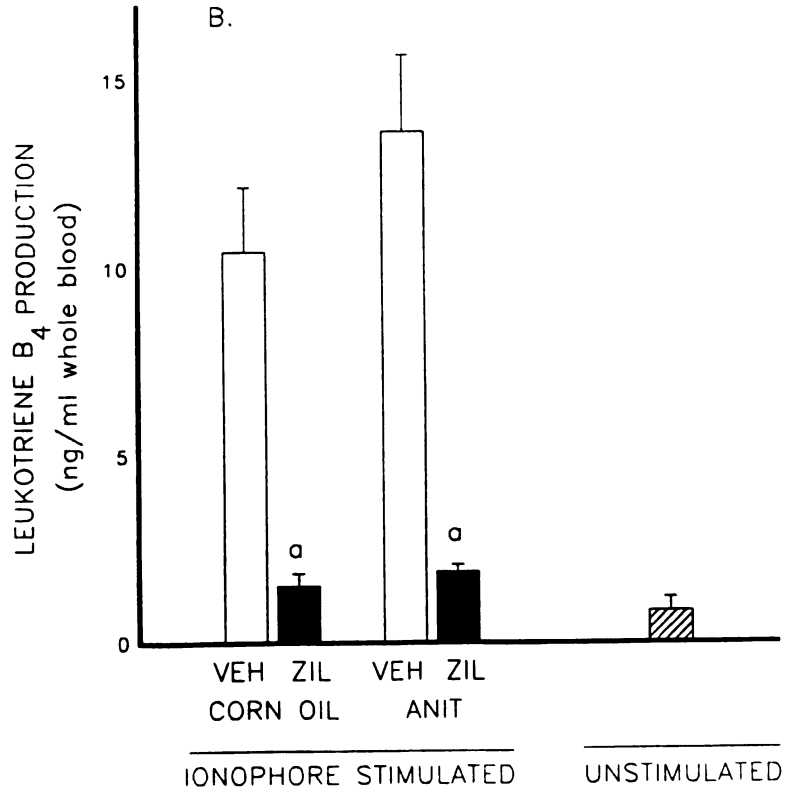

B.
LEUKOTRIENE B_4 PRODUCTION
(ng/ml whole blood)
15
10
5
0
a
a
VEH ZIL
CORN OIL
VEH ZIL
ANIT
IONOPHORE STIMULATED
UNSTIMULATED

Figure 2. Effect of A63162 on ANIT-induced liver injury.

Rats received either A63162 (50 mg/kg, po, every 8 hr) or vehicle and either ANIT (35 mg/kg, ip) or corn oil. Twenty four hours after ANIT administration, rats were killed and markers of liver injury were evaluated as described in Methods. (A) Serum ALT activity, (B) Serum total bilirubin concentration, (C) Bile flow, (D) Serum GGT activity. Bars represent means ± SE. N = 5-16. [a]Significantly different from respective corn oil control. [b]Significantly different from ANIT/VEH group.

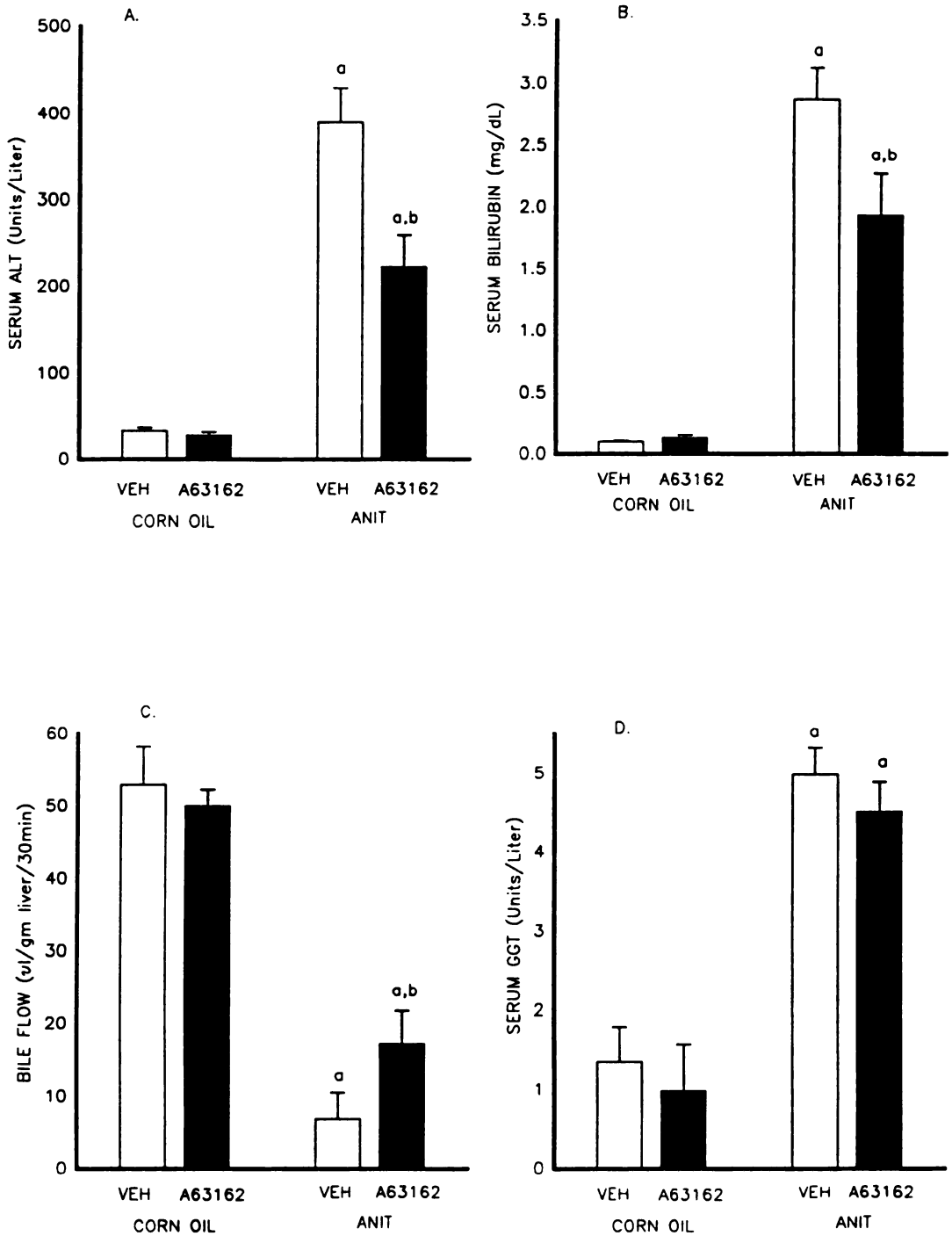
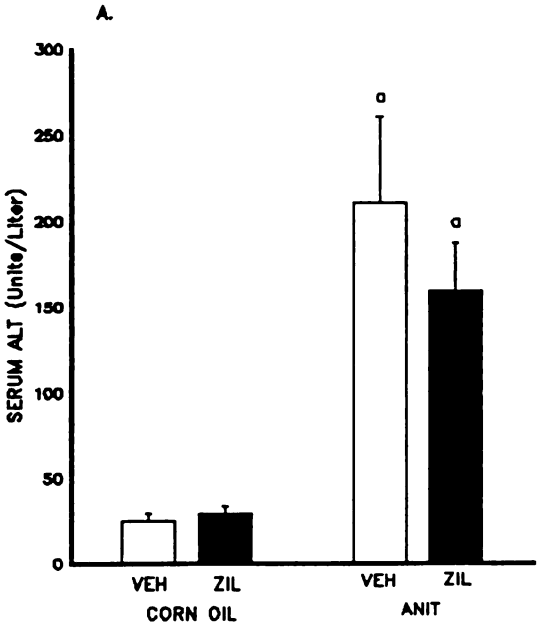
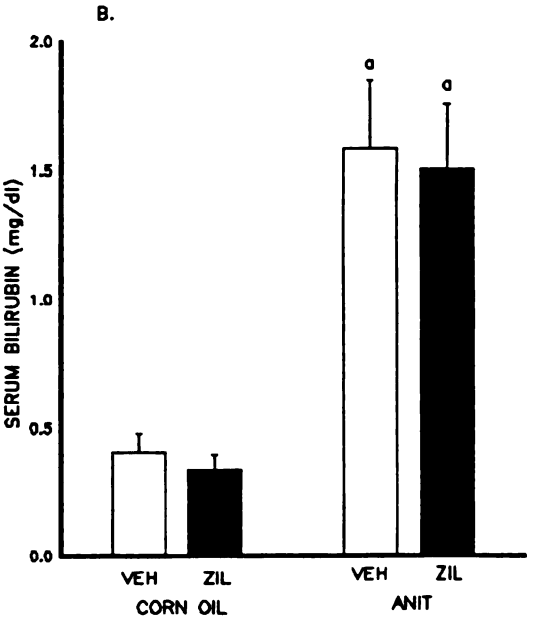

A.
SERUM ALT (Units/Liter)
500
400
300
200
100
0
a
a,b
VEH
A63162
CORN OIL
VEH
A63162
ANIT
B.
SERUM BILIRUBIN (mg/dL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b
VEH
A63162
CORN OIL
VEH
A63162
ANIT
C.
BILE FLOW (ul/gm liver/30min)
60
50
40
30
20
10
0
a
a,b
VEH
A63162
CORN OIL
VEH
A63162
ANIT
D.
SERUM GGT (Units/Liter)
5
4
3
2
1
0
a
a
VEH
A63162
CORN OIL
VEH
A63162
ANIT

Figure 3. Effect of Zileuton on ANIT-induced liver injury.

Rats received either Zileuton (40 mg/kg, po, every 6 hr) or vehicle and either ANIT (35 mg/kg, ip) or corn oil. Twenty four hours after ANIT administration, rats were euthanized and markers of liver injury were evaluated as described in Methods. (A) Serum ALT activity, (B) Serum total bilirubin concentration, (C) Serum GGT activity. Bars represent means ± SE. N = 9-15. [a]Significantly different from respective corn oil-treated group.


A.
SERUM ALT (Units/Liter)
300
250
200
150
100
50
0
a
a
VEH
ZIL
VEH
ZIL
CORN OIL
ANIT


B.
SERUM BILIRUBIN (mg/dl)
2.0
1.5
1.0
0.5
0.0
a
a
VEH
ZIL
VEH
ZIL
CORN OIL
ANIT

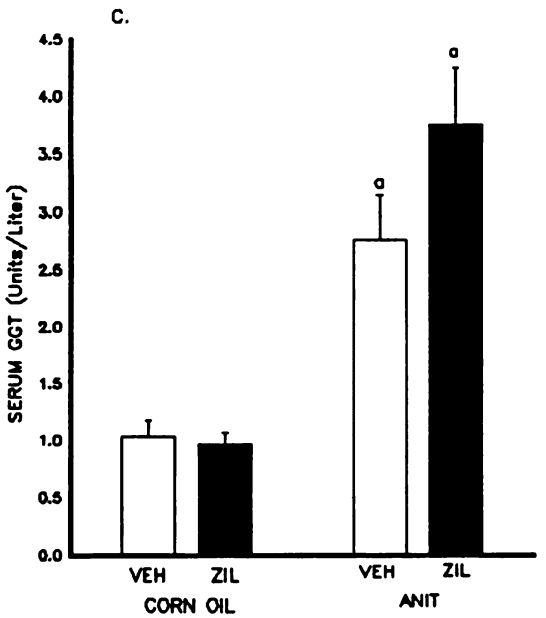
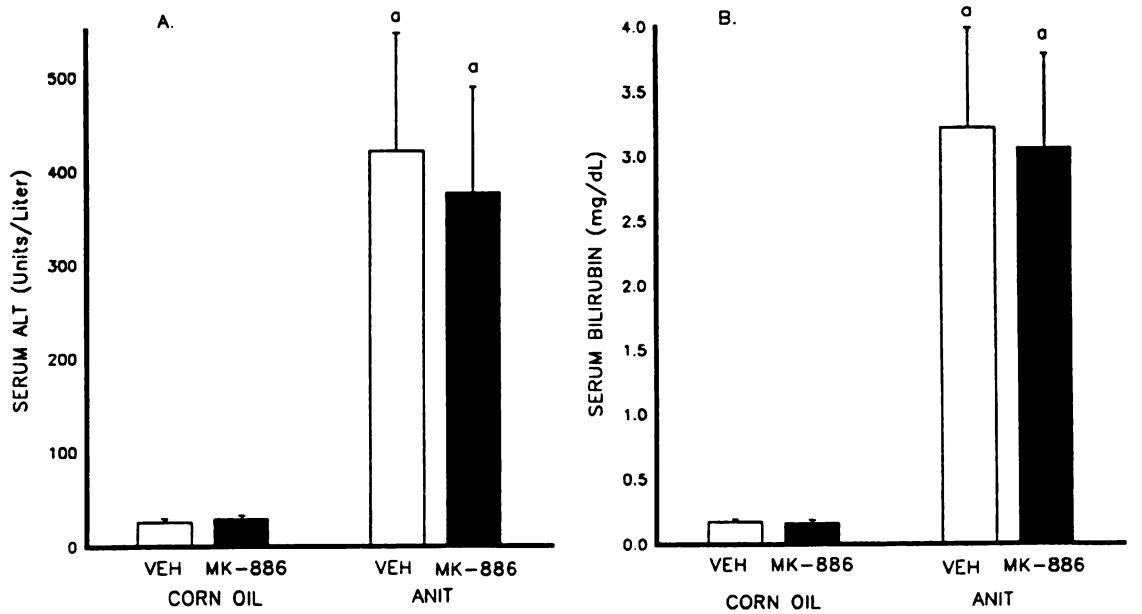

C.
SERUM GGT (Units/Liter)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH
ZIL
VEH
ZIL
CORN OIL
ANIT

Figure 4. Effect of MK-886 on ANIT-induced liver injury.

Rats received either MK-886 (5 mg/kg, ip, every 8 hr) or vehicle and either ANIT (35 mg/kg, po) or corn oil. Twenty four hours after ANIT administration, rats were killed and markers of liver injury were measured as described in Methods. (A) Serum ALT activity, (B) Serum total bilirubin concentration, (C) Bile flow, (D) Serum GGT activity. Bars represent means ± SE. N = 3-5. [a]Significantly different from respective corn oil-treated group.


A.
SERUM ALT (Units/Liter)
500
400
300
200
100
0
a
a
VEH MK-886
CORN OIL
VEH MK-886
ANIT
B.
SERUM BILIRUBIN (mg/dL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH MK-886
CORN OIL
VEH MK-886
ANIT

