ALL-TRANS-RETINOL MODULATION OF CHEMICALLY-INDUCED PULMONARY AND HEPATIC TOXICITY

by

John-Michael Sauer

A Dissertation Submitted to the Faculty of the COMMITTEE IN PHARMACOLOGY AND TOXICOLOGY (GRADUATE)

In Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by John-Michael Sauer entitled All-trans-retinol modulation of chemically-induced pulmonary and hepatic toxicity.

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: [Signature]
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# TABLE OF CONTENTS

LIST OF FIGURES ............................................................................................................. 8

LIST OF TABLES ............................................................................................................. 11

ABSTRACT ...................................................................................................................... 12

CHAPTER 1 ..................................................................................................................... 14
  CONCEPTS IN CHEMICALLY INDUCED TISSUE INJURY .................................... 14
  CHEMICALLY INDUCED LUNG TOXICITY ............................................................ 17
  CHEMICALLY INDUCED LIVER INJURY ............................................................... 23
  PHAGOCYTES OF THE LUNG AND LIVER ........................................................... 28
  ALL-trans-RETINOL MODULATION OF PHAGOCYTES
  AND CHEMICAL INDUCED INJURY ...................................................................... 36

STATEMENT OF THE PROBLEM ................................................................................ 52

CHAPTER 2 ..................................................................................................................... 55
  EFFECT OF ALL-trans-RETINOL PRETREATMENT ON CHEMICALLY
  INDUCED LUNG INJURY IN THE MALE SPRAGUE-DAWLEY RAT ................. 55

MATERIALS AND METHODS ...................................................................................... 65
  Chemicals ................................................................................................................. 65
  Animals .................................................................................................................... 65
  Experimental design ............................................................................................... 66
  Biochemical evaluation of lung injury ................................................................... 66
  Morphological evaluation of lung injury ............................................................... 67
  Statistical analysis .................................................................................................. 67

RESULTS ..................................................................................................................... 68
  1-Nitronaphthalene ............................................................................................... 68
  2-Nitronaphthalene ............................................................................................... 70
  Paraquat .................................................................................................................. 72

DISCUSSION ............................................................................................................... 82

CHAPTER 3 ..................................................................................................................... 88
  EFFECT OF ALL-trans-RETINOL ON CHEMICALLY INDUCED
  LIVER INJURY IN THE MALE SPRAGUE-DAWLEY RAT .............................. 88
# TABLE OF CONTENTS - Continued

MATERIALS AND METHODS ................................................................................. 91
   Animals ........................................................................................................... 91
   Chemicals ..................................................................................................... 91
   Experimental design .................................................................................... 92
   Morphological and biochemical evaluation of liver injury ....................... 92
   *In vitro* toxicity of 1- and 2-nitronaphthalene in primary hepatocytes .... 93
   Statistical analysis ...................................................................................... 93

RESULTS ............................................................................................................. 94
   1-Nitronaphthalene ....................................................................................... 94
   2-Nitronaphthalene ....................................................................................... 96
   Toxicity of 1- and 2-nitronaphthalene in primary hepatocytes ................. 97

DISCUSSION ..................................................................................................... 103

CHAPTER 4 ......................................................................................................... 109

EFFECT OF ALL-TRANS-RETINOL PRETREATMENT ON THE
FUNCTION OF PULMONARY ALVEOLAR MACROPHAGES
ISOLATED FROM MALE SPRAGUE-DAWLEY RATS ............................................. 109

MATERIALS AND METHODS ............................................................................. 111
   Animals ......................................................................................................... 111
   Chemicals .................................................................................................... 111
   Experimental design .................................................................................. 111
   Isolation and cell culture of alveolar macrophages .................................... 112
   Tumor necrosis factor assay ..................................................................... 113
   Leukotriene B₄ and Prostaglandin E₂ assay .............................................. 114
   Nitric oxide assay ....................................................................................... 114
   Superoxide anion assay ........................................................................... 115
   Statistical analysis ..................................................................................... 115

RESULTS ........................................................................................................... 117

DISCUSSION .................................................................................................... 123

CHAPTER 5 ......................................................................................................... 127
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY: ALL-trans-RETINOL MODULATION OF CHEMICALLY-INDUCED PULMONARY AND HEPATIC TOXICITY</td>
<td>127</td>
</tr>
<tr>
<td>Future Directions</td>
<td>133</td>
</tr>
<tr>
<td>APPENDIX A:</td>
<td>135</td>
</tr>
<tr>
<td>THE EFFECT OF ALL-trans-RETINOL PRETREATMENT ON BLOOD KINETICS AND TISSUE DISTRIBUTION OF 1-NITRONAPHTHALENE, AND CYTOCHROME P-450 2B1 LEVELS</td>
<td>135</td>
</tr>
<tr>
<td>Western Blot Analysis</td>
<td>136</td>
</tr>
<tr>
<td>Plasma Kinetics and Tissue Disposition of 1-Nitronaphthalene</td>
<td>138</td>
</tr>
<tr>
<td>APPENDIX B:</td>
<td>141</td>
</tr>
<tr>
<td>PHENOBARBITAL ALTERATION OF 1- AND 2-NITRONAPHTHALENE INDUCED IN THE MALE SPRAGUE-DAWLEY RAT</td>
<td>141</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>146</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1.1:
Stages in the progression of chemically induced tissue injury .............................................. 16

Figure 2.1:
Progression of 1-nitronaphthalene induced lung injury .......................................................... 62

Figure 2.2.
Metabolic bioactivation of 1-nitronaphthalene ........................................................................... 63

Figure 2.3:
Metabolic bioactivation of paraquat ......................................................................................... 64

Figure 2.4:
Effect of all-trans-retinol on 1-nitronaphthalene induced lung injury ........................................ 75

Figure 2.5:
Effect of all-trans-retinol on 1-nitronaphthalene-induced microscopic pathology of the lung .......................................................................................................................... 76

Figure 2.6:
Effect of all-trans-retinol on 1-nitronaphthalene-induced ultrastructural pathology of the lung .......................................................................................................................... 77

Figure 2.7:
Effect of all-trans-retinol on 2-nitronaphthalene induced lung injury ........................................ 78

Figure 2.8:
Effect of all-trans-retinol on 2-nitronaphthalene-induced microscopic pathology of the lung .......................................................................................................................... 79

Figure 2.9:
Effect of all-trans-retinol on paraquat induced lung injury ...................................................... 80

Figure 2.10:
Effect of all-trans-retinol on paraquat-induced microscopic pathology of the lung .................. 81
LIST OF FIGURES - Continued

Figure 2.11:  
Proposed mechanism of all-trans-retinol attenuation of 
chemical induced lung injury ................................................................. 87

Figure 3.1:  
Effect of gadolinium chloride pretreatment on all-trans-retinol 
potentiated 1-nitronaphthalene induced liver injury ............................... 98

Figure 3.2:  
Effect of all-trans-retinol on 1-nitronaphthalene-induced 
microscopic pathology of the liver ....................................................... 99

Figure 3.3:  
Effect of gadolinium chloride pretreatment on all-trans-retinol 
potentiated 2-nitronaphthalene induced liver injury ............................... 100

Figure 3.4:  
Effect of gadolinium chloride pretreatment on all-trans-retinol 
potentiated 2-nitronaphthalene-induced microscopic pathology 
of the liver ......................................................................................... 101

Figure 3.5:  
Effect of in vivo all-trans-retinol and phenobarbital pretreatment 
on 1- and 2-nitronaphthalene induced toxicity in primary 
cultured hepatocytes ............................................................................. 102

Figure 3.6:  
Proposed mechanism of all-trans-retinol alteration of chemical 
induced liver injury ............................................................................... 108

Figure 4.1:  
Effect of all-trans-retinol pretreatment on tumor necrosis 
factor production by isolated alveolar macrophages ............................. 118

Figure 4.2:  
Effect of all-trans-retinol pretreatment on leukotriene B₄ 
production by isolated alveolar macrophages ........................................ 119
LIST OF FIGURES - Continued

Figure 4.3: Effect of all-trans-retinol pretreatment on prostaglandin E₂ production by isolated alveolar macrophages ........................................................... 120

Figure 4.4: Effect of all-trans-retinol pretreatment on nitric oxide production by isolated alveolar macrophages ........................................................... 121

Figure 4.5: Effect of all-trans-retinol pretreatment on superoxide anion production by isolated alveolar macrophages ................................................ 122

Figure 4.6: Proposed mechanism of the functional alteration of alveolar macrophages by all-trans-retinol pretreatment .................................................. 126

Figure 5.1: Proposed mechanism of all-trans-retinol protection from chemical induced lung injury ................................................................. 132
LIST OF TABLES

Table 1.1:  
The lung as a target for toxic chemicals ................................................................. 22

Table 1.2:  
The liver as a target for toxic chemicals ................................................................. 25

Table 1.3:  
Inflammatory mediators involved in chemical induced lung injury .......................... 38

Table 1.4:  
Inflammatory mediators involved in chemical induced liver injury ......................... 39

Table 1.5:  
Effect of modifying phagocyte activity in chemical induced liver injury ................... 40

Table 1.6:  
Effect of modifying phagocyte activity in chemical induced lung injury ................... 41

Table 1.7:  
Naturally occurring retinoids and their structures ................................................... 43

Table 2.1:  
Structures of 1-nitronaphthalene, 2-nitronaphthalene, and paraquat .......................... 60

Table 2.2:  
Effect of cytochrome P-450 induction and inhibition on 1-nitronaphthalene induced toxicity in the rat ................................................................. 61

Table 2.3:  
Effect of all-trans-retinol pretreatment on chemical induced pneumonitis ................. 74
ABSTRACT

It has been previously reported that acute hypervitaminosis A in rats dramatically increases the hepatotoxicity of a number of chemicals. This potentiation appears to be mediated by the enhanced release of reactive oxygen species from retinol-primed Kupffer cells. However, in the lung it has been shown that retinol can protect against many of the inflammatory effects caused by bleomycin. Whether or not retinol pretreatment can modulate chemical-induced injury of compounds that cause both liver and lung toxicity is unknown. Therefore, the studies presented here were designed to test the hypothesis that: all-trans-retinol increases hepatic injury through a pro-inflammatory mechanism, whereas in the lung injury is attenuated through an anti-inflammatory mechanism. Male Sprague-Dawley rats were administered retinol (75 mg/kg/day) or its vehicle for up to 7 days. One day after the last dose of retinol animals were given 1-nitronaphthalene (1-NN), 2-nitronaphthalene (2-NN), or paraquat (PQ). Pulmonary and hepatic toxicity was evaluated clinically, biochemically, and morphologically. The hepatotoxicity of both 1-NN and 2-NN was significantly potentiated by retinol pretreatment. The mechanism of this potentiation was, at least in part, mediated by Kupffer cells. Furthermore, as determined from monolayers of hepatocytes, the potentiation of 1-NN and 2-NN did not appear to be caused by an alteration of hepatocyte susceptibility to these chemicals. In the lung, retinol pretreatment significantly attenuated injury caused by 1-NN, 2-NN, and PQ. With each compound, retinol significantly reduced the amount of inflammatory cell infiltration.
following chemical insult. The mechanism of pulmonary protection by retinol was apparently not directly mediated by alveolar macrophages, but instead by a down-regulation of the pro-inflammatory response. Thus, the key observation from these studies was that retinol potentiates hepatotoxicity, while it concomitantly attenuates lung injury with respect to the same chemicals. The overall mechanism by which retinol alters chemically induced lung and liver injury appears to be by modulating the progression of injury via an alteration of the inflammatory response rather than the initial injury. In the lung inflammation is attenuated and the pulmonary tissue is protected from toxicity, while in the liver inflammation is increased and chemically induced hepatic injury is potentiated.
CHAPTER 1

CONCEPTS IN CHEMICALLY INDUCED TISSUE INJURY.

All substances are poisons and are capable of causing cellular injury and even death depending on their dose. Furthermore, all organs and organ systems within the body can be injured and their functions disrupted by exposure to toxic doses of chemicals. Over the past decades, toxicologists have shown that chemically induced tissue injury and cell death is a complex issue which involves several factors. In the simplest case, a chemical is directly cytocidal upon cell contact by inhibiting vital cellular functions. However, in many instances chemically induced tissue injury is not the simple case, it involves several elements which in and of themselves are complex. These elements of chemically induced injury can be grouped into two major categories, either toxicokinetic or toxicodynamic components. Toxicokinetics involve the role of metabolism or disposition of a toxic chemical. These processes are mediated by the enzymes involved in xenobiotic activation and detoxification. Toxicodynamics include functions which are not directly involved in the metabolism or disposition of a xenobiotic, but involve a tissue's response or susceptibility to toxic injury. These functions include, among others: tissue protective factors (i.e. glutathione and vitamin E), tissue repair, hemodynamics, as well as acute and chronic inflammation.