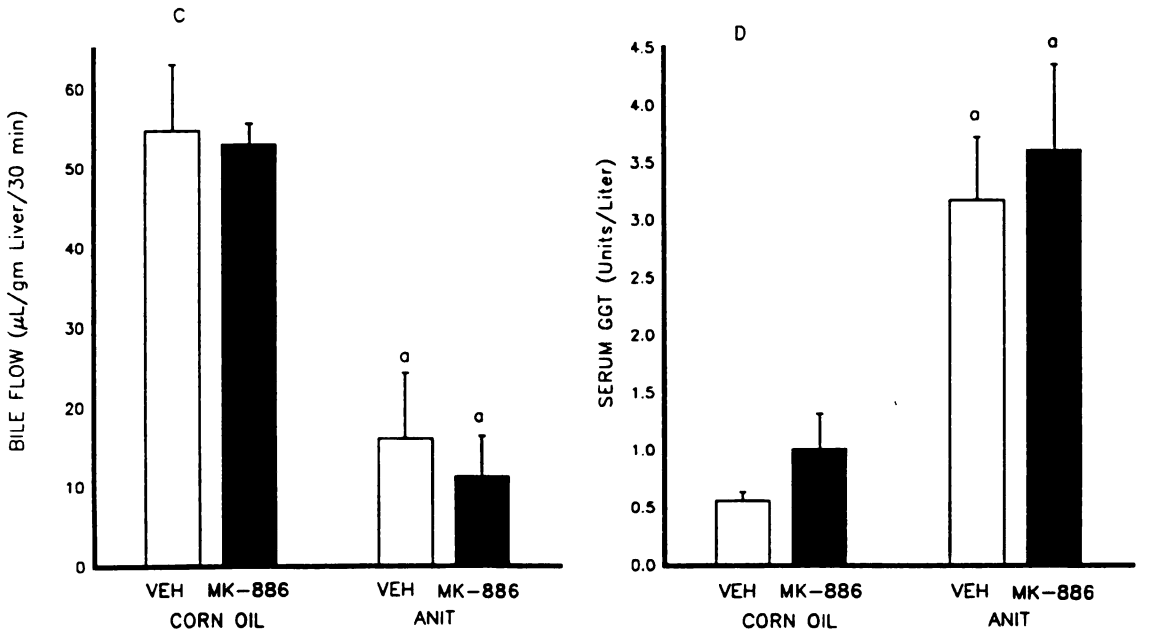
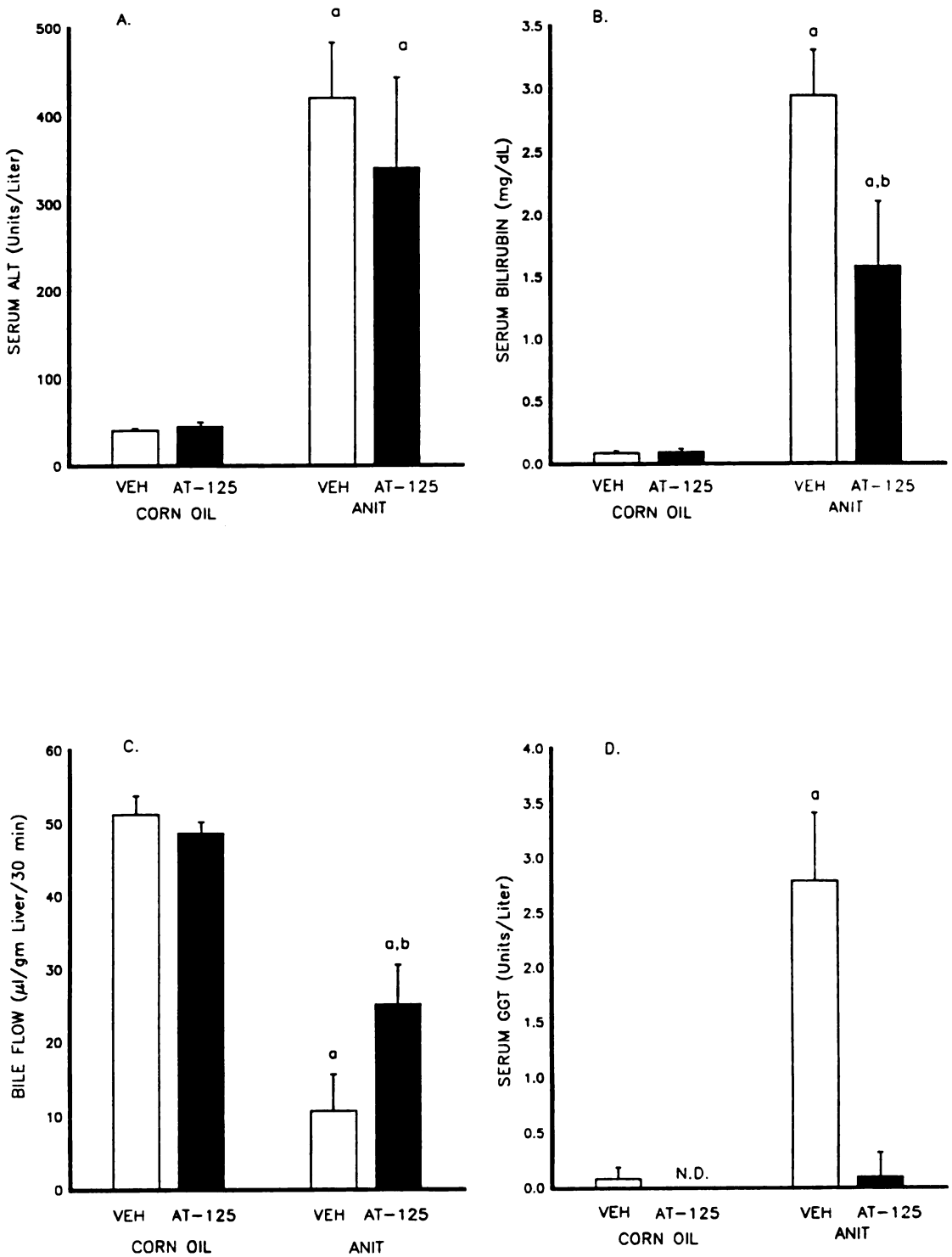

C
BILE FLOW (µL/gm Liver/30 min)
60
50
40
30
20
10
0
a
a
VEH MK-886
CORN OIL
VEH MK-886
ANIT
D
SERUM GGT (Units/Liter)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH MK-886
CORN OIL
VEH MK-886
ANIT

Figure 5. Effect of AT-125 on ANIT-induced liver injury.

Rats received either AT-125 (10 mg/kg, ip) or sterile water and either ANIT (35 mg/kg, po) or corn oil. Twenty four hours after ANIT treatment, rats were killed and markers of liver injury were assessed as described in Methods. (A) Serum ALT activity, (B) Total serum bilirubin concentration, (C) Bile flow, (D) Serum GGT activity. Bars represent means ± SE. N = 9-14. [a]Significantly different from respective corn oil treated group. [b]Significantly different from ANIT/VEH group.


A.
SERUM ALT (Units/Liter)
500
400
300
200
100
0
a
a
VEH
AT-125
VEH
AT-125
CORN OIL
ANIT
B.
SERUM BILIRUBIN (mg/dL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b
VEH
AT-125
VEH
AT-125
CORN OIL
ANIT
C.
BILE FLOW (µl/gm Liver/30 min)
60
50
40
30
20
10
0
a
a,b
VEH
AT-125
VEH
AT-125
CORN OIL
ANIT
D.
SERUM GGT (Units/Liter)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
N.D.
a
VEH
AT-125
VEH
AT-125
CORN OIL
ANIT

Discussion

Two important features of ANIT-induced liver injury suggested that LTs might play a role in ANIT hepatotoxicity. First, neutrophils are requisite for expression of ANIT-induced liver injury (Dahm and Roth, 1991). Although neutrophils do not appear to produce cysteinyl LTs directly, they may act in concert with other cells, such as platelets, to produce the cysteinyl LTs (Maclouf et al., 1990). In addition, neutrophils readily form LTB_4 and their function is modulated by LTs. Also, the cysteinyl LTs produce alterations in vascular tone, vascular leak and edema. These changes, coupled with the effects of LTB_4 on leukocytes, could account for many of the hepatic changes caused by ANIT. Secondly, GSH is needed for the expression of ANIT hepatotoxicity. Since this tripeptide is required for and is rate limiting in the production of cysteinyl LTs (Austen and Soberman, 1988), it seemed possible that LTs were important soluble inflammatory mediators of ANIT-induced hepatitis.

Because drugs vary in selectivity but are probably never specific, several pharmacologically effective drug treatment regimens were used to inhibit LT biosynthesis. The partial protection provided by A63162 and AT-125 was consistent with the hypothesis that the cysteinyl LTs were mediators of ANIT-induced liver injury. However, two other agents, Zileuton and MK-886, also produced a marked reduction in the biosynthesis of LTs but did not ameliorate ANIT hepatotoxicity. The lack of protection seen with Zileuton and MK-886 suggests that the modest protection afforded by A63162 and AT-125 is likely due

to effects unrelated to their actions on LT formation. Although we cannot rule out the possibility that MK-886 and Zileuton did not achieve sufficient concentrations in the liver to inhibit LT biosynthesis effectively there, these drugs are highly lipid soluble and should distribute readily from blood into liver cells after oral administration.

Our results suggest that LTs do not, by themselves, produce the hepatic insult that follows ANIT treatment. However, the process by which cellular and soluble components contribute to tissue injury is likely complex and may involve the interaction of numerous mediators. For example, LTs interact with a variety of cell signaling systems including interleukins and other cytokines. Yoshikawa and Goto (1992) recently demonstrated that independent blockade of the actions of LTs or PAF was not sufficient to ameliorate the systemic effects caused by administration of bacterial endotoxin. However, if the actions of both LTs and PAF were inhibited simultaneously, the systemic effects of endotoxin were attenuated. This suggests that endotoxin-induced pathophysiologic changes are dependent on a network of soluble mediators. The results of that study suggest that inhibition of an individual component of a cell signaling network may not be sufficient to overcome the effects of such a system. There may be a similar network of inflammatory mediators at work in ANIT-induced liver injury. Thus, amelioration of ANIT hepatotoxicity may require intervention at numerous sites in the network, particularly if the system comprises several mediators with redundant effects.

Numerous inflammatory mediators are capable of producing changes consistent with those seen after ANIT treatment (Cotran et al., 1989). The potential role of other inflammatory mediators in ANIT-induced hepatic insult remains to be addressed. Although LTs may be capable of producing pathophysiologic changes in liver in other animal models (Tiegs and Wendel, 1988; Wendel and Tiegs, 1987; Keppler et al., 1985; Hagmann et al., 1987), the results of this study suggest that LTs are not solely responsible for expression of liver injury that follows ANIT administration in rats.

CHAPTER III

PLATELETS AND α-NAPHTHYLISOTHIOCYANATE-INDUCED LIVER INJURY

Summary

Administration of *α*-naphthylisothiocyanate (ANIT) to rats results in periportal cholangiolitic hepatopathy. Inflammation is a hallmark of the liver injury, and expression of toxicity is dependent on blood neutrophils. The role of other cellular mediators of inflammation in ANIT-induced hepatic insult is unknown. We hypothesized that platelets participate in the expression of ANIT hepatotoxicity. To test this, circulating platelets were decreased by administration of anti-rat platelet serum (PAb) prior to treatment of rats with ANIT. The PAb treatment regimen effectively reduced circulating thrombocytes over the course of the experiment. Twenty-four hours after oral ANIT administration, rats were euthanized and liver injury was estimated by increases in serum alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) activities. Cholestasis was assessed by measurement of serum total bilirubin concentration and bile flow. Reduction in platelet numbers was associated with attenuation of the increases in plasma ALT activity and bilirubin concentration seen after ANIT administration. However, PAb treatment did not attenuate the increase in plasma GGT, a marker of biliary epithelial cell injury. ANIT-induced changes in platelet function were assessed by evaluating platelet aggregation responses in platelet-rich plasma from rats treated with ANIT in vivo. ANIT treatment of rats modestly decreased ex vivo platelet aggregation in response to ADP and collagen stimuli. To address further the role of platelet-derived cyclooxygenase products in ANIT hepatotoxicity, rats were treated with aspirin (ASA) or ibuprofen (IB). Neither pretreatment ameliorated ANIT-induced hepatic insult. These results suggest that platelets contribute to the

expression of ANIT-induced liver injury, but they do not appear to act through the production of cyclooxygenase metabolites.

Introduction

ANIT is an experimental hepatotoxicant that causes cholestasis and injury to biliary epithelial cells and hepatic parenchymal cells after oral administration in rats (Goldfarb et al., 1962; McLean and Rees, 1958). A prominent feature of acute ANIT hepatotoxicity is marked periportal edema and inflammatory cell infiltrate, comprising primarily neutrophils. Depletion of circulating neutrophils attenuates ANIT-induced hepatotoxicity, emphasizing the importance of the inflammatory reaction in the expression of the hepatic injury (Dahm et al., 1991). Although neutrophils appear to be important in ANIT-induced liver injury, the roles of other inflammatory cells have not been addressed.

Platelets are a potential source of inflammatory mediators, inasmuch as they release chemotactic agents and proteolytic factors which may contribute to inflammation (Weiss, 1982). In addition, platelets modulate vasoactivity through the release of thromboxane A_2 (TxA_2) and other agents (Weiss, 1982; Cotran et al., 1989). Lastly, platelets may alter vascular permeability through the release of histamine or a "permeability factor" (Weiss, 1982).

Since platelets are capable of contributing to an inflammatory response, the role of platelets in ANIT-induced liver injury was addressed in rats by reducing circulating platelet numbers. The effect of ANIT administration on platelet aggregation was also evaluated. Finally, to determine if platelet-derived prostanoids were important in the pathology of ANIT-induced liver injury, the effect

of cyclooxygenase inhibition on ANIT hepatotoxicity was evaluated.

Materials and Methods

Materials.

Adenosine diphosphate (ADP), aspirin (ASA), collagen, ibuprofen (IB), and Dulbecco's phosphate-buffered saline (endotoxin free) were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were of the highest quality commercially available. Silicon-treated cuvettes for platelet aggregometry were purchased from Biodata (Hatboro, PA), and disposable stir bars were obtained from Sienco (Morrison, CO). Planimetry was performed using Sigmascan software (Jandel Scientific, San Raphael, CA). Goat anti-rat platelet serum was generated as previously described (White et al., 1989)

Animals.

Male, Sprague-Dawley rats (VAF/plus, CF:CD(SD)BR, Charles River, Portage MI) weighing 225-350g were housed and maintained as described in Methods Chapter II.

Methods

Effect of platelet depletion on ANIT-induced liver injury.

Anti-platelet serum (PAb, 0.7ml/animal, ip) or control serum (CAb) was administered to rats 12 hr prior to ANIT (35 mg/kg, 2ml/kg, po) or corn oil vehicle and 6 hr afterwards. Twenty-four hours after ANIT administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and placed on a heating

pad to maintain body temperature. A midline laparotomy was performed, and the bile duct was cannulated for measurement of bile flow as previously described (Dahm and Roth, 1991). Rats were killed via exsanguination from the descending aorta, and blood collected in sodium citrate (0.38% final concentration) was used for measurement of plasma alanine aminotransferase (ALT) and γ-glutamyl transferase (GGT) activities, plasma bilirubin concentration and blood platelet numbers. Plasma ALT activity, measured spectrophotometrically by a modification of the method of Bergmeyer et al. (1978) using Sigma Kit 59, was assumed to reflect loss of hepatic parenchymal cell integrity. Plasma GGT activity was assessed by a modification of the method of Szaz (1969) and was used as a marker of biliary epithelial cell integrity. Plasma total bilirubin concentration was measured spectrophotometrically as previously described (Dahm and Roth, 1991) and used as a marker of cholestasis. Circulating platelet numbers were assessed using a hemocytometer as previously described (White et al., 1989). Rats that received PAb but did not demonstrate a reduction in circulating platelet numbers below $3x10^5$ platelets/μl blood were excluded from the study. Liver sections were collected and fixed in 10% phosphate-buffered formalin for histologic analysis.

Aspirin and Ibuprofen treatment.

Aspirin (450 mg/kg, po) or corn oil vehicle (2 ml/kg) was administered 1 hr prior to ANIT (35 mg/kg, ip) or corn oil vehicle (2ml/kg). Efficacy of the aspirin treatment was verified in a preliminary experiment by evaluating platelet aggregation 1 hr and 24 hr after ASA in response to collagen. In a separate study,

ibuprofen (17.5 mg/kg, 0.5 ml/kg, ip) or DMSO vehicle was administered 1 hr prior to ANIT (35 mg/kg, po) or corn oil vehicle and every 8 hr thereafter (Ganey and Roth, 1987). Twenty-four hr after ANIT administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and processed as described above, except that serum rather than plasma was used to assess markers of liver injury.

Effect of ANIT administration in vivo on ex vivo platelet aggregation.

Rats were treated with ANIT (35 mg/kg, ip) or corn oil vehicle, and 1, 4, 8 or 24 hr later platelets were harvested as described by Killam and Cohen (1991). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and the chest was opened via a midline thoracotomy. Blood was withdrawn from the right ventricle into a syringe containing heparin (1:10 dilution; final concentration, 5 U/ml heparin). The anticoagulated whole blood was spun in a centrifuge at 100 x g for 20 min and platelet rich plasma (PRP) was removed. The remaining blood fraction was spun again at 2000 x g for 15 min and platelet poor plasma (PPP) was collected. PPP was used as the reference for aggregometry and as diluent for PRP. Platelet aggregometry was carried out as described by Killam and Cohen (1991) as originally presented by Born (1962). PRP (360 μl) was added to silicon-treated cuvettes and allowed to warm to 37° C for 2 min. The appropriate volumes of phosphate-buffered saline and aggregating agonist were added to bring the final volume of the reaction mixture to 444 μl. Aggregation was measured in a Chonolog 570-VS, 4 channel whole blood aggregometer (Chronolog Corp. Havertown, PA). Response to the aggregation stimulus was estimated as the area

under the curve of a 5 min aggregation response. Results were expressed as minute x %, where 100% represented the chart pen deflection between PRP and PPP. ADP and collagen were used as stimuli for aggregation. Three concentrations (ADP- 10^{-7}, $3x10^{-7}$, 10^{-6} M; collagen- 0.05, 0.1, 0.2 mg/ml) of each stimulus were used.

Statistical analysis.

Results are expressed as mean ± SE. Statistical analysis was performed as described in Methods Chapter II. Where appropriate, the Student's-t test with Bonferonni's correction was also used for comparison of treatment means.

Results

Effect of PAb on circulating platelet numbers.

The efficacy of PAb in depleting platelets from blood was assessed 24 hr after ANIT exposure. ANIT administration caused no alteration in circulating platelet numbers, however, treatment with PAb reduced the number of circulating platelets in both corn oil- and ANIT- treated rats to 13 % of control (Fig. 1).

Platelet depletion and ANIT-induced liver injury.

ANIT administration caused hepatic parenchymal and biliary epithelial insult as demonstrated by significant elevations in plasma ALT and GGT activities, respectively (Fig. 2A,D). ANIT-induced cholestasis was reflected as a decrease in bile flow and an increase in plasma total bilirubin concentration (Fig. 2B,C). Platelet depletion attenuated the ANIT-induced increase in plasma ALT activity (Fig. 2A) and the elevation in total plasma bilirubin concentration (Fig. 2B). In contrast, PAb administration did not significantly alter ANIT-induced changes in bile flow or plasma GGT activity (Fig. 2C,D). Histologic analysis of livers confirmed the lack of attenuation of ANIT-induced biliary epithelial cell necrosis by platelet depletion.

Effect of ASA and IB treatment on ANIT-induced hepatotoxicity.

ASA and IB modulate platelet function in vivo and were used to address the role of platelet-derived cyclooxygenase products in ANIT hepatotoxicity. To confirm drug effectiveness, blood was collected from rats 1 and 24 hr after ASA treatment. Ex vivo platelet aggregation in response to collagen was completely inhibited by

ASA treatment at both times (data not shown). The treatment regimen used for IB has previously been shown to abolish the platelet aggregation response to arachidonate (Ganey and Roth, 1987). ANIT treatment caused increases in serum ALT and GGT activities and serum total bilirubin concentration (Fig 3,4). Pretreatment with either ASA or IB did not alter ANIT-induced hepatotoxicity. This was reflected in the lack of attenuation of any of the markers of liver injury evaluated.

ANIT treatment and platelet aggregation.