In the 1960’s and 1970’s the importance for the role of biotransformation in hepatic and extrahepatic chemically induced tissue injury was consolidated (Reid et al.,
This research led to the belief that foreign compounds are biochemically modified (biotransformation) to more water soluble forms by specialized cellular processes, and in some cases these modifications can lead to the formation of reactive intermediates which cause tissue damage. Furthermore, other processes (conjugation) are thought to act to protect the cell from the insult of reactive intermediates (Brodie et al., 1971; Mitchell et al., 1973). In the late 1980's the idea that inflammatory cells play a role in some types of chemically induced injury gained acceptance (Laskin and Pilaro, 1986; Sipes et al., 1989). This concept gathered further support in the 1990's with the advent of several pharmacological interventions which elucidated the critical and almost pinnacle role for inflammatory cells in chemical induced tissue injury (Gunawardhana et al., 1993; ElSisi et al., 1993a; Edwards et al., 1993; Laskin and Pendino, 1995).

Currently it is understood that tissue injury by xenobiotics can involve both direct and indirect target cell damage. While chemicals may act directly on a target cell causing toxicity via biotransformation, they may also act indirectly by recruiting, as well as activating resident and inflammatory phagocytes. Phagocytes participate in the pathogenesis of tissue injury by releasing pro-inflammatory and cytotoxic cytokines/growth factors, bioactive lipids, hydrolytic enzymes, as well as reactive oxygen and nitrogen species. A graphical outline of both the direct and indirect divisions involved with chemically induced tissue injury are shown in Figure 1.1. It has been previously shown that pretreatment with all-trans-retinol (vitamin A) can modulate the
Figure 1.1. Stages in the progression of chemically induced tissue injury. Currently it is accepted that there are various stages involved with chemical induced tissue injury. These stages include the bioactivation of the chemical to a toxic metabolite, the induction of an inflammatory response, the resolution of inflammatory response and the repair of tissue following toxic injury. The model shown above illustrates these various stages over time.
indirect effect or progression of injury caused by several toxicants in the liver. Retinol has been shown to exacerbate the hepatotoxicity of several chemicals including; carbon tetrachloride, acetaminophen, allyl alcohol, ethanol, and endotoxin (Sipes et al., 1989; ElSisi et al., 1993a). In this introductory chapter, the ability of chemicals to cause hepatic and pulmonary injury will be reviewed, as well as the current evidence showing roles for inflammatory cells in chemically induced tissue injury. Special emphasis has been placed on reviewing the current information available on ability of retinol to modulate the progression of both lung and liver injury.

CHEMICALLY INDUCED LUNG TOXICITY

The anatomy and function of the lung: There have been 40 different cell types identified within the lung, at least 12 of which are found in the epithelial population that line the airways (for review see, Breeze and Wheeldon, 1977; Jeffery and Reid, 1975). The lung's airways are subdivided into various regions. In the rat, the regions are named by their size and location in respect to the trachea. At the distal-most end of the airways, furthest from the trachea, are the alveolar sacs where oxygen is exchanged for carbon dioxide in the blood. Bronchioles can be defined as the most distal branches of the conduction system of the airway, an area between the bronchi and alveolar sacs. Bronchi are more proximal than bronchioles and are distal to the trachea, but like the trachea are supported by collagen rings.
Many of the epithelial cell types are restricted to specific regions of the airway and have specialized functions due to their location. The alveolar sacs are composed of supporting cells and those which make surfactant, type I and II cells respectively. The luminal surface of the bronchioles is lined with two types of simple cuboidal epithelium consisting of a mixture of ciliated cells and nonciliated secretory cells. The bronchi contain a complex epithelial lining made up of basal cells, ciliated cells, mucous goblet cells, and a variety of intermediate forms.

From a toxicological point, one of the most important cells is the nonciliated secretory cell, known as the Clara cell. Clara cells are found in the distal bronchioles of both mammalian and avian lungs. These cells are arranged within bronchioles in linear arrays between the ciliated cells and have unique club-shaped surface projections, known as apicals (Plopper et al., 1980). From these projections are secreted membrane-bound granules of choline-based phospholipid. The constituents of these granules are thought to form a protective lining layer which is unrelated to alveolar surfactant (Stinson and Loosli, 1978). The major function of this phospholipid layer may be to allow proper function of the cilia and thereby prevent bronchiole blockage. The apical surface of the Clara cell is covered by microvilli; but, since these cells do not have cilia, they are referred to as non-ciliated bronchiolar epithelial cells.

Several years ago, a 10 Kd protein was found to be associated with the granules of Clara cells in the lung (Gupta and Hook, 1987). This protein was found to have the same structure and activity as uteroglobin, a protein associated with the urogenital tract of
rabbits which has anti-inflammatory functions (Singh et al., 1990). This lung associated protein has been appropriately named the Clara cell secretory protein (CCSP) and is commonly referred to as CC10. The Clara cell secretory protein has a similar structure to both uteroglobin and the polychlorinated binding protein found in humans, as well as rodents. This family of proteins can inhibit phospholipase A2 (PLA2) and are substrates for transglutaminase (Peri et al., 1994). The inhibition of PLA2 is a plausible mechanism by which CC10 could regulate the inflammatory response in the lung. Hay and co-workers have speculated that CC10 is a determining factor in inherited susceptibility to asthma (Hay et al., 1995). From a toxicological standpoint, it has been shown that CC10 increases significantly following repeated exposures to non-toxic concentrations of ozone (Dodge et al., 1994). This group speculated that CC10 acts as anti-inflammatory factor in the lung and is, in part, responsible for ozone tolerance. Although the complete role of this protein has not completely elucidated, CC10 appears to act as an anti-inflammatory protein in the lung. Thus, the production of this protein by Clara cells may be important in the physiological and pathophysiological regulation of pulmonary inflammation.

**Pulmonary xenobiotic metabolism:** Aside from being responsible for the production of phospholipid granules, Clara cells contain the largest reserve of cytochrome P-450 in the lung (Boyd, 1977). These enzymes, in some cases, are able to carryout and even exceed the xenobiotic activation abilities associated with hepatocytes (Boyd, 1980). Cytochrome P-450 (CYP) isoenzymes 1A1, 1A2, 2B1, and 2E1 are localized in Clara cells and are
known to be involved in xenobiotic metabolism (Lacy et al., 1992; Martin et al., 1990). The pulmonary P-450 system is also present in the bronchial and alveolar epithelium (type II cells), but immunohistochemical, electrophoretic and metabolic evidence shows that its specific activity is much less than in the Clara cells (Voigt et al., 1989). Clara cells are also a reserve for GSH in the lung, likely because of their high oxidative potential (Lacy et al., 1992; Martin et al., 1990). The high xenobiotic-metabolizing capacity of Clara cells may explain, in part, their high susceptibility to environmental chemicals.

The lung as a target for chemical-induced toxicity: Although the lung is exposed to environmental toxicant predominantly through inhalation, it can also be damaged by chemicals and drugs which are ingested or systemically administered. The mechanism by which systemic toxicants act has been well defined, both biochemically and toxicologically. Boyd (1980) introduced three possible mechanisms of bioactivation which a systematically administered chemical could undergo to cause pulmonary toxicity. The first of Boyd's mechanisms depict a reaction in which an "inert" parent chemical is metabolized in situ by the lung to an ultimate toxicant. The ultimate toxicant may cause damage to the cell in which it was formed or to other adjacent cells in the lung. 4-Ipomeanol is an excellent example of a compound which is acted upon by this mechanism (Boyd and Reznik-Schuller, 1984). In the second mechanism, a compound which is primarily metabolized in the liver is transported to the lung were it finds its target and causes cell toxicity. An example of a compound acted upon by this mechanism
is naphthalene (Cho et al., 1995). Finally, the third mechanism represents a pathway followed by a compound which undergoes cyclic reduction/oxidation metabolism. The reduction/oxidation cycling within pulmonary cells results in the production of reactive oxygen species and compromises cellular defense mechanisms due to decreased energy pools. The relatively high oxygen tension of the lung could contribute to its preferential damage by agents acting through this mechanism. An example of a compound which is acted upon by this mechanism is paraquat (Foth, 1995).

These mechanisms are highly simplified representations of early events which could occur in the complex steps leading to lung damage by chemical agents. These mechanisms do not take into account factors such as detoxification pathways, other cellular defense mechanisms, and inflammatory processes. However, these mechanisms do represent a starting point for understanding chemical induced lung injury. Aside from the metabolic activation of chemical toxicants, the lung has several other features which predispose it to chemically induced injury. Some of these features are listed in Table 1.1.

Responses of the lung to chemical injury: From a morphological aspect, the cells which comprise the lung can only react to toxicant-induced damage in a limited number of ways. These responses are categorized as; 1) pulmonary edema, including both interstitial and alveolar edema, which are reversible conditions; 2) cell cytotoxicity, which in the early stages (cell swelling) is reversible but can progress to irreversible necrosis; 3) pulmonary inflammation or pneumonitis, in which inflammatory cells migrate into a
Table 1.1

The lung as a target for toxic chemicals

<table>
<thead>
<tr>
<th>Feature</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Anatomical and physiological</td>
<td>• Exposure to inhaled and blood borne chemicals</td>
</tr>
<tr>
<td>2. Direct exposure to environment</td>
<td>• High O₂ tension</td>
</tr>
<tr>
<td></td>
<td>• Exposure to inhaled chemicals</td>
</tr>
<tr>
<td>3. Epithelium contain multiple drug-metabolizing enzyme systems (cytochrome P-450, FMO)</td>
<td>• Formation of toxic metabolites, reactive intermediates</td>
</tr>
<tr>
<td>4. Multiple cell types</td>
<td>• Modulation of function of one cell type (example: Alveolar macrophage) can influence the response of another cell type (example: Clara cell)</td>
</tr>
<tr>
<td>5. Polyamine uptake system</td>
<td>• Promotes uptake of chemicals into lung</td>
</tr>
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</table>
localized portion of the lung; 4) type II cell hyperplasia, a condition usually associated with inflammation in which type II cells become the predominant cell type in the alveolus; 5) pulmonary fibrosis, in which parenchymal necrosis and deposition of collagen fibers disrupt normal pulmonary architecture and function; and 6) the development of pulmonary neoplasia (for review see, Windsor et al., 1993).

CHEMICALLY INDUCED LIVER INJURY

The anatomy and function of the liver: The liver is a complex organ, composed of various cell types which interact with each other, but are adapted to performing specific functions. Parenchymal cells (hepatocytes) comprise approximately 80% of the liver's total volume with 250 billion hepatocytes found in normal human adults (Arias et al., 1988). The sinusoidal space and cells associated with the sinusoids comprise the remaining liver volume. The endothelial cells total 44% of the sinusoidal cell volume, while 22% are Ito cells, and 33% are Kupffer cells (Arias et al., 1988; Blouin, 1977). Lymphocytes and pit (natural killer) cells comprise the remaining 1% of the sinusoidal cell volume.

Although it is impossible to assign orders of importance to specific cell types, it is the hepatocytes or parenchymal cells that carry out the functions generally associated with the liver. Hepatocytes extract nutrients and other chemicals from the blood, produce bile to aid the digestive process and promote the absorption of lipids, secrete metabolic
products into bile, synthesize proteins for hepatic and non-hepatic use, store energy-rich products such as fat and glycogen, and among many other functions, metabolize drugs and chemicals. The non-parenchymal cells also have important properties. Kupffer cells are the resident macrophages of the liver and serve to phagocytize blood-borne toxicants and particulates from the circulation. The endothelial cells form a leaky barrier between the parenchymal cells and the hepatic blood flow. These fenestrated cells act as a sieve to prevent red blood cells and other cellular components from interacting with hepatocytes while allowing access to all other substances in the blood. The Ito cell (or fat storing cell) is an important cell type that stores vitamin A (and other fat soluble vitamins) and can also synthesize collagen. It plays a major role in the development of cirrhosis (Ramadori, 1991). Other non-parenchymal cells include lymphocytes and natural killer (pit) cells, which may be important in the identification and elimination of neoplastic cells.