Ex vivo platelet aggregometry was used to evaluate platelet function in PRP from rats treated with ANIT in vivo. When the highest concentration of ADP was used as the aggregating stimulus, a modest decrease in aggregation occurred 1hr after ANIT administration. No other significant effect on platelet aggregation was detected prior to 24hr (Table 1). However, by 24hr, ADP-induced platelet aggregation was moderately depressed. When collagen was used as the stimulus, ANIT treatment similarly caused a modest depression of the platelet aggregation response at 1 hr after administration (Table 2). The response of platelets to collagen was also depressed 24hr after ANIT administration. At no time after ANIT treatment were platelets more responsive to aggregating agents.

TABLE 1: EFFECT OF ANIT TREATMENT IN VIVO ON EX VIVO PLATELET AGGREGATION IN RESPONSE TO ADP.

TIME Rx
Aggregation Response (Min. x %)

		ADP ($1x10^{-7}$ M)	ADP ($3x10^{-7}$ M)	ADP ($1x10^{-6}$ M)
1 HR	CO	2.1 ± 1.6	245 ± 44	360 ± 13
	ANIT	3.2 ± 1.2	179 ± 26	302* ± 11
4 HR	CO	2.6 ± 1.3	206 ± 26	305 ± 34
	ANIT	5.3 ± 1.9	174 ± 35	320 ± 31
8 HR	CO	3.2 ± 1.6	117 ± 24	319 ± 35
	ANIT	6.0 ± 2.3	159 ± 24	317 ± 11
24 HR	CO	4.9 ± 1.9	117 ± 21	334 ± 16
	ANIT	2.6 ± 0.6	90 ± 29	196* ± 20

Fasted rats were treated with ANIT (35 mg/kg, po) or corn oil (CO) vehicle and euthanized 24hr later. PRP and PPP were prepared from heparin-anticoagulated blood, and ADP-induced aggregation of platelets was measured as described in Methods. Values are means ± SE and represent the area under the curve for a 5 min. aggregation reaction. N= 3-8 rats. * Significantly different from corn oil-treated controls, $p \leq 0.05$.

TABLE 2: EFFECT OF ANIT TREATMENT IN VIVO ON EX VIVO PLATELET AGGREGATION IN RESPONSE TO COLLAGEN.

TIME	Rx	Aggregation Response (Min. x %)		
		COLLAGEN 0.05 mg/ml	COLLAGEN 0.1 mg/ml	COLLAGEN 0.2 mg/ml
1 HR	CO	46 ± 46	353 ± 16	356 ± 13
	ANIT	70 ± 28	181* ± 54	319 ± 18
4 HR	CO	123 ± 59	297 ± 36	311 ± 39
	ANIT	48 ± 34	190 ± 56	329 ± 24
8 HR	CO	90 ± 73	280 ± 42	336 ± 31
	ANIT	114 ± 46	218 ± 49	311 ± 12
24 HR	CO	311 ± 76	372 ± 30	368 ± 31
	ANIT	65* ± 41	105* ± 65	202 ± 70

Fasted rats were treated with ANIT (35 mg/kg, po) or corn oil (CO) vehicle and euthanized 24hr later. PRP and PPP were prepared from heparin-anticoagulated blood, and collagen-induced aggregation of platelets was measured as described in Methods. Values are means ± SE and represent the area under the curve of a 5 minute aggregation reaction. N= 3-8 rats. * Significantly different from corn oil-treated controls, $p \leq 0.05$.

Figure 1. Effect of PAb and ANIT on circulating platelet numbers.

Rats received 2 injections of either PAb or CAb and a single, oral administration of ANIT or corn oil vehicle as outlined in Methods. Determination of circulating platelet numbers was carried out 24hr after treatment with ANIT or corn oil vehicle. Bars represent means ± SE. N = 6-15. *Significantly different from respective CAb-treated group.

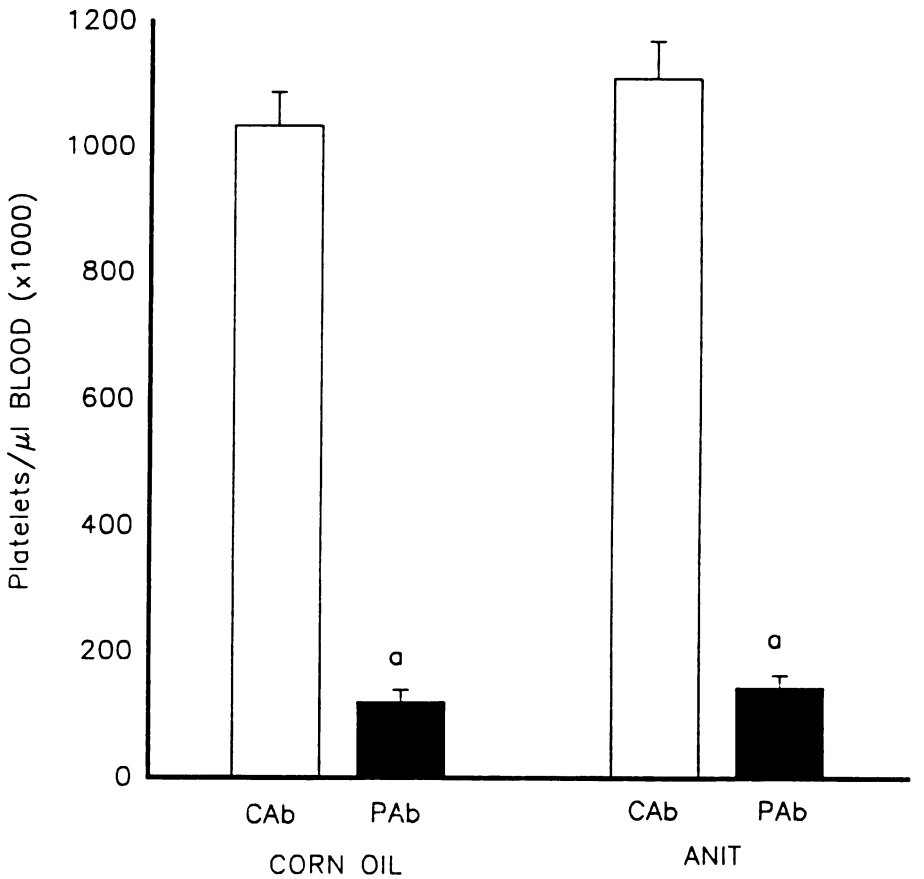

1200
1000
800
600
400
200
0
Platelets/μl BLOOD (x1000)
a
a
CAb
PAb
CAb
PAb
CORN OIL
ANIT

Figure 2. Effect of platelet depletion on markers of ANIT-induced hepatotoxicity.

Rats received 2 injections of either PAb or CAb and a single, oral administration of ANIT or corn oil vehicle as outlined in Methods. Twenty-four hours after ANIT or vehicle administration, rats were euthanized and indices of liver injury were assessed. A. Plasma alanine aminotransferase (ALT) activity. B. Plasma total bilirubin concentration. C. Bile flow. D. Plasma gamma-glutamyl transferase (GGT) activity. Bars represent means ± SE. N= 6-15. [a]Significantly different from respective corn oil treated group. [b]Significantly different from ANIT/CAb treated group.

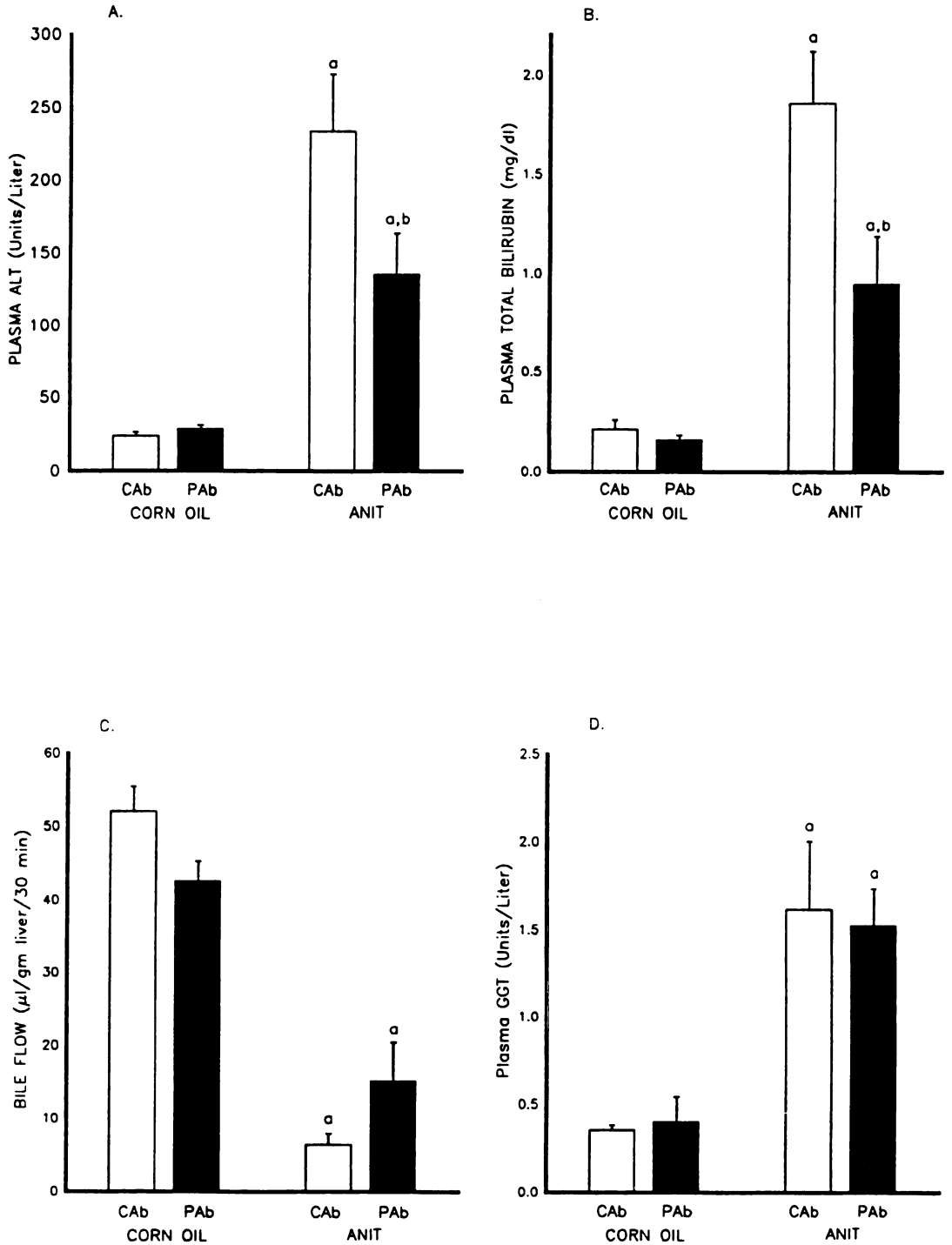
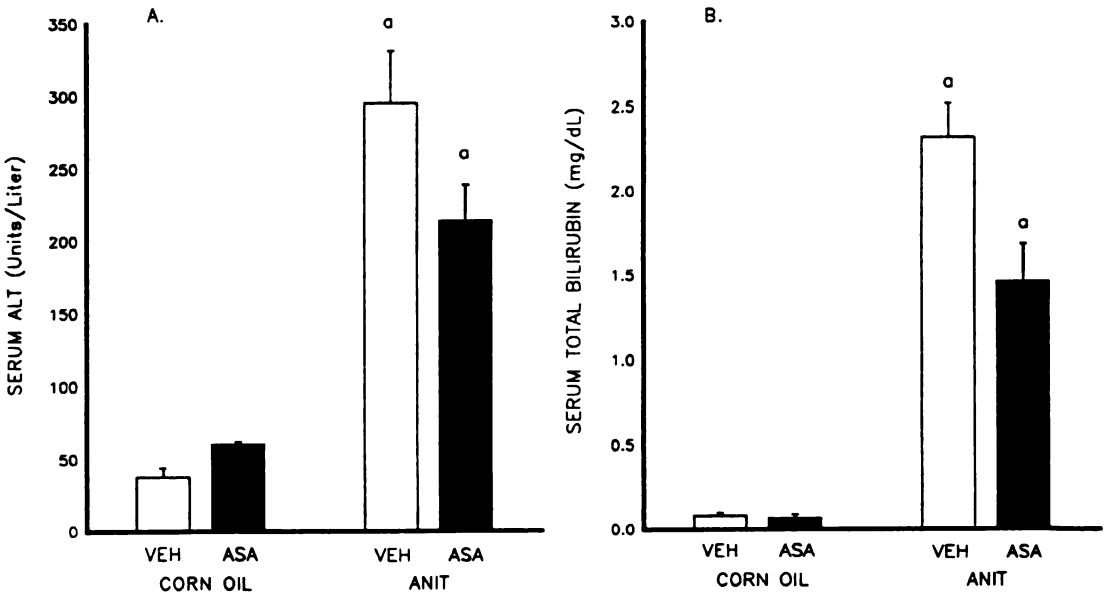

A.
PLASMA ALT (Units/Liter)
300
250
200
150
100
50
0
a
a,b
CAb
PAb
CAb
PAb
CORN OIL
ANIT
B.
PLASMA TOTAL BILIRUBIN (mg/dl)
2.0
1.5
1.0
0.5
0.0
a
a,b
CAb
PAb
CAb
PAb
CORN OIL
ANIT
C.
BILE FLOW (µl/gm liver/30 min)
60
50
40
30
20
10
0
a
a
CAb
PAb
CAb
PAb
CORN OIL
ANIT
D.
Plasma GGT (Units/Liter)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
CAb
PAb
CAb
PAb
CORN OIL
ANIT

Figure 3. Effect of aspirin (ASA) on indices of ANIT-induced liver injury. Rats received a single, oral administration of ASA or vehicle 1 hr prior to ANIT or corn oil vehicle administration as outlined in Methods. Twenty-four hours after ANIT or vehicle administration, rats were killed and markers of liver injury were evaluated. A. Serum ALT activity. B. Serum total bilirubin concentration. C. Serum GGT activity. Bars represent means ± SE. N = 6-15. *Signficantly different from respective corn oil treated group.


A.
SERUM ALT (Units/Liter)
350
300
250
200
150
100
50
0
a
a
VEH
ASA
CORN OIL
VEH
ASA
ANIT
B.
SERUM TOTAL BILIRUBIN (mg/dL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH
ASA
CORN OIL
VEH
ASA
ANIT

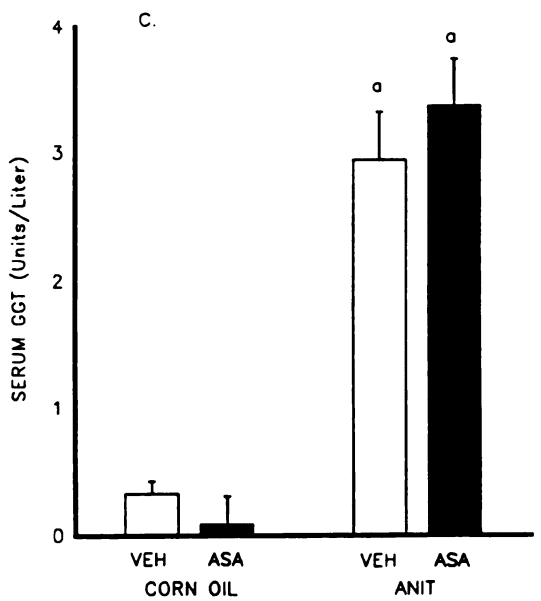
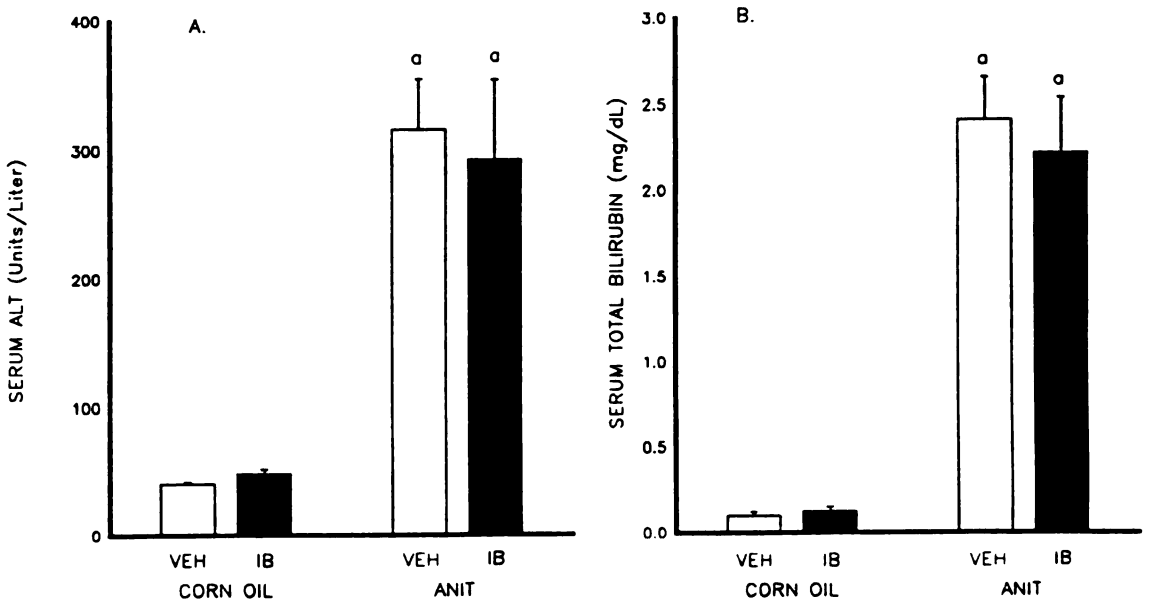
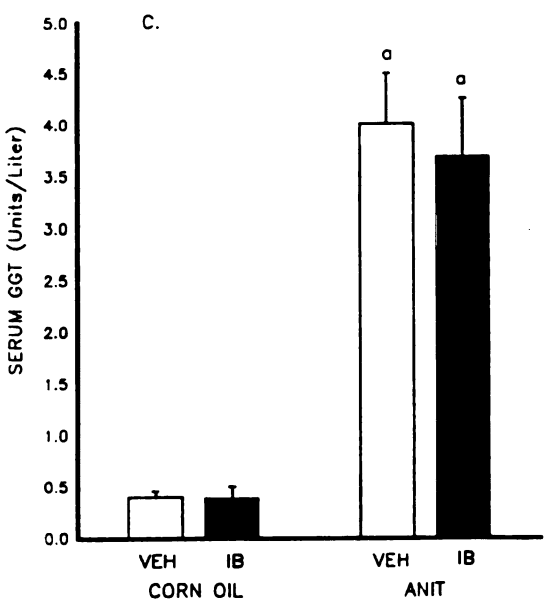

C.
SERUM GGT (Units/Liter)
4
3
2
1
0
a
a
VEH
ASA
CORN OIL
VEH
ASA
ANIT

Figure 4. Effect of ibuprofen (IB) on markers of ANIT-induced hepatotoxicity. IB or vehicle was administered 1 hr prior to ANIT or corn oil vehicle and every 8 hr thereafter. Twenty-four hours after ANIT or vehicle administration, rats were killed and markers of liver injury were assessed. A. Serum ALT activity. B. Serum total bilirubin concentration. C. Serum GGT activity. Bars represent means ± SE. N= 6-15. *Significantly different from respective corn oil treated group.