A number of features (Table 1.2) predispose the liver to chemically induced tissue injury. Because the liver's predominant blood supply has first passed through the intestines it is low in oxygen content but highly enriched in nutrients. Likewise it also contains endotoxin (lipopolysaccharide products of intestinal bacteria), metabolic waste products, absorbed chemicals, and other cell debris which may all enter the liver via the portal circulation and present a risk of toxicity to the liver. Thus, because the liver receives blood directly from the intestines, it delivers chemicals directly to the liver. Following oral ingestion, these toxicants can achieve high concentrations (Koporec et al., 1995). Toxic chemicals can also be delivered to the liver in the arterial blood that mixes
Table 1.2

The liver as a target for toxic chemicals

<table>
<thead>
<tr>
<th>Feature</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Anatomical and physiological relationship to G.I. tract</td>
<td>• Exposure to orally ingested chemicals</td>
</tr>
<tr>
<td>2. Blood supply predominantly from portal vein</td>
<td>• Lower O₂ tension</td>
</tr>
<tr>
<td></td>
<td>• Exposure to orally ingested chemicals</td>
</tr>
<tr>
<td>3. Leaky endothelial barrier</td>
<td>• Promotes exposure of hepatocytes to chemicals</td>
</tr>
<tr>
<td>4. Hepatocyte structure; microvilli, proximity to sinusoid</td>
<td>• Promotes uptake of chemicals into hepatocytes</td>
</tr>
<tr>
<td>5. Hepatocytes contain multiple drug-metabolizing enzyme systems (cytochrome P-450, alcohol dehydrogenase)</td>
<td>• Formation of toxic metabolites, reactive intermediates</td>
</tr>
<tr>
<td>6. Biliary excretory function</td>
<td>• Disruption in bile flow damages hepatocytes</td>
</tr>
<tr>
<td>7. Multiple cell types</td>
<td>• Modulation of function of one cell type (example: Kupffer cell) can influence the response of another cell type (example: hepatocyte)</td>
</tr>
</tbody>
</table>
with venous blood in the sinusoids. For example, compounds that are absorbed via inhalation can be delivered directly to the liver, which receives 29% of the resting cardiac output (Guyton, 1991). Once present in the blood of the sinusoidal space, chemicals can be readily extracted from blood into the hepatocyte. This is primarily facilitated through the leaky capillary system (i.e. sinusoids) of the liver, which permits plasma to enter the space of Disse (space between hepatocytes and endothelial cells) where it has direct contact with the hepatocyte. Furthermore, microvilli on the sinusoidal surface of the hepatocyte greatly increases absorption.

**Hepatic xenobiotic metabolism:** Another feature of the liver that predisposes it to chemical induced injury is its ability to biotransform or metabolize chemicals. This important process is catalyzed by numerous enzymes which converts lipophilic compounds into more hydrophilic metabolites. These can be more readily excreted in the urine and feces. The liver is the most important organ of biotransformation, due largely to its high content and large diversity of enzymes capable of metabolizing foreign, as well as endogenous, chemicals. These enzymes include UDP-glucuronosyltransferase, glutathione-S-transferase, cytochrome P-450, FAD-containing monooxygenase, as well as others. However, with respect to biotransformation the cytochromes P-450 represent the most important family of enzymes in the liver. The main function of this family of enzymes is to add or expose functional groups on a substrate. Addition of polar groups is usually achieved by introduction of oxygen into the substrate. Cytochrome P-450, by its
ability to reduce oxygen to a highly reactive chemical form, introduces oxygen into a wide variety of chemicals. The resulting products can be excreted or further metabolized (usually conjugated with an endogenous molecule such as glutathione, glucuronic acid, sulfate, etc.). On occasion, a chemical can accept electrons from cytochrome P-450. The resulting product may be a highly reactive free radical. Although biotransformation of chemicals is a critical process that works to prevent the accumulation of lipophilic chemicals in the body, the process of biotransformation is not always innocuous. Toxic metabolites, some of which are chemically reactive, often can be produced.

Toxic or reactive metabolites can initiate a series of events that ultimately result in liver injury. Such events include initiation of lipid peroxidation, covalent modification of critical cellular molecules, consumption and ultimate depletion of important cellular components, mutations in DNA, and inhibition of protein synthesis among others (Hinson et al., 1994). At low rates of formation, reactive metabolites can be detoxified by conjugation with endogenous molecules or their damage repaired. However, if the rate of utilization of these endogenous molecules exceeds their synthesis, they will ultimately be depleted. At this stage, the hepatocyte becomes extremely vulnerable to damage by reactive metabolites of chemicals.

**Responses of the liver to chemical injury:** From a morphological aspect the cells which comprise the liver can only react to toxicant-induced damage in a limited number of ways. Therefore, there are only a few, rather well-defined responses to a multitude of
toxic chemicals. These responses are categorized as; 1) fatty change (or steatosis), which is a reversible condition; 2) cell cytotoxicity, which in the early stages (cell swelling) is reversible but can progress to irreversible coagulative necrosis; 3) cholestasis, in which bile flow slows or ceases; 4) fibrosis and cirrhosis, in which hepatocyte necrosis and deposition of collagen fibers disrupt normal hepatic architecture and function; and 5) the development of liver neoplasia (for review see, Moslen, 1996).

**PHAGOCYTES OF THE LUNG AND LIVER**

Phagocytes are defined as cells capable of ingesting particulate matter. However, the term usually refers to polymorphonuclear leukocytes (neutrophils, basophils, and eosinophils) and mononuclear phagocytes (macrophages and monocytes). These cells ingest microorganisms, as well as other particulate antigens that are coated with antibody or complement (opsonized), a process that is mediated by specific cell-surface receptors (Fc receptors and complement receptors). Other cell types exhibit phagocytosis, but it is not specific to phagocytosis of opsonized particulates. To avoid confusion, in this dissertation phagocytes will refer to monocytes, tissue macrophages, and neutrophils.

Polymorphonuclear leukocytes are a family of cells including neutrophils, basophils, and eosinophils, all originating from a single pluripotent progenitor cell. These cells are granular leukocytes possessing a nucleus with three to five lobes connected by slender threads of chromatin, and cytoplasm connecting fine inconspicuous granules.
These cells have the properties of chemotaxis, adherence to immune complexes, and phagocytosis of opsonized and nonspecific particulates. Macrophages are any of the mononuclear phagocytes normally found in tissue (Papadimitriou and Ashman, 1989). Mononuclear phagocytes arise from hematopoietic stem cells in the bone marrow. After passing through the monoblast and promonocyte stages to the monocyte stage, they enter the blood, circulating for about 40 hours. Monocytes then enter tissue were they increase in size, volume, phagocytic activity and lysosomal enzyme content, and become macrophages. The morphology of macrophages varies among different tissues and between normal and pathological states. However, most macrophages are large cells with round or indented nuclei, a well-developed Golgi apparatus, abundant endocytotic vacuoles, lysosomes, phagolysosomes, and a plasma membrane covered with ruffles or microvilli (Papadimitriou and Ashman, 1989). Among the functions of macrophages are nonspecific phagocytosis and pinocytosis, specific phagocytosis of opsonized microorganisms, killing of ingested microorganisms, digestion and presentation of antigens to T and B lymphocytes, and the secretion of a large number of products. These secreted products include enzymes (lysozyme, collagenases, elastase, acid hydrolases), complement components and coagulation factors, prostaglandins and leukotrienes, reactive oxygen species and several regulatory molecules (TNF-α, IL-1, and interferons). Macrophages and neutrophils contribute greatly to chemical induced tissue injury. They appear to function in a concerted manner controlled by signaling molecules. Because of
the importance of these cells to this dissertation, this section describes pulmonary alveolar macrophages, Kupffer cells and neutrophils in greater detail.

**Kupffer cells (KC):** Kupffer cells, like all macrophages, are recruited from the stem cells of the bone marrow and differentiate under the influence of specific signals (cytokines, interleukins, etc.) to mature tissue resident macrophages (for reviews see, Decker, 1990; Wake et al., 1989). Although all KC are ultimately derived from the same source (bone marrow), they may in some instances also propagate at the site of their final destination in the liver (Jones and Summerfield, 1988). Kupffer cells represent the highest concentration of fixed-mononuclear phagocytes in the body (80 to 90% of the total) (Laskin et al., 1986). They are distributed over zones 1, 2, and 3 of the liver acinus in a ratio of 4:3:2 (Sleyster and Knook, 1982). Kupffer cells are highly phagocytic members of the reticuloendothelial system, and together with other sinusoidal cells, play a critical role in the maintenance of normal liver function. The major functions of KC include phagocytosis of particular matter from portal circulation, removal of tumor cells from the circulation, regulation of hepatic microvascular circulation, detoxification/clearance of endotoxin, release of reactive mediators to modulate host defense, as well as uptake and catabolism of lipids and glycoproteins (Nolan, 1981; Phillips, 1989). Despite their functional specialization, KC have at least four major functions in common with other macrophages. These functions include presenting antigens, phagocytosis, unspecified immune response and biochemical attack (Decker, 1990).
Kupffer cells are usually stellate (star-shaped) and are easily distinguished from the fenestrated endothelial cells. Kupffer cells are medium to large (8.7 to 9.1 μm), irregularly shaped cells which contain one horseshoe-shaped nucleus, and abundant cytoplasm filled with large numbers of rod-shaped mitochondria, pinosomes, and lysosomes (Knook and Sleyster, 1976). Immunohistochemically, KC stain for endogenous peroxidase, nonspecific esterase, acid phosphatase and glucose-6-phosphate (Knook et al., 1980; De Leeuw et al., 1983). Furthermore, KC are enriched with a large number of lysosomal enzymes including cathepsin D, and N-acetyl glucosaminidase (Knook and Sleyster, 1980). Ultrastructurally, the Kupffer cell is irregularly shaped and possesses a surface covered with numerous microvilli, filopodia, and lamellopodia. The cytoplasm contains a well-developed golgi zone, dark staining lysosomal vacuoles and a complex cytoskeleton of microfilaments, intermediate filaments and microtubules (McCuskey and McCuskey, 1990). The complexity of the cytoplasm and its organelles increases markedly with the maturation and activation of these cells. The complex structure lends to the cell's ability as a secretory cell, producing a variety of defined molecular products. Kupffer cells in zone 1 of the hepatic acinus differ from those located in zone 2 and 3 in four distinct ways. They tend to be larger in size, have larger lysosomes, and possess higher phagocytic and lysosomal enzyme activities (Sleyster and Knook, 1982). Kupffer cells stay attached to endothelial cells by adhering to cellular adhesion molecules via CD11/CD18. These cells also elicit adhesion molecules (ICAM-1), which other immune cells can attach.
Kupffer cells can be stimulated by several chemical factors including zymosan (Bouwens and Wisse, 1985), endotoxin (Nolan, 1981), cytokines (Decker, 1985), estrogen (Ikejiri and Tanikawa, 1977), large doses of vitamin A (Mobley et al., 1991) as well as others. These stimulated or activated cells show unique metabolic changes characterized by increased oxygen and glucose uptake and utilization, as well as increased production of activated oxygen via the NADPH oxidase system (Bhatnagar et al., 1981). Kupffer cells, like all macrophages, possess an NADPH oxidase enzyme located on the plasma membrane to generate a respiratory burst when the cell is activated. The major products of the respiratory burst are superoxide anion, hydrogen peroxide, hypochlorous acid, and chloramine (Bhatnagar et al., 1981). The production of reactive oxygen species during the respiratory burst is usually coupled with increased phagocytic activity, as well as morphological changes associated with the plasma membrane (i.e. the formation of vermiform processes and increased cell size).

During activation, several cytokines and eicosanoids are released by KC, including interleukin-1 (IL-1), interleukin-6 (IL-6), prostanoids, transforming growth factor-beta, platelet activating factor, leukotrienes, gamma-interferon, and tumor necrosis factor-alpha (TNF-α). These agents can both effect nonparenchymal and parenchymal cells (Decker, 1990). For example, IL-1 causes KC to remain in an activated state, while stimulating hepatocyte fibronectin production, DNA synthesis, and glucose and lipid metabolism (Katsumoto et al., 1989; Feingold et al., 1989; Butterwith and Griffin, 1989).
Pulmonary alveolar macrophages (PAM): Four types of macrophages are located in the lung; PAM, interstitial macrophages, intravascular macrophages, and dendritic cells (for review see; Lohmann-Matthes et al., 1994). Interstitial macrophages are located in the connective tissue of the lung. This macrophage subtype elicits high levels of MHC class II receptor and is involved in antigen presentation. Antigen presentation is an important function in basic host immune responses against pathogenic viruses and bacteria. Although these cells make very little reactive oxygen species or TNF compared to other macrophages (i.e. PAM), they still possess the ability to eliminate pathogenic microorganisms (Prokhorova et al., 1994). Dendritic cells, like interstitial macrophages, are located in the interstitial areas of the lung and are involved only in antigen presentation. Intravascular macrophages are located in the blood vessels of the lungs, and like the Kupffer cell, are involved in clearance of particulate material blood (Chitko-McKown and Blecha, 1992). However, unlike the other pulmonary macrophages, intravascular macrophages are not found in rodents. Finally, one of the most active and dynamic macrophages is the PAM located throughout the lung primarily in the alveolar spaces.