A.
SERUM ALT (Units/Liter)
400
300
200
100
0
a
a
VEH
IB
VEH
IB
CORN OIL
ANIT
B.
SERUM TOTAL BILIRUBIN (mg/dL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH
IB
VEH
IB
CORN OIL
ANIT


C.
SERUM GGT (Units/Liter)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH
IB
VEH
IB
CORN OIL
ANIT

Discussion

Inflammation appears to play a prominent role in the expression of ANIT hepatotoxicity. The histologic lesion caused by ANIT administration consists of periportal inflammatory cell infiltrate, edema, biliary epithelial cell necrosis and hepatic parenchymal cell injury. Neutrophils are requisite for ANIT-induced liver injury (Dahm et al., 1991), but the mechanism(s) of neutrophil infiltration into periportal regions and how they contribute to toxicity have not been elucidated. This study demonstrates that platelet depletion attenuates the hepatic parenchymal cell insult and hyperbilirubinemia caused by ANIT administration. However, plasma enzyme analysis and histologic analysis of livers indicated that ANIT-induced biliary epithelial cell injury was not diminished by platelet depletion. Thus, platelets seem to play a role in injury to hepatic parenchymal cells but not to bile duct epithelium in this model.

Generally, markers of hepatic parenchymal cell injury, biliary epithelial cell insult and cholestasis change in concert after ANIT administration. Previous reports of interventions which modulate ANIT toxicity altered all markers of liver injury assessed (Dahm and Roth, 1991; Dahm et al., 1991; Traiger et al., 1984). To our knowledge, this is the first evidence of dissociation of hepatic parenchymal cell injury and biliary epithelial injury in this model. The dissociation of two related pathophysiologic changes caused by ANIT suggests that the toxicity may involve several mechanisms, at least one of which requires platelets. Although platelets apparently participate in causing or facilitating ANIT-induced liver injury, the

mechanism by which platelets contribute to the expression of toxicity remains unknown.

Bile flow and plasma bilirubin concentration are markers of cholestasis. Although platelet depletion ameliorated ANIT-induced hyperbilirubinemia, it did not attenuate the decrease in bile flow caused by ANIT. These disparate effects on hyperbilirubinemia and bile flow may reflect the different effects that platelet depletion had on injury to the two major hepatic cell types affected by ANIT. As noted above, platelet depletion partially protected from ANIT-induced hepatic parenchymal cell injury, but it did not protect biliary epithelial cells. Each of these cell types contribute in a different way to bile formation. The hepatic parenchymal cell contributes most of the solutes in bile, including bile acids, salts and bilirubin, whereas the biliary epithelium provides bicarbonate and approximately 50% of the bile fluid (Berne and Levy, 1988). It is possible that the effect of ANIT on hepatic parenchymal cells leads to reduced transport of bilirubin into bile with consequent reflux of this solute into the blood, whereas ANIT's effect on biliary epithelium contributes to the reduction in bile flow. If so, selective protection of hepatic parenchymal cells would be expected to result in lowered plasma bilirubin concentration without affecting the impairment of bile flow.

Platelets are a rich source of vasoactive and inflammatory mediators, including TxA_2, 5-hydroxytryptamine, epinephrine, histamine and 12-HPETE (Cohen, 1980; Holmsen et al., 1969). These agents contribute to inflammation and tissue injury

by inducing inflammatory cell chemotaxis and activation. They can also modulate vascular tone and permeability, thereby altering regional blood flow and extravascular fluid accumulation. Thus, platelets may be important in ANIT-induced liver injury through release of inflammatory mediators or modulation of liver cell function.

The prostanoids, a group of arachidonic acid metabolites produced by cyclooxygenases, are important in controlling a variety of physiologic responses including platelet aggregation, vasoactivity and vascular permeability. For example, cyclooxygenase acts on arachidonate released from membranes of stimulated platelets to produce PGH_2, which is further metabolized in platelets to TxA_2. TxA_2 promotes platelet aggregation and causes vasoconstriction (Moncada *et al.,* 1985) and may alter regional blood flow by these actions. Thus, it seemed plausible that platelets contribute to ANIT-induced liver injury through the formation and release of this prostanoid. ASA and IB modify platelet function through an inhibitory action on cyclooxygenase enzymes and consequent inhibition of synthesis of TxA_2. However, pretreatment of rats with these drugs, at doses demonstrated to modulate platelet function, did not attenuate ANIT-induced hepatotoxicity. In addition, ANIT treatment in vivo did not enhance platelet responses to collagen, a stimulus that causes aggregation through the formation of TxA_2. Taken together, these results suggest that platelet-derived prostanoids are not of primary importance in ANIT hepatotoxicity.

In summary, platelet depletion provides partial protection from ANIT-induced liver injury. This finding implicates platelets as important for the expression of ANIT hepatotoxicity. Although platelets appear to play a role in ANIT-induced hepatic parenchymal cell injury, they do not apparently contribute to the biliary epithelial cell insult that follows ANIT administration. The lack of effect of cyclooxgenase inhibitors suggests that prostanoids do not participate in causing injury. The mechanism(s) by which platelets contribute to ANIT hepatotoxicity and the possible interactions of platelets with other inflammatory mediators remain to be elucidated.

CHAPTER IV

PLATELET ACTIVATING FACTOR RECEPTOR BLOCKADE ALONE OR IN COMBINATION WITH LEUKOTRIENE SYNTHESIS INHIBITION DOES NOT AMELIORATE α-NAPHTHYLISOTHIOCYANATE-INDUCED HEPATOTOXICITY

Summary

Alpha-naphthylisothiocyanate (ANIT) is a cholangiolitic hepatotoxicant that causes periportal edema, hepatic parenchymal and biliary epithelial cell necrosis, and cholestasis in the rat. A hallmark of ANIT hepatotoxicity is periportal inflammation that includes neutrophil infiltration. Neutrophils are requisite for the expression of ANIT-induced liver injury, however the mechanism(s) of neutrophil accumulation in the liver and the role of these cells in ANIT hepatotoxicity are incompletely understood. Platelet activating factor (PAF) is an inflammatory agent capable of producing many pathophysiologic changes consistent with those seen after ANIT intoxication. Therefore, we evaluated the role of PAF in ANIT-induced liver injury. Rats were treated with the PAF receptor antagonist WEB-2086 (WEB) to determine if it afforded protection from ANIT hepatotoxicity. In a separate study, a combination of WEB and the leukotriene synthesis inhibitor Zileuton (ZIL) was used to address the possible interaction of PAF and leukotrienes in ANIT-induced liver injury. Treatment of rats with WEB, alone or in combination with Zileuton, did not attenuate ANIT-induced liver injury as assessed by increases in serum alanine aminotransferase or gamma-glutamyl transferase activities. In addition, neither treatment ameliorated ANIT-induced cholestasis, assessed as increased plasma bilirubin concentration. These results suggest that PAF, alone or in combination with products of the 5-lipoxygenase enzyme, do not contribute to ANIT-induced liver injury.

Introduction

Inflammation is a prominent feature of the hepatotoxicity produced by *α*-naphthylisothiocyanate (ANIT). Rats treated with ANIT exhibit a marked accumulation of neutrophils in the periportal region of the liver, periportal edema, hepatocellular necrosis and cholestasis. Further, depletion of circulating neutrophils ameliorates ANIT-induced hepatic insult, suggesting that neutrophils play a pivotal role in the expression of ANIT hepatotoxicity (Dahm et al., 1991). The inflammatory response raises the possibility that host-derived, soluble inflammatory mediators may contribute to the liver lesion.

Platelet activating factor (acetyl glycerol ether phosphocholine, PAF) is a lipid mediator produced by leukocytes, platelets and endothelial cells (Badr et al., 1989). It is a potent inflammatory agent capable of producing platelet activation and aggregation, leukocyte chemotaxis and activation, increased vascular permeability and changes in vascular tone (Snyder, 1990; Hosford et al., 1993; Bone, 1992). PAF also activates cells to produce other soluble mediators of inflammation including eicosanoids, cytokines and superoxide anion (Snyder, 1990; Hosford et al., 1993; Yue et al., 1991; Olson et al., 1990). This mediator appears to contribute to a variety of pathologic conditions, and PAF antagonism affords protection in some models of tissue injury (Yue et al., 1991).

Administration of PAF to animals results in increased hepatic vascular resistance and increased glucose production, oxygen uptake and free radical production in

the liver (Zhou et al., 1992; Lapointe and Olson, 1989). Since PAF is produced by inflammatory cells and can generate pathophysiologic alterations consistent with those observed after ANIT administration, the role of PAF in ANIT-induced hepatic insult was assessed using the PAF receptor antagonist, WEB-2086.

Many of the pathophysiologic changes associated with PAF, including the inflammatory response, edema formation and alterations in vascular tone, can also be produced by metabolites of arachidonic acid such as the leukotrienes (LTs). In certain pathologic conditions, the biosynthesis of LTs appears to be linked to PAF (Olson et al., 1990). Moreover, with regard to the inflammation and pathophysiology of endotoxin shock, the changes can be generated by either LTs or PAF. Thus, in endotoxin shock in rats, increased survival requires abrogation of the actions of both mediators (Yoshikawa and Goto, 1992). Since a redundancy of effects of PAF and LTs exists in at least one model of systemic injury, the possibility of this relationship existing in ANIT hepatotoxicity was addressed using a combined treatment with WEB and the leukotriene biosynthesis inhibitor, Zileuton.

Materials and Methods

Materials.

PAF was purchased from CalBiochem (San Diego, CA). WEB-2086 was a gift from Boehringer Ingelheim Corp. (Ridgefield, CT). Zileuton was a gift from Abbott Laboratories (Abbott Park, IL).

Animals.

Male, Sprague-Dawley rats (VAF/plus, CF:CD(SD)BR, Charles River, Portage MI) weighing 150-250g were maintained as described in Methods Chapter II.

Methods.

The effect of PAF receptor antagonism on ANIT-induced liver injury was addressed by administration of WEB (10 mg/kg, 3 ml/kg, ip) or saline vehicle to rats 1 hr prior to giving either ANIT (35 mg/kg, 2 ml/kg, po) or corn oil vehicle. They received WEB or saline every 8 hr thereafter. To evaluate the possible redundancy of PAF and LTs, rats were treated with a combination of WEB and Zileuton. In experiments assessing the combined effect of WEB and Zileuton, both groups of rats received ANIT (35 mg/kg, po). One group of rats received WEB (10 mg/kg in saline) and Zileuton (40 mg/kg) 1 hr prior to administration of ANIT and thereafter every 8 or 6 hr, respectively. The control group of rats received saline vehicle (3 ml/kg,ip) and methylcellulose vehicle (4 ml/kg,po) 1 hr prior to ANIT adminstration and 8 or 6 hr thereafter, respectively.

Twenty-four hours after ANIT administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and placed on a heating pad to maintain body temperature. A midline laparotomy was performed for measurement of bile flow as previously described (Dahm and Roth, 1991). Rats were euthanized via exsanguination from the descending aorta, and collected blood was used for measurement of markers of liver injury. Serum total bilirubin content and gamma-glutamyl transferase (GGT) activity were measured as previously described (Dahm and Roth, 1991). Serum alanine aminotransferase (ALT) activity was measured based on the method of Bergmeyer et al. using Sigma Kit 59-20 (1978). Liver sections were collected and fixed in 10% phosphate-buffered formalin for histologic analysis.

Statistical analysis:

Results are expressed as the mean ± SE. Statstistical analysis and data manipulation were carried out as described in Methods Chapter II

Results and Discussion

ANIT administration caused hepatic parenchymal and biliary epithelial insult as demonstrated by increases in serum ALT and GGT activities, respectively (Fig.1). This was confirmed by histologic analysis of livers from ANIT-treated rats. ANIT-induced cholestasis was reflected by increased serum total bilirubin concentration and decreased bile flow. These changes are consistent with previous reports of ANIT-induced liver injury (Dahm et al, 1991).

To test whether PAF has a role in ANIT hepatotoxicity, rats were pretreated with WEB, a PAF receptor antagonist, prior to and after administration of ANIT. In preliminary studies, efficacy of PAF receptor antagonism over the course of 8 hr was demonstrated by attenuation of systemic hypotension after iv administration of PAF to rats (data not shown). Pretreatment with WEB failed to obtund ANIT-induced hepatotoxicity. This was reflected in histologic evidence of hepatic insult and the lack of attenuation of any of the markers of liver injury evaluated (Fig.1).

The recent demonstration that blockade of the actions of both PAF and cysteinyl leukotrienes are requisite for attenuation of pathophysiologic changes in a rat model of endotoxin shock suggests a redundancy of actions of these two mediators of inflammation (Yoshikawa and Goto, 1992). It seemed plausible that this relationship also exists in the ANIT model of hepatotoxicity. To test this, the effects of both mediators were abrogated with WEB and Zileuton, an inhibitor of LT biosynthesis.

The efficacy of Zileuton inhibition of leukotriene biosynthesis has been verified using in vivo treatment with Zileuton and ex vivo stimulation of whole blood with the calcium ionophore A23187. Six hours after Zileuton treatment, LTB_4 production was inhibited by at least 85%. Cotreatment with Zileuton and WEB failed to ameliorate ANIT-induced liver injury. This was reflected in histologic evidence of hepatic insult and the lack of attenuation of any of the serum markers of liver injury evaluated after ANIT administration (Table 1).

PAF is capable of producing many of the pathophysiologic changes associated with ANIT intoxication, including leukocyte chemotaxis and activation, platelet activation, alterations in vascular resistance and enhanced fluid extravasation (Snyder, 1990; Hosford et al., 1993; Cotran et al., 1989). Further, at least one model of tissue insult demonstrates redundant effects of LTs and PAF. The findings of the present study suggest that PAF, alone or in combination with products of the 5-lipoxygenase enzyme, does not contribute to ANIT-induced liver injury. However, numerous other mediators of inflammation have been implicated in tissue injury, and the role of these agents in ANIT hepatotoxicity remains to be addressed.

Figure 1. Effect of WEB on ANIT-induced liver injury.

Rats were treated with ANIT (35 mg/kg, po) or corn oil. In addition, each rat received either WEB (10 mg/kg, ip) or saline 1 hr prior to ANIT and every 8 hr thereafter. Twenty-four hours after ANIT administration, rats were euthanized, and markers of liver injury were evaluated as described in Methods. (A) Serum ALT activity, (B) Serum total bilirubin concentration, (C) Serum GGT activity, (D) Bile flow. Bars represent means ± SE. N = 6-11. *Significantly different from respective corn oil treated group.

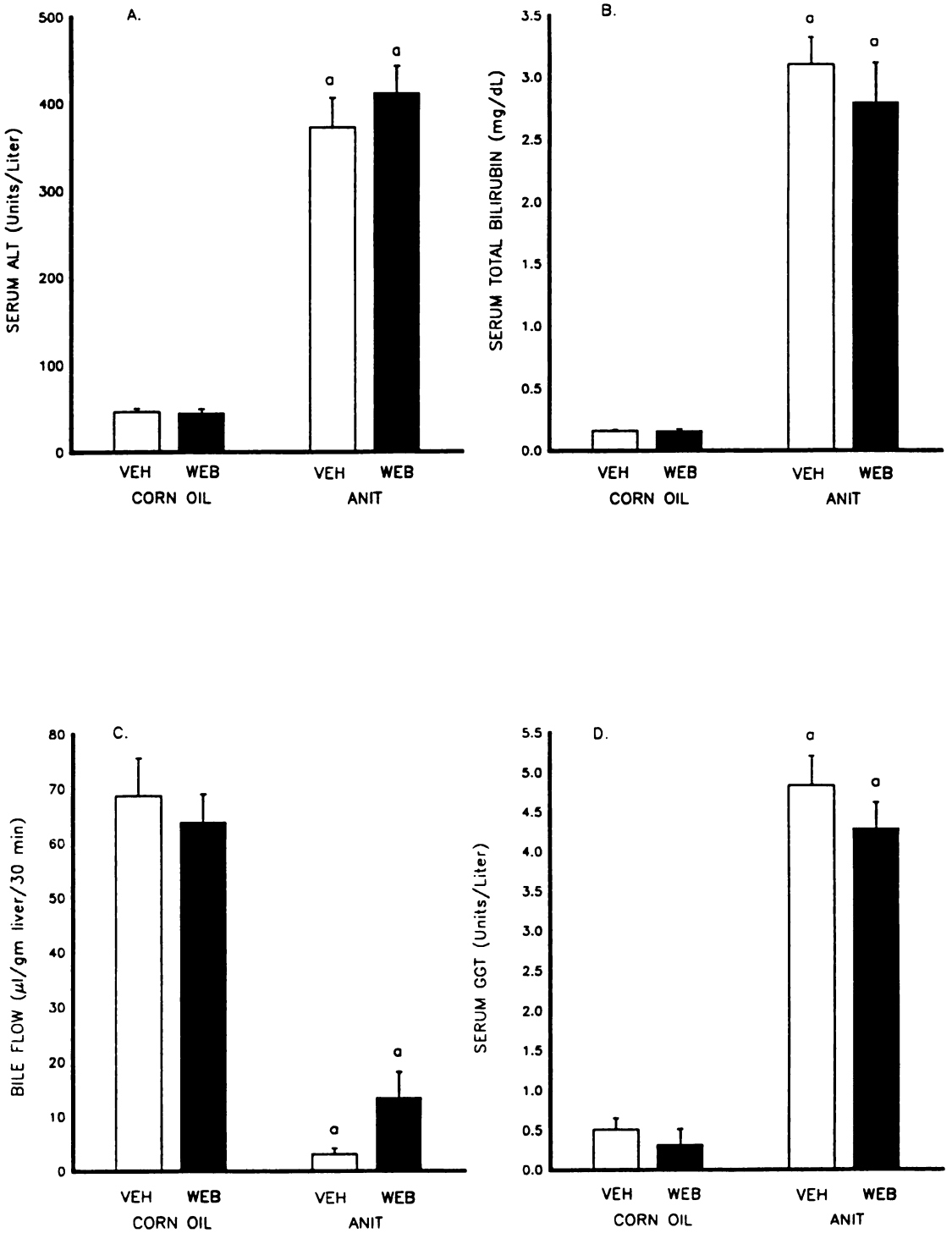

A.
SERUM ALT (Units/Liter)
500
400
300
200
100
0
a
a
VEH
WEB
CORN OIL
VEH
WEB
ANIT
B.
SERUM TOTAL BILIRUBIN (mg/dL)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH
WEB
CORN OIL
VEH
WEB
ANIT
C.
BILE FLOW (µl/gm liver/30 min)
80
70
60
50
40
30
20
10
0
a
a
VEH
WEB
CORN OIL
VEH
WEB
ANIT
D.
SERUM GGT (Units/Liter)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH
WEB
CORN OIL
VEH
WEB
ANIT

TABLE 1. EFFECT OF COMBINED TREATMENT WITH WEB-2086 AND ZILEUTON ON ANIT-INDUCED LIVER INJURY

	ALT	GGT	BILIRUBIN
ANIT+VEH	385 ± 39	2.7 ± 0.6	2.9 ± 0.2
ANIT+WEB/ZIL	285 ± 31	3.2 ± 0.4	3.2 ± 0.2

Rats were treated with ANIT (35 mg/kg, po). In addition, each rat received either WEB (10 mg/kg, ip) and Zileuton (40 mg/kg, po) or the appropriate vehicles (saline and methylcellulose) 1 hr prior to ANIT and every 8 hr and 6 hr thereafter, respectively. Twenty-four hours after ANIT administration, rats were euthanized and serum markers of liver injury were assessed as described in Methods. Values represent means ± SE. N = 4-6.

CHAPTER V

VITAMIN A OR ZYMOSAN PRETREATMENT ATTENUATES α-NAPHTHYLISOTHIOCYANATE-INDUCED LIVER INJURY

Summary

α-Naphthylisothiocyanate (ANIT) is a cholangiolitic hepatotoxicant that causes periportal hepatic insult in the rat that is neutrophil-dependent. Since macrophages have recently been implicated as participants in chemically induced hepatotoxicities, we evaluated the role of these cells in ANIT-induced hepatic insult. Rats were treated with either gadolinium chloride ($GdCl_3$), an agent which decreases hepatic macrophage numbers and activity, zymosan, an agent which increases hepatic macrophage numbers, or vitamin A, which increases hepatic macrophage activity. $GdCl_3$ did not ameliorate ANIT-induced hepatotoxicity, as demonstrated by a lack of attenuation of any of the markers of hepatic insult evaluated. In contrast, either zymosan or vitamin A pretreatment decreased ANIT hepatotoxicity. To investigate the protective mechanism(s) of zymosan, blood leukocyte numbers and peritoneal neutrophil numbers were assessed after intraperitoneal glycogen administration. Zymosan caused a systemic leukopenia and neutropenia. Furthermore, zymosan decreased the influx of neutrophils into the peritoneum after intraperitoneal glycogen administration, a result which suggests that an alteration in neutrophil numbers or function may mediate the protection afforded by zymosan. To determine if macrophages were important in the protection by vitamin A, rats were cotreated with $GdCl_3$ and vitamin A. $GdCl_3$ did not alter the protection from ANIT hepatotoxicity afforded by vitamin A. Vitamin A treatment decreased biliary ANIT and glutathione concentrations at 1 and 4 hr after ANIT administration, while having only a minimal effect on plasma ANIT concentration. In summary, pretreatment of rats with zymosan or vitamin A but not

$GdCl_3$ attenuated ANIT-induced liver injury. The protection afforded by zymosan may relate to its effect on neutrophil numbers or responsiveness. The protection by vitamin A appears to result from its effect on the transport of ANIT into bile. The results suggest that hepatic macrophages are not required for the manifestation of ANIT hepatotoxicity.

Introduction

α-Naphthylisothiocyanate (ANIT) is an experimental, cholangiolitic hepatotoxicant that causes cholestasis, hyperbilirubinemia, biliary epithelial cell necrosis and modest hepatic parenchymal cell injury after oral administration to rats (McLean and Rees, 1958; Goldfarb et al., 1962; Ungar et al., 1962). A prominent feature of the hepatic lesion caused by ANIT is neutrophil infiltration associated with the areas of hepatic parenchymal and biliary epithelial cell insult (McLean and Rees, 1958; Goldfarb et al., 1962; Ungar et al., 1962). Depletion of circulating neutrophils or platelets ameliorates ANIT-induced liver injury, suggesting that these inflammatory cells are critical for the expression of this chemical insult (Dahm et al., 1991; Roth et al., 1994). However, the role of other cells which contribute to inflammation such as hepatic macrophages (eg, Kupffer cells) has not been addressed.

Recently, the macrophage has gained attention as a potential contributor to chemically induced liver injury. Of interest are both Kupffer cells and migratory, blood-borne macrophages. Macrophages produce a variety of physiologically active, soluble mediators, many of which are proinflammatory and can contribute to tissue injury (Hagmann and Keppler, 1988; Decker, 1990; Cotran et al., 1989). Furthermore, these cells have the capacity to produce reactive oxygen metabolites and to release cytolytic enzymes. Accordingly, the macrophage, like the neutrophil, has the capacity to produce tissue injury.

Vitamin A is an agent that enhances Kupffer cell function and magnifies the

hepatotoxicity of carbon tetrachloride (ElSisi et al., 1993a). Enhancement of carbon tetrachloride hepatotoxicity by vitamin A can be eliminated by treatment with methyl palmitate, an agent which inhibits Kupffer cell activity (ElSisi et al., 1993a). Vitamin A pretreatment also increases the hepatotoxicity of allyl alcohol and bacterial endotoxin (ElSisi et al., 1993b). These results suggest that Kupffer cells can enhance the liver injury caused by low doses of certain hepatotoxicants. Further evidence for the potential involvement of Kupffer cells in chemically induced liver injury is demonstrated by studies using $GdCl_3$, an agent which decreases Kupffer cell function and numbers (Hardonk et al., 1992). $GdCl_3$ has been reported to ameliorate allyl alcohol- and carbon tetrachloride-induced liver injury in certain treatment paradigms, further supporting the possibility that macrophages may contribute to hepatotoxicity (Przybocki et al., 1992; Edwards et al., 1993).

The observation that neutrophil or platelet depletion attenuates ANIT-induced liver injury suggests that the inflammatory response may be critical for the full expression of ANIT hepatotoxicity (Dahm et al., 1991; Roth et al., 1994). However, the involvement of Kupffer cells or migratory macrophages in ANIT-induced hepatic insult has not been addressed. Accordingly, pharmacologic manipulations with agents that increase hepatic macrophage numbers or modify Kupffer cell function were used to assess the contribution of these cells to ANIT hepatotoxicity.

Materials and Methods

Materials:

ANIT, gamma-glutamylglutamate, iodoacetic acid, glutathione, L-gamma-glutamyl-p-nitroanilide, Kit 605-D for bilirubin determination, Kit 59 for alanine aminotransferase activity, $GdCl_3$ and zymosan were purchased from Sigma Chemical Co. (St. Louis, MO). Vitamin A (Aquasol A) was purchased from Astra Pharmaceutical Products, Inc. (Westborough, MA). High-performance Liquid Chromatography (HPLC) reagent grade methanol and acetonitrile were purchased from VWR Scientific (Chicago, IL). All other reagents were of the highest quality commercially available.

Animals:

Male, Sprague-Dawley rats (VAF/plus, CF:CD(SD)B, Charles River, Portage MI) weighing 200-300g were maintained as described in Methods Chapter II.

Methods:

Gadolinium chloride pretreatment and ANIT hepatotoxicity:

$GdCl_3$ (10mg/kg, 0.5ml/kg, iv) or saline vehicle was administered to rats 24hr prior to ANIT (35mg/kg, 2ml/kg, po) or corn oil vehicle. Twenty-four hours after ANIT administration, rats were anesthetized with sodium pentobarbital (50mg/kg, ip) and placed on a heating pad to maintain body temperature. A midline laparotomy was performed, and the bile duct was cannulated for measurement of bile flow as previously described (Dahm and Roth, 1991). After bile collection, rats were

exsanguinated via the descending aorta, and collected blood was used for the measurement of markers of liver injury. Serum total bilirubin content was measured spectrophotometrically as previously described (Dahm and Roth, 1991) and used as an index of cholestasis. Serum gamma-glutamyltransferase (GGT) activity was evaluated by a modification of the method of Szaz (1969) and was assumed to reflect biliary epithelial integrity. Enzymatic evaluation of hepatic parenchymal cell integrity was carried out using spectrophotometric analysis of serum ALT activity (Sigma kit 59) based on the method of Bergmeyer et al. (1978). Liver sections were collected and fixed in 10% phosphate-buffered formalin for histologic analysis.

Vitamin A administration and ANIT hepatotoxicity:

For 7 days prior to ANIT (35mg/kg, 2ml/kg, po) or corn oil administration, rats received vitamin A (250,000 IU/kg, 5ml/kg, po) or vehicle (7% tween 20, 10% propylene glycol in distilled water) daily. Animals were denied access to food after the last dose of vitamin A or vehicle, and 24 hr later ANIT was administered. This dosing regimen was based on previously reported protocols (ElSisi et al., 1993a) Twenty-four hours after ANIT administration, rats were anesthetized and processed as described above.

Effect of zymosan pretreatment on ANIT-induced liver injury:

Rats received zymosan (25mg/kg, 1.5ml/kg, iv) or saline vehicle 48 and 24 hr prior to ANIT (35mg/kg, 2ml/kg, po) or corn oil administration. The dosing regimen with zymosan was a modification of previously reported protocols (Bouwens et al., 1984; Bouwens and Wisse, 1985; Bouwens et al., 1986). Twenty-four hours after ANIT administration, rats were anesthetized and processed as described above.

Zymosan and leukocyte numbers:

To determine the effect of zymosan on circulating leukocyte numbers and the capacity of glycogen to recruit neutrophils into the peritoneum, rats were treated with zymosan (25mg/kg, 1.5ml/kg, iv) or saline vehicle 48 and 24 hr prior to glycogen (1%, 30ml/rat, ip) or saline administration. Four hours after glycogen treatment, rats were anesthetized and blood was collected via the descending aorta into sodium citrate (0.38% final concentration). Total leukocyte numbers in the blood samples were determined on a Coulter Counter Model ZM. Differential leukocyte counts were assessed on blood smears stained with Wright-Giemsa stain.

In a separate set of experiments, rats were treated with zymosan or saline and with glycogen as described above. Four hours after glycogen administration, peritoneal neutrophils were harvested by peritoneal lavage as previously described (Dahm et al., 1991) and quantified by hemocytometric enumeration.

Effect of Vitamin A pretreatment on ANIT disposition and bile glutathione status:

Rats were treated with vitamin A and ANIT as described above. At one and 4hr after ANIT administration, rats were anesthetized and bile was collected into 250ul of 10% perchloric acid containing 1mM bathophenanthroline disulfonic acid for HPLC measurement of bile ANIT and glutathione concentration. Rats were exsanguinated, and citrated blood (0.38% final citrate concentration) was collected for determination of plasma ANIT concentration.

Glutathione was quantified as described by Fariss and Reed (1987) using high performance liquid chromatographic (HPLC) separation followed by integration of the area under the resulting peaks. Bile was spun in a centrifuge (15,000 x g, 1 min), and an aliquot of the supernatant (0.1ml) was mixed with 50ul iodoacetic acid (20 mg/ml) in 0.2mM m-cresol. The pH was adjusted to greater than 8.0 with 2M KOH/2.4M $KHCO_3$. After 30 min, 1-fluoro-2,4-dinitrobenzene (1% in absolute ethanol) was added, and the samples were stored in the dark at room temperature for 16-24 hr. Glutathione content of liver was determined by homogenizing frozen liver in 4 ml 10% perchloric acid containing 1mM bathophenanthroline disulfonic acid, spinning the homogenate in a centrifuge to remove debris (400xg, 10 min) and assaying the supernatant fluid (0.3 ml) as described for bile.

ANIT concentration in bile samples was determined by mixing 0.3 ml of bile with 1.2 ml 40% acetonitrile containing 0.1% glacial acetic acid. Plasma samples were

prepared by mixing 0.5 ml plasma with 1.0 ml of 60% acetonitrile containing 0.1% glacial acetic acid. The mixtures were allowed to stand for 30 min at 4° C, and precipitated material was subsequently removed by centrifugation (15,000 x g, 2 min). Resulting supernatant fluids were injected onto a 4mm X 250mm, reverse phase, 5 micron ODSII column (Custom LC, Houston TX) and eluted (1.0ml/min) with a linear gradient of acetonitrile containing 0.1% glacial acetic acid (51% to 90% acetonitrile from 0 to 5 min, 90% acetonitrile from 5-18 min). Detection of ANIT was performed by measuring the absorbance at 308 nm. A standard curve was constructed from the areas of peaks obtained after injection of known amounts of ANIT. Identification of ANIT in biologic samples was based on retention time comparisons (samples with and without added ANIT) and absorbance characteristics (Carpenter-Deyo et al., 1991).

HPLC analysis was carried out using a system comprising two Waters HPLC 510 pumps, a Waters 717 WISP autosampler and a Waters 486 variable wavelength detector. Millennium 2010 Chromatography Manager software (Millipore Corp., Milford, MA) was used for the control of instrument operation and peak integration.

Effect of $GdCl_3$ on vitamin A-treated rats:

To determine if the effect of vitamin A on ANIT-treated rats was related to an action on Kupffer cells, rats were treated with vitamin A and $GdCl_3$. Rats received vitamin A pretreatment as described above. Twenty-four hours prior to ANIT treatment

(35mg/kg, 2ml/kg, po), rats received either $GdCl_3$ (10mg/kg, 0.5ml/kg, iv) or vehicle. Twenty-four hours after ANIT treatment, they were anesthetized and processed for determination of liver injury as described above.

To ensure that $GdCl_3$ administration reduced Kupffer cell function, the half-life of colloidal carbon in blood was determined in vitamin A treated rats given $GdCl_3$ using a modification of the method of Way et al., (1985). Under pentobarbital anesthesia (50 mg/kg, ip), a midline laparotomy was performed and colloidal carbon was injected into the inferior vena cava of rats. At 1,2,3,4,5,10 and 15 min after carbon administration, blood samples (50 μl) were collected from a non-obstructive, indwelling catheter (25 ga) placed in the descending aorta and mixed with a 0.1% Na_2CO_3 solution. The absorbance of hemolyzed blood at 640nM was determined and compared to a blank (blood drawn before the administration of carbon). The changes in absorbance with time were used to determine the half-life of carbon.

Analysis of Data:

Results are expressed as the mean ± SE. Homogeneity of variance was tested with the F-max test. Prior to further analysis, a log-transformation was performed on nonhomogeneous data. Data were analyzed with a completely random, two-way analysis of variance (ANOVA) or a one-way ANOVA, as appropriate. Comparisons between treatment means were carried out using Tukey's omega test, Student's T-test or the least significant difference test as appropriate (Steel

and Torrie, 1980). The criterion for significance was $p \leq 0.05$.