The PAM is localized in the surfactant film which lines the lungs and is the only macrophage in the body to be directly exposed to air. Thus, the PAM represents a primary defense against inhaled agents. This unique pulmonary cell type has a high phagocytic and microbicidal potential. Following bronchioalveolar lavage the alveolar macrophage comprises 90% of cells found in the wash of normal lungs. These cells have their origin
from recruited blood monocytes or from local replication of other alveolar macrophages (Tarling and Coggle, 1982). Like all macrophages, the PAM has important receptors located on the plasma membrane. These include Fc receptors, complement receptors, and lectin receptors. Fc receptors bind to the Fc portion of antibodies with great affinity, while the complement receptors bind various fragments of complement. Both of these receptors are involved in the phagocytosis of opsinized particles. The lectin receptor recognizes and binds to lectins located on the outer membrane of microorganisms. Aside from the receptors that facilitate phagocytosis, PAM have receptors for a variety of cytokines (IL-1, IL-2, IL-4, IL-10, TNFα, INFγ, etc.). These cells also elicit adhesion molecules (ICAM-1), as well as receptors for the adhesion molecules (i.e. CD11/CD18 and MAC-1) (Lohmann-Matthes et al., 1994).

One of the most important functions of PAM is the maintenance of sterility in the alveolar septum. This basic process is accomplished by the release of reactive oxygen species and the mounting of an inflammatory response which results the killing of invading microorganisms (Lohmann-Matthes et al., 1994). The acute inflammatory response is initiated by the release of arachidonic acid metabolites and cytokines. Alveolar macrophages recruit and activate polymorphonuclear leukocytes to participate in the inflammatory response. Alveolar macrophages can release interleukin-1 (IL-1), prostanoids, transforming growth factor-beta, platelet activating factor, leukotrienes, and
tumor necrosis factor-alpha (TNF-α). Thus, PAM are, in part, responsible for the orchestration of cell mediated pulmonary defense.

Neutrophils: Neutrophils are complex mature hemopoietic cells that provide a defense for the body against infection by microorganisms. They also contribute to tissue repair following injury (for review see; Ferrante et al., 1992). Neutrophils are distinguished from other members of the family of polymorphonuclear leukocytes by differences in granule composition. The life span of a neutrophil from stem cell to its removal in tissue is approximately 12 to 14 days. However, the half-life of a blood borne neutrophil is 4 to 7 hr, therefore these cells must be replaced at a great rate (Price and Dale, 1977). The mature neutrophils move from the bone marrow into the circulation where they are distributed between the circulating pool and marginating pool. However, the bone marrow retains a significant number of mature neutrophils, which constitutes an important reserve pool during neutrophil consumption. Unlike tissue macrophages, neutrophils are not resident in tissue. Instead these cells are recruited from the blood to participate in inflammatory processes. Several bioactive substances possessing chemoattractive properties for neutrophils are produced and released by parenchymal cells as well as tissue macrophages. These pro-inflammatory chemokines include interlukin-8 (IL-8, human), GRO (human), macrophage inflammatory protein (MIP, rodent), as well as leukotriene B₄ (LTB₄, human and rodent). The activities and actions of these compounds have been discussed in reviews by Driscoll (1994), and Miller and
Krangel (1992). Furthermore, neutrophil tissue emigration is regulated by the expression of adhesion molecules by endothelial cells. Adhesion molecules are regulated by several cytokines and bioactive lipid mediators (Albelda et al., 1994). The roles of adhesion molecules, ICAMs and integrins, in tissue injury have been reviewed by Selby and MacNee (1993).

Neutrophils are the first non-tissue associated cell to accumulate at a site of inflammation, and function to engulf tissue debris (phagocytosis), as well as to kill microorganisms. As with other phagocytes, phagocytosis is normally accompanied by a respiratory burst. This respiratory burst mediated by NADPH oxidase located on the plasma membrane is similar to monocytes. However, unlike macrophages, neutrophils release extraordinarily large amounts of proteases and other cytotoxic factors that are contained in cytoplasmic granules (Ferrante et al., 1992). These factors contribute to the lethal cell injury caused by neutrophils. Thus neutrophils can be thought of as mobile soldiers of the immune system, migrating to target sites, killing invading pathogens, participating in tissue injury, and mediating inflammatory processes.

**ALL-trans-RETINOL MODULATION OF PHAGOCYTES AND CHEMICAL INDUCED INJURY**

**Role of Phagocytes in Chemical Induced Tissue Injury:** Tissue injury by chemicals can involve both direct and indirect target cell damage. While xenobiotics may act directly on
a target cell causing toxicity (i.e. following bioactivation), they may also act indirectly by recruiting, as well as activating resident and inflammatory phagocytes. These cells participate in the pathogenesis of tissue injury by releasing bioactive mediators (i.e. TNF, superoxide anion, nitric oxide, and proteases). A brief summary of chemicals in which the mediators of phagocytes have been shown to participate in lung and liver injury is shown in Tables 1.3 and 1.4. The role of phagocytes in chemical induced lung and liver injury has been reviewed by Laskin and Pendino (1995), Laskin (1990), and Willoughby and Willoughby (1984).