Results

Effect of $GdCl_3$ on ANIT hepatotoxicity:

Previous studies have demonstrated that $GdCl_3$ effectively attenuates macrophage function as assessed by colloidal carbon clearance (Ganey et al., 1993). ANIT administration caused hepatic parenchymal and biliary epithelial injury as reflected in increases in serum ALT and GGT activities, respectively (Figs. 1,2,3). Injury to these cells was confirmed by histologic analysis of livers from ANIT-treated rats. Additionally, cholestasis was reflected as increased serum total bilirubin and/or decreased bile flow (Figs. 1,2,3). $GdCl_3$ treatment failed to attenuate any of the markers of ANIT-induced liver injury (Fig.1). In fact, an increase in GGT activity was observed in rats receiving ANIT and $GdCl_3$ (Fig. 1C).

Zymosan treatment and ANIT hepatotoxicity:

Zymosan-induced increases in hepatic macrophage content were confirmed by histologic evaluation of liver sections taken from zymosan-treated rats. Zymosan caused a marked increase in hepatic macrophage numbers, primarily in periportal and midzonal regions. Animals that received zymosan demonstrated smaller increases in serum ALT activity and serum bilirubin concentration than did controls treated with ANIT alone (Fig. 2). However, zymosan did not attenuate the ANIT-induced elevation in serum GGT activity.

Zymosan treatment and leukocyte numbers:

Blood taken from rats receiving zymosan demonstrated leukopenia with a prominent neutropenia (Table 1). Zymosan treatment also markedly reduced the number of neutrophils recovered in peritoneal lavage fluid from glycogen-treated rats (Table 2). Indeed, glycogen elicited only 1/5 as many neutrophils from zymosan-treated rats compared to controls. This decrease was evident whether neutrophil recovery was expressed on a whole animal or per gm body weight basis (Table 2).

Vitamin A effect on ANIT-induced liver injury:

Previous reports indicate that vitamin A treatment modulates Kupffer cell function and alters the hepatotoxicity of certain compounds (ElSisi et al., 1993a). Vitamin A pretreatment attenuated ANIT-induced hepatic parenchymal necrosis, biliary epithelial insult and cholestasis. This was evident as a decrease in all markers of liver injury (Fig. 3).

Vitamin A pretreatment and ANIT disposition:

One and 4 hr after ANIT administration, bile ANIT concentration was approximately 6μM, whereas plasma ANIT concentration was 0.7μM and 2.0μM, respectively (Fig. 4A). This result is consistent with previous findings that ANIT is concentrated in bile (Jean et al., 1994). Vitamin A pretreatment caused a marked decrease in bile ANIT concentration; however, plasma ANIT concentration was unaltered at 1 hr and only slightly elevated by vitamin A pretreatment at 4 hr (Fig. 4B). One and

4 hr after ANIT administration, biliary GSH concentration was 3.4mM and 3.9mM, respectively. Vitamin A pretreatment tended to decrease the concentration of GSH in bile after ANIT administration, although this change was not statistically significant at 1 hr (Fig. 4C). Bile flow was not significantly altered by vitamin A pretreatment (Fig. 4D).

Effect of $GdCl_3$ on vitamin A attenuation of ANIT hepatotoxicity:

$GdCl_3$ inhibition of Kupffer cell function in the presence of vitamin A was confirmed by the disappearance of colloidal carbon from plasma. The half life of colloidal carbon in blood of vitamin A-treated rats was 12.9 ± 1.3 min, while the half life in $GdCl_3$/vitamin A-treated rats was increased to 23.0 ± 2.7 min ($p \leq 0.05$, N=5-7). Furthermore, histologic evaluation of livers from $GdCl_3$/vitamin A treated rats demonstrated less carbon in phagocytic cells associated with the hepatic sinusoid, indicating decreased Kupffer cell phagocytosis of carbon. ANIT treatment alone caused the expected rise in serum ALT activity and bilirubin concentration and a decrease in bile flow (Fig 5). Vitamin A pretreatment attenuated ANIT-induced liver injury as assessed by these markers. However, treatment with $GdCl_3$ did not alter the protection afforded by vitamin A pretreatment (Fig 5).

Discussion

Hepatic macrophages may contribute to the pathologic alterations associated with a variety of models of chemically induced liver injury (ElSisi et al., 1993; Edwards et al., 1993; Przybocki et al., 1992; Laskin et al., 1986). Inflammation is a prominent feature of ANIT-induced hepatic lesions (McLean and Rees, 1958;

Goldfarb et al., 1962; Ungar et al., 1962; Dahm et al., 1991), and neutrophils contribute to the liver injury caused by ANIT (Dahm et al., 1991). Inasmuch as macrophages are activated during inflammation and products of activated macrophages interact with neutrophils in certain models of hepatic insult, we sought to determine if hepatic macrophages contribute to ANIT-induced liver injury.

Pretreatment of rats with $GdCl_3$, an agent which decreases Kupffer cell numbers and function, failed to influence ANIT hepatotoxicity, suggesting that macrophages are not required for the expression of ANIT injury. Vitamin A and zymosan have been shown to increase hepatic macrophage function or numbers, respectively, and therefore might be expected to enhance liver injury that is dependent on macrophages (ElSisi et al., 1993; Bouwens and Wisse 1985; Bouwens et al., 1984,1986). However, pretreatment of rats with either of these agents protected from, rather than enhanced, ANIT hepatotoxicity. We used a vitamin A dosing regimen shown by others to enhance carbon clearance in rats (ElSisi et al., 1993). In our studies, this regimen failed to alter significantly the disposition of colloidal carbon (data not shown), raising the possibility that a different mechanism was involved in the reduction of toxicity we observed. Thus, neither the findings with vitamin A nor those with $GdCl_3$ support a contribution of macrophages to ANIT-induced hepatic insult.

Since the finding that pharmacologic interventions with vitamin A or zymosan ameliorated ANIT hepatotoxicity was unexpected, we investigated mechanisms by

which these agents may have altered liver injury. To determine if the protective effect of vitamin A was related to alterations in macrophage function, $GdCl_3$ was administered to vitamin A-treated rats. Although $GdCl_3$ attenuated carbon clearance in vivo in vitamin A treated rats, it failed to alter the capacity of vitamin A to ameliorate ANIT toxicity, suggesting that vitamin A does not act via hepatic macrophages in decreasing the hepatotoxicity of ANIT.

Recently, Rosengren and Sipes (1994) demonstrated attenuation of carbon tetrachloride-induced hepatotoxicity in mice after vitamin A pretreatment. These investigators found that vitamin A decreases microsomal cytochrome P450IIE1 activity, the monooxygenase isoform responsible for carbon tetrachloride bioactivation. They hypothesized that vitamin A decreased carbon tetrachloride-induced liver injury by altering bioactivation rather than through its effect on Kupffer cell function (Rosengren and Sipes, 1994). Although the role of bioactivation in ANIT toxicity is incompletely understood, some evidence suggests that ANIT might require metabolism/bioactivation for complete manifestation of hepatotoxicity (Roberts and Plaa, 1965; El-Hawari and Plaa, 1977). Accordingly, the possibility exists that vitamin A provides protection from ANIT-induced hepatic insult by either decreasing ANIT bioactivation or by increasing ANIT inactivation.

Recent evidence suggests that GSH is important for the expression of ANIT hepatotoxicity (Dahm and Roth, 1991; Carpenter-Deyo et al., 1991; Jean et al., 1994). ANIT appears to accumulate rapidly in bile, and ANIT administration is

associated with a rapid increase in bile glutathione concentration (Jean et al., 1994). Glutathione may be important in this model as a substrate for conjugation to ANIT (Carpenter-Deyo et al., 1991), leading to enhanced transport of ANIT into bile. Since this increase in the bile concentration of ANIT may be important for the expression of ANIT toxicity, we evaluated the effect of vitamin A on the disposition of ANIT and GSH in bile. Vitamin A pretreatment diminished the accumulation of ANIT and glutathione in bile 1 and 4 hr after ANIT administration. Thus, the protection afforded by vitamin A may result from its ability to prevent the transport of ANIT into the bile.

Zymosan treatment causes an increase in the number of hepatic macrophages (Bouwens et al., 1986). However, zymosan also effects a variety of other inflammatory changes, including activation of neutrophils (Lesch et al., 1991; Levy et al., 1993). To address the mechanism of protection associated with zymosan administration, the influence of this compound on neutrophils was explored. Zymosan treatment of rats caused decreased peritoneal neutrophil accumulation and a marked leukopenia and neutropenia (Table 1,2). These findings suggest that zymosan alters neutrophil numbers and/or chemotactic responsiveness. Neutrophils are requisite for the expression of ANIT hepatotoxicity in vivo (Dahm et al., 1991) and ANIT activates neutrophils in vitro (Roth and Hewett, 1990). Therefore, zymosan may afford protection from ANIT-induced hepatic insult by diminishing circulating neutrophil numbers, depressing neutrophil chemotaxis and/or by altering the response of neutrophils to other priming or activating

agents.

In summary, our results suggest that macrophages do not contribute to ANIT-induced liver injury. Pretreatment with either vitamin A or zymosan ameliorates ANIT hepatotoxicity. The protective effects of vitamin A and zymosan are likely unrelated to their actions on hepatic macrophages. Vitamin A may attenuate ANIT-induced liver injury by reducing the accumulation of ANIT in bile. In contrast, zymosan may diminish ANIT hepatotoxicity by an action on neutrophil numbers or function. These results lend further support for a role of PMNs in ANIT injury and suggest that agents which alter the biliary disposition of ANIT modulate its toxicity.

TABLE 1. EFFECT OF ZYMOSAN AND GLYCOGEN TREATMENT OF RATS ON LEUKOCYTE NUMBERS IN BLOOD

cells/μL blood

	Total Leukocytes	Neutrophils	Lymphocytes	Monocytes
Saline/Saline	16470 ± 2425	1609 ± 276	14613 ± 2502	189 ± 126
Saline/Glycogen	15232 ± 960	1255 ± 443	13455 ± 809	159 ± 80
Zymosan/Saline	[a]4418 ± 455	[a]668 ± 118	[a]3700 ± 557	36 ± 8
Zymosan/Glycogen	[a]4375 ± 653	[a]353 ± 99	[a]3986 ± 715	29 ± 16

Rats received zymosan or saline and were then given glycogen or saline as described in Methods. Rats were euthanized, and citrated blood was collected for determination of total leukocyte numbers and differential cell counting. Values represent means ± SE. N = 3. [a]Significantly different from respective control group

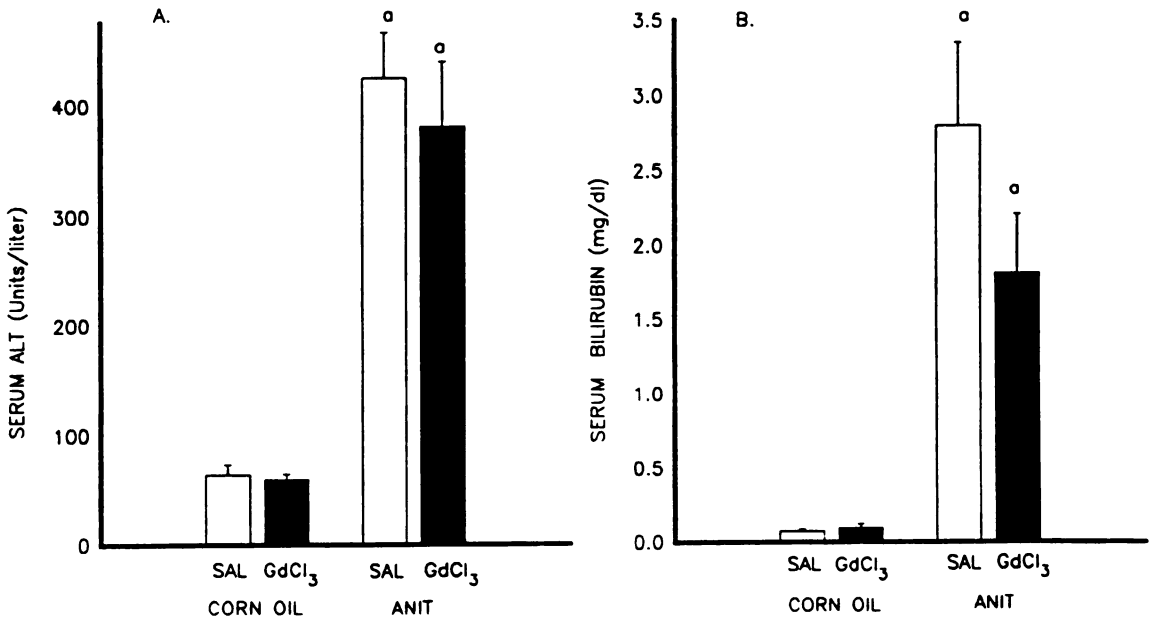
TABLE 2. EFFECT OF ZYMOSAN TREATMENT ON GLYCOGEN-INDUCED NEUTROPHIL ACCUMULATION IN RAT PERITONEUM.

	Saline	Zymosan
Neutrophils (10^6/rat)	150 ± 30	*32 ± 8
Neutrophils (10^6/gm body wt)	0.27 ± 0.06	*0.06 ± 0.02

Rats received either zymosan (25mg/kg, 1.5ml/kg iv) or saline vehicle and were then given glycogen (1% in saline, 40ml/rat, ip) as described in Methods. Four hours after glycogen administration, peritoneal lavage was carried out as previously described (Dahm et al., 1991). Neutrophil numbers were quantified by hemocytometry. Values represent means ± SE. N = 5. *Significantly different from saline group.

Figure 1. Effect of gadolinium chloride on ANIT hepatotoxicity.

Rats received $GdCl_3$ or vehicle as described in Methods. Twenty-four hours after ANIT administration, rats were killed and markers of liver injury were assessed. A) Serum ALT activity, B) Serum total bilirubin concentration, C) Serum GGT activity. Bars represent means ± SE. N = 4-8. [a]Significantly different from respective corn oil group. [b]Significantly different from Sal/ANIT group.


A.
SERUM ALT (Units/liter)
400
300
200
100
0
a
a
SAL GdCl3
CORN OIL
SAL GdCl3
ANIT
B.
SERUM BILIRUBIN (mg/dl)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
SAL GdCl3
CORN OIL
SAL GdCl3
ANIT

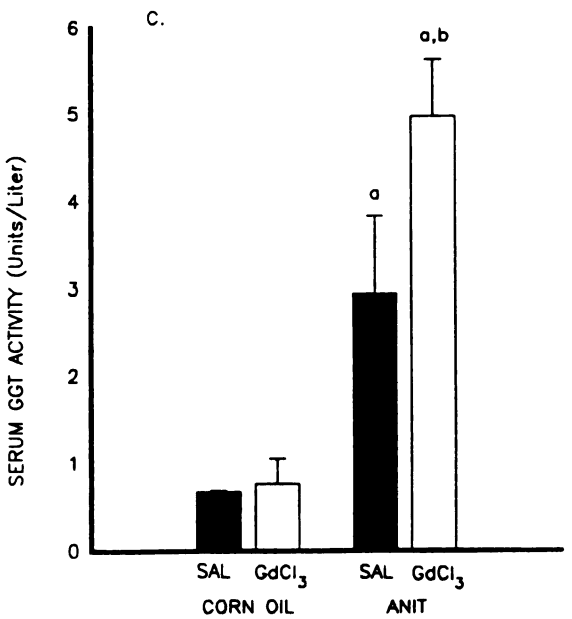
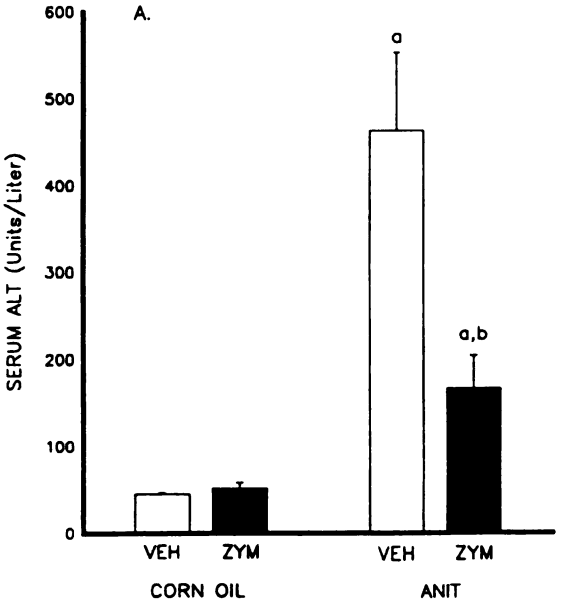
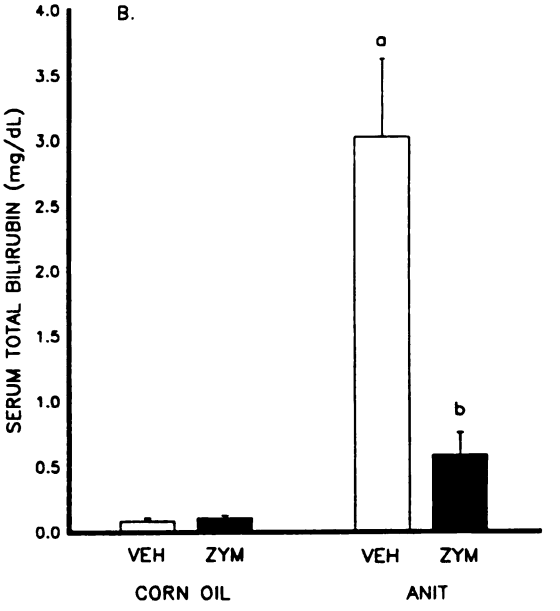

C.
SERUM GGT ACTIVITY (Units/Liter)
6
5
4
3
2
1
0
a
a,b
SAL GdCl3
CORN OIL
SAL GdCl3
ANIT

Figure 2. Zymosan treatment and ANIT hepatotoxicity.

Rats received zymosan or vehicle 48 and 24 hr prior to administration of ANIT as described in Methods. Twenty-four hours after ANIT, rats were euthanized and liver injury was determined. A) Serum ALT activity, B) Serum total bilirubin concentration, C) Serum GGT activity. Bars represent means ± SE. N = 6-8. [a]Significantly different from respective corn oil group. [b]Significantly different from VEH/ANIT.


A.
SERUM ALT (Units/Liter)
600
500
400
300
200
100
0
a
a,b
VEH
ZYM
VEH
ZYM
CORN OIL
ANIT


B.
SERUM TOTAL BILIRUBIN (mg/dL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
VEH
ZYM
VEH
ZYM
CORN OIL
ANIT

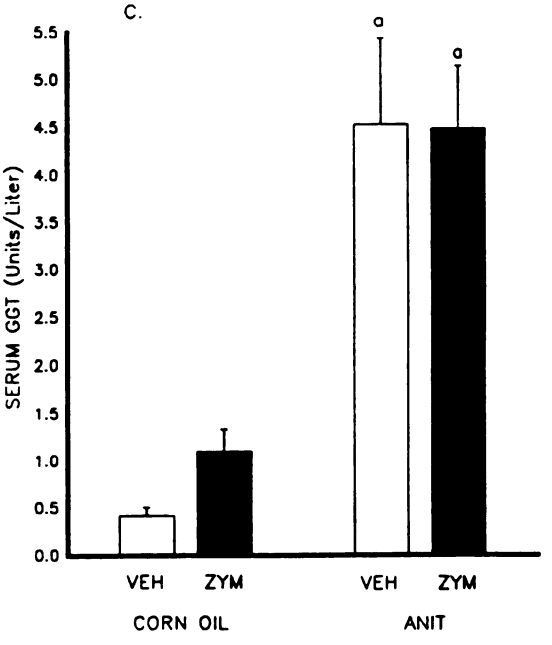
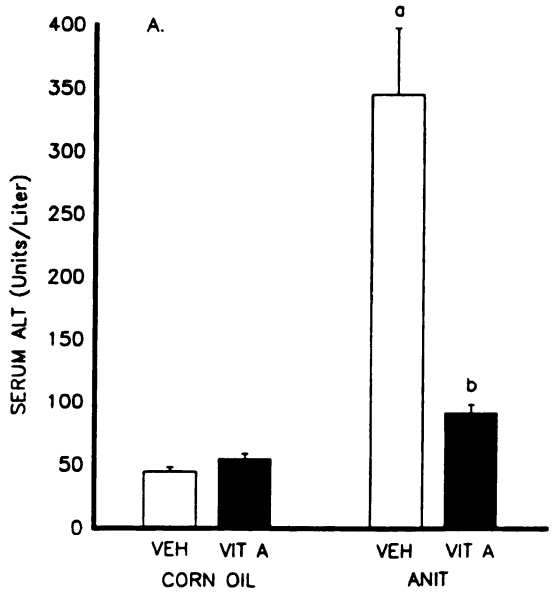
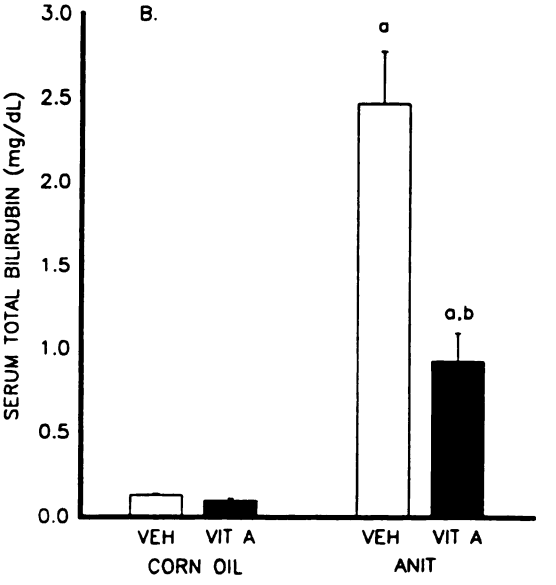

C.
SERUM GGT (Units/Liter)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
VEH
ZYM
VEH
ZYM
CORN OIL
ANIT

Figure 3. Effect of vitamin A on ANIT-induced liver injury.

Rats received vitamin A or vehicle on each of 7 days prior to ANIT or vehicle administration as outlined in Methods. Twenty-four hours after ANIT, rats were euthanized and markers of liver injury were evaluated. A) Serum ALT activity, B) Serum total bilirubin concentration, C) Serum GGT activity. Bars represent means ± SE. N = 5-12. [a]Significantly different from respective corn oil group. [b]Significantly different from VEH/ANIT group.


A.
SERUM ALT (Units/Liter)
400
350
300
250
200
150
100
50
0
a
b
VEH
VIT A
VEH
VIT A
CORN OIL
ANIT


B.
SERUM TOTAL BILIRUBIN (mg/dL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a,b
VEH
VIT A
VEH
VIT A
CORN OIL
ANIT

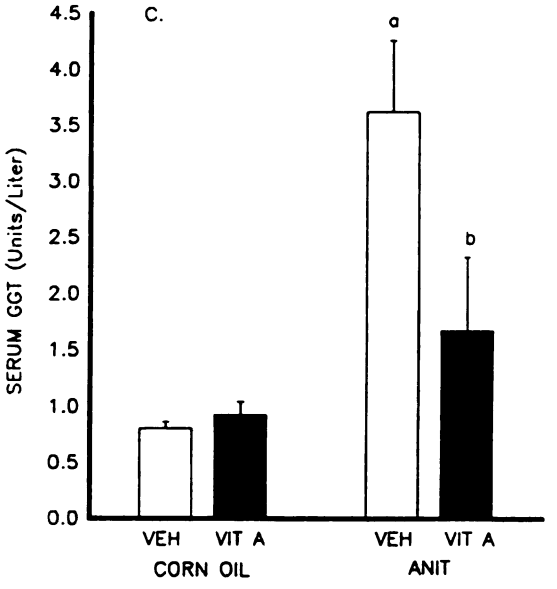
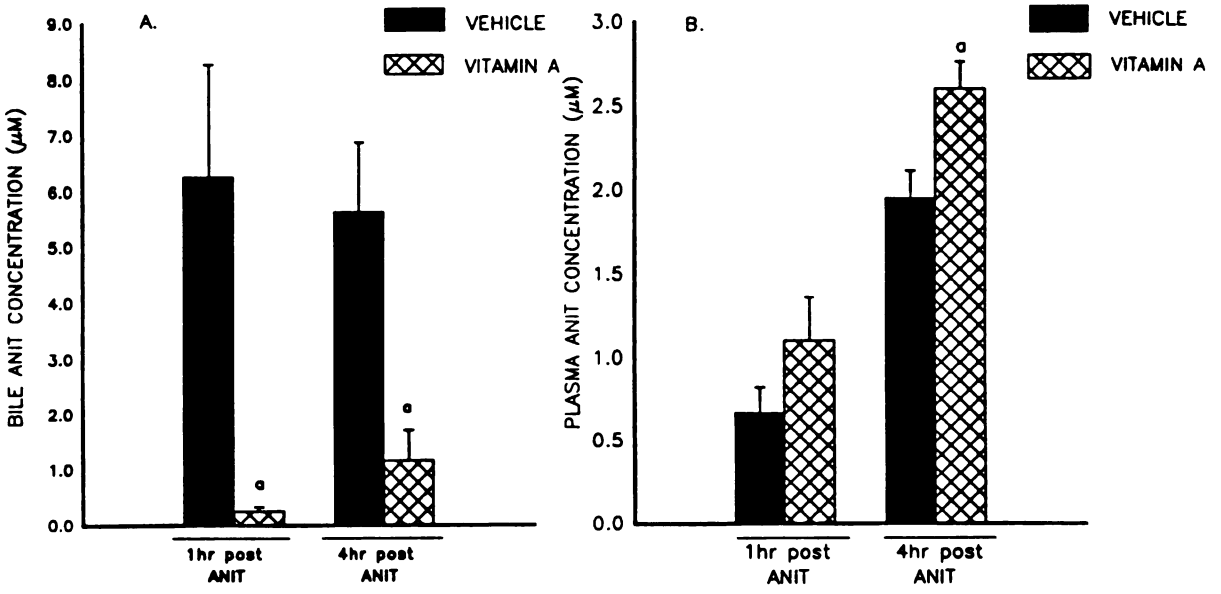

C.
SERUM GGT (Units/Liter)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
VEH
VIT A
VEH
VIT A
CORN OIL
ANIT

Figure 4. Effect of Vitamin A on ANIT disposition:

Rats were treated with vitamin A or vehicle and with ANIT as described in Methods. One and 4 hr after ANIT administration, rats were anesthetized and bile and blood were collected for measurement of ANIT and GSH. A) Bile ANIT concentration. B) Plasma ANIT concentration. C) bile GSH concentration. D) Bile flow. Bars represent means ± SE. N = 5-8. *Significantly different from vehicle treated control.


A.
VEHICLE
VITAMIN A
BILE ANIT CONCENTRATION (μM)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
a
1hr post ANIT
4hr post ANIT
B.
VEHICLE
VITAMIN A
PLASMA ANIT CONCENTRATION (μM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
1hr post ANIT
4hr post ANIT

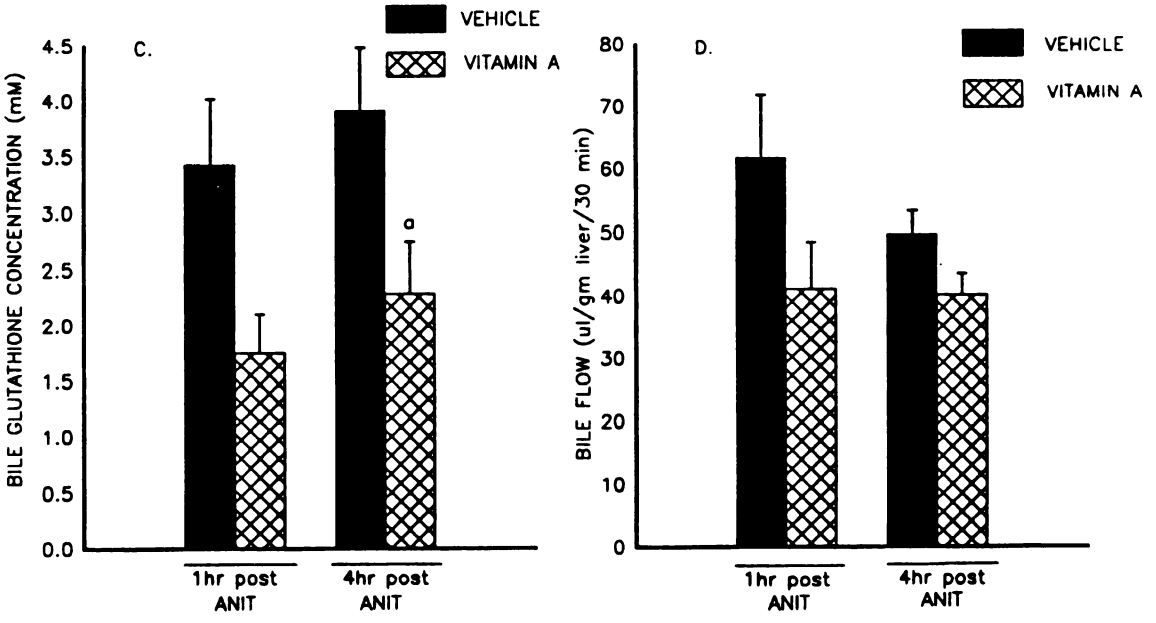
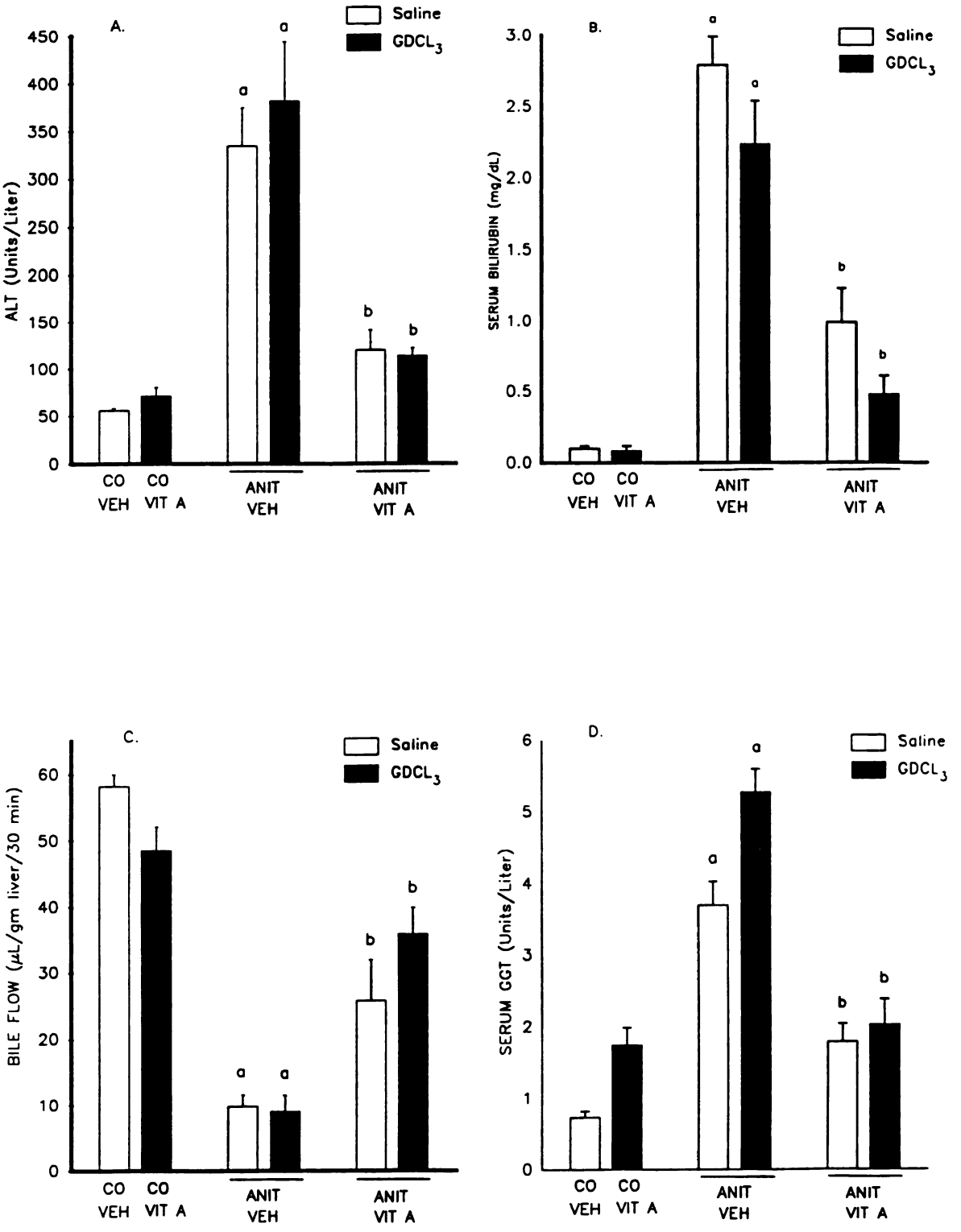

C.
VEHICLE
VITAMIN A
BILE GLUTATHIONE CONCENTRATION (mM)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
1hr post ANIT
4hr post ANIT
D.
VEHICLE
VITAMIN A
BILE FLOW (ul/gm liver/30 min)
80
70
60
50
40
30
20
10
0
1hr post ANIT
4hr post ANIT

Figure 5. $GdCl_3$ effect on vitamin A attenuation of ANIT hepatotoxicity.

In this experimental paradigm 6 groups were included. Four groups of rats received either $GdCl_3$ or saline and either vitamin A or vehicle as described in Methods. Each rat in these groups also received ANIT (35mg/kg, po) 24 hr prior to measurement of markers of liver injury. In the other two groups, rats were treated with corn oil (CO), the vehicle for vitamin A and saline or received corn oil, vitamin A and $GdCl_3$. These two groups served as negative controls. A) Serum ALT activity, B) Bile Flow, C) Serum total bilirubin concentration, D) Serum GGT activity. Bars represent means ± SE. N = 4-10. *Significantly different from respective ANIT/VEH group.


A.
Saline
GDCL3
ALT (Units/Liter)
450
400
350
300
250
200
150
100
50
0
a
a
b
b
CO VEH
CO VIT A
ANIT VEH
ANIT VIT A
B.
Saline
GDCL3
SERUM BILIRUBIN (mg/dL)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
b
CO VEH
CO VIT A
ANIT VEH
ANIT VIT A
C.
Saline
GDCL3
BILE FLOW (µL/gm liver/30 min)
60
50
40
30
20
10
0
a
a
b
b
CO VEH
CO VIT A
ANIT VEH
ANIT VIT A
D.
Saline
GDCL3
SERUM GGT (Units/Liter)
6
5
4
3
2
1
0
a
a
b
b
CO VEH
CO VIT A
ANIT VEH
ANIT VIT A

CHAPTER VI

DISCUSSION

Lack of involvement of leukotrienes in ANIT hepatotoxicity.

In the rat, oral administration of ANIT causes periportal cholangiolitic hepatitis. Recent reports suggest that GSH is requisite for the expression of ANIT-induced liver injury. Since GSH is required and is a rate limiting substrate for the synthesis of cysteinyl LTs, it seemed plausible that LTs might be a primary mediator of ANIT toxicity.

The findings of the present study suggest that LTs are not causal in ANIT hepatotoxicity. Treatment with two of three agents that selectively and effectively antagonize the 5-lipoxygenase enzyme did not ameliorate the hepatotoxic effects of ANIT. Although treatment with A63162, a 5-lipoxygenase inhibitor, or AT-125, a gamma-glutamyl transferase inhibitor, did provide attenuation of some of the markers of ANIT hepatotoxicity, these results are likely related to other, non-specific effects of these agents. Although the efficacy of LT synthesis inhibition by both Zileuton and MK-886 was verified in whole blood, the possibility that these agents were ineffective at inhibiting 5-lipoxygenase activity in the liver cannot be ruled out. However, the weight of the evidence in this study suggests that cysteinyl LTs are not requisite for ANIT-induced liver injury.

Since cysteinyl LTs are probably not involved in the mechanism of action of ANIT, the question of how GSH is related to the expression of liver injury in this model remains unanswered. Recently Carpenter-Deyo and coworkers (Carpenter-Deyo et al., 1991) demonstrated that treatment of isolated hepatocytes with ANIT caused

et al., 1991) demonstrated that treatment of isolated hepatocytes with ANIT caused a time- and concentration-dependent export of GSH into the extracellular medium. These investigators showed that ANIT forms a reversible conjugate with GSH (GS-ANIT) and this GS-ANIT caused GSH depletion and cytotoxicity similar to that observed for native ANIT. This result in vitro led to the hypothesis that ANIT forms a reversible conjugate with GSH in vivo which can be exported into bile, allowing for accumulation of ANIT in the biliary tract, thereby exposing biliary epithelium to potentially cytotoxic concentrations of ANIT (Carpenter-Deyo et al., 1991). Accordingly, this "GSH shuttle" may enhance biliary epithelial cell exposure to ANIT.

The observation that reversible conjugation of a xenobiotic to GSH can enhance toxicity is not without precedent. Methyl isocyanate is an agent which undergoes reversible conjugation to GSH (Pearson et al., 1990). The resulting GSH conjugate has been proposed as a possible transport molecule allowing for the expression of toxicity in organs distant from the site of exposure (Pearson et al., 1990). Thus, it is conceivable that ANIT conjugation to GSH serves a similar transport function.

Evidence supporting a possible "ANIT-GSH shuttle" in vivo has recently been revealed. In vivo measurement of biliary GSH and ANIT concentrations 1 hr and 4 hr after oral ANIT demonstrate a marked accumulation of ANIT and an increase of GSH in bile (Jean et al., 1994). Interestingly, the rise in bile GSH content (on a molar basis) is greater than the rise in biliary ANIT concentration. Since ANIT

bound to biliary macromolecules and is therefore not observable in bile. Alternatively, since ANIT is highly lipid soluble it may be reabsorbed into hepatic parenchymal cells or biliary epithelium. Regardless, these data support the existence of a "GSH shuttle" in vivo which may concentrate ANIT in bile and thereby contribute to the mechanism of toxicity. It should be noted that BNIT, a non-hepatotoxic congener of ANIT is concentrated in bile to an even greater extent than ANIT (Paul Jean, personal communication), suggesting that the difference in ANIT and BNIT hepatotoxicity is not related to dispositional differences (ie, a lack of exposure of biliary epithelium to BNIT). Since ANIT but not BNIT can stimulate and prime neutrophils, one might propose that this action on neutrophils, and possibly other cells, is a critical difference for the expression of toxicity.

Role of platelets in ANIT-induced liver injury.

Since neutrophils are requisite for the expression of ANIT hepatotoxicity, the possibility exists that other circulating cells are also important in the mechanism(s) of action of this compound. Studies presented in this thesis suggest that platelets contribute, at least in part, to the hepatic insult caused by ANIT. Platelet depletion ameliorated ANIT-induced increases in serum ALT activity and bilirubin concentrations but not increases in serum GGT activity, suggesting that the protection is specific for hepatic parenchymal cells.

Pharmacologic inhibition of platelet cyclooxygenase activity does not afford protection from ANIT hepatotoxicity, implying that platelet-derived cyclooxygenase

Pharmacologic inhibition of platelet cyclooxygenase activity does not afford protection from ANIT hepatotoxicity, implying that platelet-derived cyclooxygenase products are not involved in this insult. Because platelets are also a source of other vasoactive and inflammatory mediators such as 5-hydroxytryptamine, epinephrine, histamine, a vascular permeability factor, a compound which activates complement, 12-HPETE and 12-HETE (Cohen, 1980; Weiss, 1982; Carey et al., 1989) it is plausible that one or more of these mediators may play a role in ANIT hepatotoxicity.

Platelets are of primary importance in the activation of compounds involved in coagulation (Weiss, 1982). Thus, the function of platelets in ANIT-induced hepatic insult may relate to their ability to either activate or participate in coagulation. Microthrombi or platelets have not been identified in the hepatic lesions caused by ANIT. This may, however, reflect a lack of detailed histologic evaluation of livers from ANIT-treated animals. To the knowledge of this investigator, specific determinations of platelet accumulation or microthrombosis in the livers of ANIT-treated rats have not been carried out. Thus, the issue of how platelets participate in ANIT-induced hepatotoxicity remains to be thoroughly addressed.

PAF and ANIT-induced hepatic insult.

Since inflammation appears to play a key role in the expression of ANIT hepatotoxicity, and because PAF can produce many of the pathophysiologic

hepatotoxicity. Although PAF is a potential player in certain models of tissue injury, this compound does not appear to participate in ANIT-induced hepatitis.

The list of plausible soluble inflammatory mediators which might be involved in the mechanism(s) of ANIT hepatotoxicity is extensive. Likely candidates include, but are not limited to, components of the complement system, histamine or bradykinins, cytokines, 12 or 15 lipoxygenase products and recently identified arachidonate metabolites such as the lipoxins. Complement, histamine and certain cytokines (specifically TNF-α, and some of the interleukins) are considered important mediators of inflammation and tissue injury in a variety of experimental models and pathologic conditions. The possible involvement of these mediators in ANIT hepatotoxicity warrants further investigation.

Vitamin A, zymosan, gadolinium chloride and ANIT hepatotoxicity.

Evaluation of the effects of vitamin A, zymosan and gadolinium chloride on ANIT-induced hepatic insult was undertaken to address the possibility that macrophages contribute to liver injury in this model. Results obtained from these experiments suggest that macrophages are not necessary for manifestation of liver injury in this model of chemical insult. Gadolinium chloride, an agent which decreases Kupffer cell function and numbers (Hardonk et al., 1992), did not ameliorate the liver injury caused by ANIT. If hepatic macrophages were important in the expression of ANIT hepatotoxicity, it might be expected that vitamin A and zymosan, agents which enhance hepatic macrophage function (ElSisi et al., 1993a; ElSisi et al., 1993b) or

cell function and numbers (Hardonk et al., 1992), did not ameliorate the liver injury caused by ANIT. If hepatic macrophages were important in the expression of ANIT hepatotoxicity, it might be expected that vitamin A and zymosan, agents which enhance hepatic macrophage function (ElSisi et al., 1993a; ElSisi et al., 1993b) or number (Bouwens et al., 1984; Bouwens and Wisse, 1985), would augment ANIT toxicity. Exactly the opposite occurred; these agents attenuated liver injury in this model. These findings further support the contention that macrophages do not contribute to ANIT hepatotoxicity.

To determine if the protection provided by vitamin A was a consequence of its action on hepatic macrophages, rats were cotreated with vitamin A and gadolinium chloride. The results obtained from this experimental paradigm suggest that the protective effect of vitamin A on ANIT hepatotoxicity is probably not related to an effect on Kupffer cells. Studies of ANIT disposition revealed that vitamin A diminished the biliary accumulation of ANIT. One might speculate that this attenuation of biliary ANIT accumulation is the mechanism by which vitamin A affords protection in this model.

Recently, Rosengren and Sipes (1994) reported that vitamin A pretreatment in Swiss Webster mice ameliorates carbon tetrachloride-induced liver injury. In this treatment paradigm, vitamin A caused a reduction in microsomal aniline hydroxylase activity, a marker of cytochrome P-450IIE1. These investigators

hypothesized that since this enzyme is responsible for the bioactivation of carbon tetrachloride, vitamin A pretreatment attenuated hepatotoxicity by decreasing carbon tetrachloride bioactivation (Rosengren and Sipes, 1994). Although the role of bioactivation in ANIT-induced liver injury is still incompletely defined, some investigators have suggested that ANIT metabolism is necessary for expression of toxicity (Roberts and Plaa, 1965; El-Hawari and Plaa, 1977; Traiger et al., 1984; Connolly et al., 1988). If bioactivation of ANIT is requisite for toxicity, it is conceivable that vitamin A diminishes ANIT bioactivation and thereby exerts its protective effect. Conversely, vitamin A may enhance certain hepatic metabolic activities and afford protection from ANIT hepatotoxicity by increasing its inactivation and elimination. The role of metabolism in ANIT-induced liver injury warrants further investigation.

The mechanism by which zymosan ameliorates ANIT injury remains an enigma. Zymosan is known to modulate neutrophil function (Lesch et al., 1991; Levy et al., 1993) and it might be hypothesized that since neutrophils are required for the expression of ANIT hepatotoxicity, this is the mechanism by which zymosan attenuates ANIT hepatotoxicity. Evidence from this study supports the possibility that zymosan alters circulating neutrophil numbers or function. When administered to rats, zymosan diminished the ability of glycogen to elicit peritoneal neutrophil accumulation. Furthermore, zymosan administration caused a marked leukopenia with prominent neutropenia. From these findings one can theorize that zymosan

can markedly alter neutrophil distribution, both in the circulation and in tissues. Thus, zymosan may be protective in the ANIT model of hepatitis via its actions on neutrophil numbers or function.

Another hypothesis for the protection afforded by vitamin A and zymosan in this model of liver injury is their potential effects on inflammatory cell production of PGE_2. In a myriad of liver injury models, including ANIT-induced liver injury, administration of an analog of PGE_2 attenuates tissue insult (Ruwart et al., 1984; Sinclair et al., 1990). Most compounds which activate Kupffer cells stimulate prostanoid production, including PGE_2 synthesis (Decker, 1990a; Decker, 1990b). Zymosan is known to cause macrophage activation leading to PGE_2 synthesis (Decker, 1990a). Furthermore, biologic response modifiers, such as bacterial endotoxin, propionibacterium acnes or OK-423, enhance Kupffer cell production of PGE_2 somewhat selectively (Kawada et al., 1992). It can be envisioned that treatment with either vitamin A or zymosan, in the presence of ANIT, might cause an increased production of hepatic PGE_2 leading to attenuation of the hepatotoxic effects of ANIT.

Although potentially unrelated to the mechanism of toxicity of ANIT, the observations that zymosan and vitamin A ameliorate the hepatic insult caused by ANIT are interesting. The preceding speculations pertaining to this protective mechanism may lead to investigations which could further our knowledge of

hepatic pathophysiology. With regard to ANIT hepatotoxicity, many questions remain unanswered.

Hypothetical mechanism of ANIT hepatotoxicity.

Previous work has demonstrated that both neutrophils and GSH are requisite for the expression of ANIT injury. More recent evidence suggests that GSH may act to facilitate transport of ANIT from plasma into bile and allow high concentrations of ANIT to be achieved in bile. Work presented in this dissertation suggests that platelets may augment ANIT hepatotoxicity. One might speculate that ANIT injury is manifest when the following events occur (Fig VI-1). 1) ANIT diffuses from plasma into hepatocytes where it is conjugated to GSH and actively transported into bile. 2) In bile the ANIT-GSH conjugate dissociates, liberating free ANIT. 3) As a result of lipid solubility and the concentration gradient between bile and surrounding fluids, ANIT in bile freely diffuses out of bile into the surrounding interstitium. Alternatively, the high bile concentration of ANIT may compromise biliary epithelium, allowing for the release of bile, intracellular constituents, ANIT or unidentified chemotactic factors into the interstitium (Space of Mall). 4) The presence of these factors in the interstitium causes the chemotaxis and activation of neutrophils. 5) Neutrophils emigrate into periportal regions and cause direct biliiary epithelial and hepatocellular injury through the release of cytotoxic agents. 6) Platelets, in response to an unidentified factor, adhere to endothelium and either directly or through interactions with neutrophils contribute to hepatic parenchymal

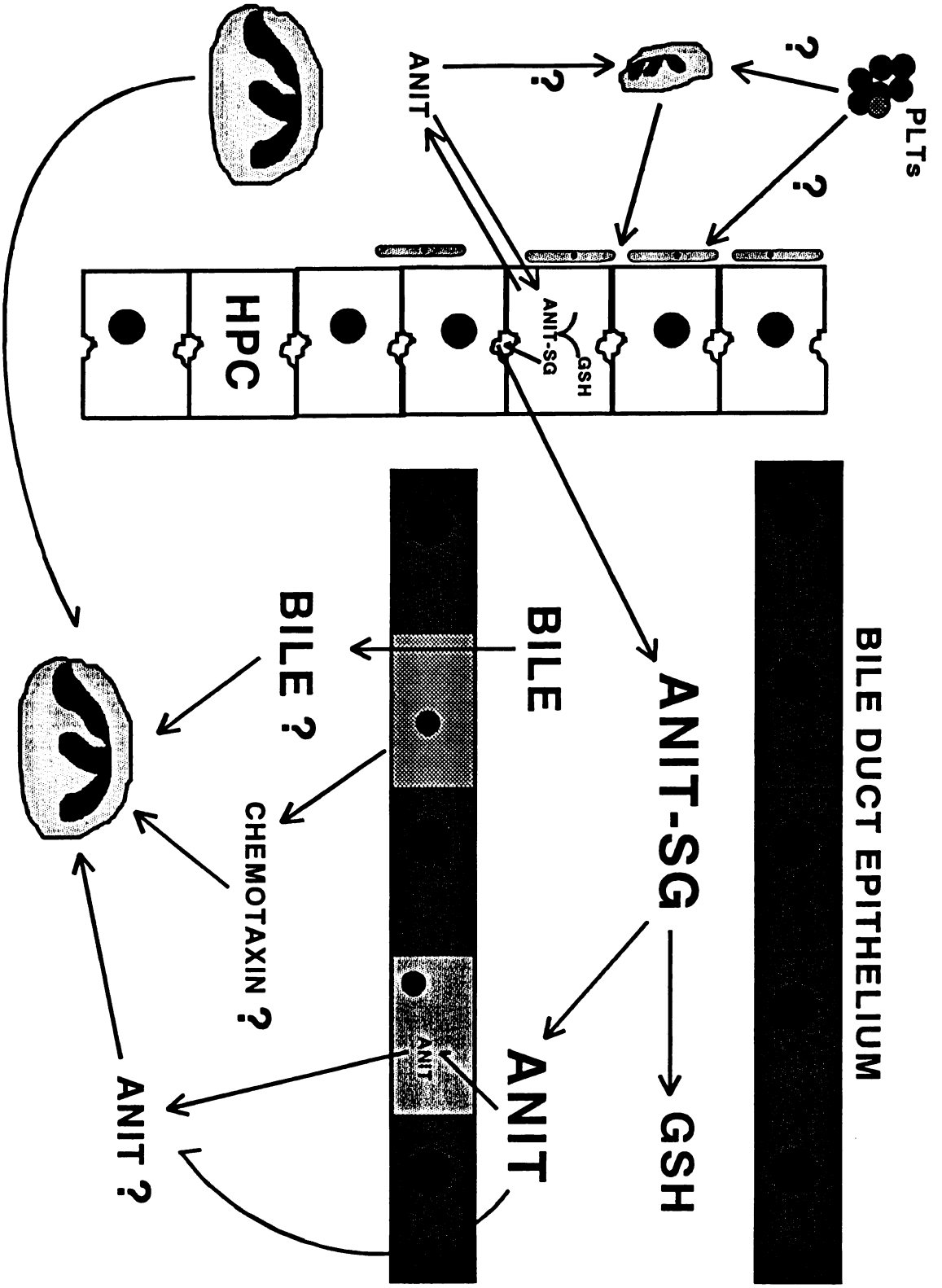
injury. This might be due to the formation of microthombi and subsequent alteration of local perfusion or via the release of activating or cytotoxic agents. Although purely speculative, this scenario accounts for most of the observations made during ANIT hepatotoxicity.

Future directions.

The mechanism(s) by which platelets are involved in the expression of this chemically induced insult are unclear. The work presented here rules out certain mediators in ANIT hepatotoxicity (ie, PAF, leukotrienes, TxA_2, cyclooxygenase products and macrophages), but the role of other soluble mediators of inflammation remains to be addressed. Furthermore, the primary target cell and the initiating event in ANIT hepatotoxicity have not been conclusively identified. Lastly, the signal for neutrophil chemotaxis and activation in this model requires elaboration.

The possibility that inflammatory cells and soluble mediators contribute to chemically induced liver injury is generating increased interest. Certain pharmaceutical companies are currently pursuing investigations into the mechanism of toxicity of compounds under development which cause an hepatic insult similar to that of ANIT (personal communications). It seems likely that diverse examples of chemically induced liver injury with an inflammatory component will arise. Understanding the mechanism(s) of ANIT hepatotoxicity will

provide insight for the study of these agents.


PLTs
?
?
?
ANIT
HPC
ANIT-SG
GSH
BILE DUCT EPITHELIUM
ANIT-SG
GSH
BILE
BILE ?
CHEMOTAXIN ?
ANIT
ANIT
ANIT ?

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