It has been shown that Kupffer cells are responsible for the progression of injury associated with the hepatotoxicity of carbon tetrachloride (ElSisi et al., 1993; Edwards et al., 1992). Furthermore, some investigators have shown evidence that Kupffer cells may be directly responsible for the hepatic damaged caused by endotoxin (Roerdrink et al., 1981). In the lung, the inflammatory response has been shown to be important in the elicitation of injury by several systemic and directly acting toxicants. For example, phagocytes play an important role in both acute and chronic ozone toxicity, as well as the toxicity associated with bleomycin and silica (Pino et al., 1992a; Hyde et al., 1992; Joad et al., 1993; Jones et al., 1994; Driscoll et al., 1990). Neutrophils have been shown to be especially destructive in the lung, participating in adult respiratory distress syndrome and causing almost complete obliteration of the lung parenchyma (Windsor et al., 1993).

Many chemicals have been shown to modulate the function of phagocytes, and thereby the outcome of chemically induced lung and liver injury (Table 1.5 and 1.6). It
<table>
<thead>
<tr>
<th>Mediator</th>
<th>Toxicant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive oxygen intermediates</td>
<td>Ozone</td>
<td>Pendino et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Asbestos</td>
<td>Roney and Holian 1989</td>
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<tr>
<td></td>
<td>Amiodarone</td>
<td>Zitnik et al., 1992</td>
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<td></td>
<td>Bleomycin</td>
<td>Habib et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Paraquat</td>
<td>Fukushima et al., 1993</td>
</tr>
<tr>
<td>Reactive nitrogen intermediates</td>
<td>Ozone</td>
<td>Pendino et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Ravinovici et al., 1991</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>Blackford et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Paraquat</td>
<td>Berisha et al., 1994</td>
</tr>
<tr>
<td>Hydrolytic enzymes</td>
<td>Silica</td>
<td>Ryrfeld et al., 1993</td>
</tr>
<tr>
<td>Bioactive lipids</td>
<td>Ozone</td>
<td>Madden et al., 1991</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Ravinovici et al., 1991</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>Koren et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Bleomycin</td>
<td>Scheule et al., 1992</td>
</tr>
<tr>
<td>Interlukin-1</td>
<td>Ozone</td>
<td>Pendino et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Cigarette Smoke</td>
<td>Soliman and Twigg, 1992</td>
</tr>
<tr>
<td></td>
<td>Amiodarone</td>
<td>Wilson and Lippmann, 1993</td>
</tr>
<tr>
<td></td>
<td>Bleomycin</td>
<td>Scheule et al., 1992</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>Ozone</td>
<td>Pendino et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Endotoxin</td>
<td>Kips et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Bleomycin</td>
<td>Scheule et al., 1992</td>
</tr>
<tr>
<td>Macrophage inflammatory protein</td>
<td>Ozone</td>
<td>Driscoll et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>Driscoll et al., 1990</td>
</tr>
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### Table 1.4

Inflammatory mediators involved in chemical induced liver injury

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Toxicant</th>
<th>Reference</th>
</tr>
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<td>Reactive oxygen intermediates</td>
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<td></td>
<td>Acetaminophen</td>
<td>Laskin and Pilaro, 1986</td>
</tr>
<tr>
<td></td>
<td>Galactosamine</td>
<td>Al-Tuwajri et al., 1981</td>
</tr>
<tr>
<td></td>
<td>CCl4</td>
<td>ElSisi et al., 1993</td>
</tr>
<tr>
<td></td>
<td>1,2-DCB</td>
<td>Gunawardhana et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>Laskin et al., 1988</td>
</tr>
<tr>
<td>Reactive nitrogen intermediates</td>
<td>Endotoxin</td>
<td>Lysz et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Acetaminophen</td>
<td>Laskin, 1992</td>
</tr>
<tr>
<td></td>
<td>CCl4</td>
<td>Pizcueta et al., 1992</td>
</tr>
<tr>
<td>Hydrolytic enzymes</td>
<td>Endotoxin</td>
<td>Tanner et al., 1983</td>
</tr>
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<td>Bioactive lipids</td>
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<td>Keppler et al., 1985</td>
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<td></td>
<td>Galactosamine</td>
<td>Keppler et al., 1985</td>
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<tr>
<td>Interlukin-1</td>
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<td>Feder et al., 1993</td>
</tr>
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<td></td>
<td>Acetaminophen</td>
<td>Laskin et al., 1993</td>
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<td>Tumor necrosis factor</td>
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<td>Silva et al., 1990</td>
</tr>
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<td></td>
<td>Acetaminophen</td>
<td>Laskin et al., 1993</td>
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<tr>
<td></td>
<td>Galactosamine</td>
<td>Hishinuma et al., 1990</td>
</tr>
<tr>
<td>Macrophage inflammatory protein</td>
<td>Cadmium</td>
<td>Kayama et al., 1995</td>
</tr>
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1,2-DCB = 1,2-Dichlorobenzene  
CCl4 = Carbon tetrachloride
Table 1.5

Effect of modifying phagocyte activity in chemical induced liver injury

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Pretreatment</th>
<th>Toxicity</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Acetaminophen</td>
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<td>↑</td>
<td>Sipes et al., 1989</td>
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<tr>
<td></td>
<td>Gadolinium chloride</td>
<td>↓</td>
<td>Laskin et al., 1995</td>
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<td></td>
<td>Dextran sulfate</td>
<td>↓</td>
<td>Laskin et al., 1995</td>
</tr>
<tr>
<td>Allyl Alcohol</td>
<td>Gadolinium chloride</td>
<td>↓</td>
<td>Przybocki et al., 1992</td>
</tr>
<tr>
<td>CCl4</td>
<td>All-trans-retinol</td>
<td>↑</td>
<td>ElSisi et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Gadolinium chloride</td>
<td>↓</td>
<td>Edwards et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Methyl palmitate</td>
<td>↓</td>
<td>ElSisi et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Lipopolysaccharide</td>
<td>↑</td>
<td>Nolan and Leibowitz, 1978</td>
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<td></td>
<td>Lipopolysaccharide</td>
<td>↓</td>
<td>Williams, 1988</td>
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<tr>
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<td>Polymyxin B</td>
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<td></td>
<td>Lipopolysaccharide</td>
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<td></td>
<td>SOD</td>
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<td>Anti-TNF antibody</td>
<td>↓</td>
<td>Hishinuma et al., 1990</td>
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<td></td>
<td>Glucan</td>
<td>↑</td>
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<td>Gadolinium chloride</td>
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<td>Anti-TNF-α antibody</td>
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<td>Silva et al., 1990</td>
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<tr>
<td>1,2-DCB</td>
<td>Gadolinium chloride</td>
<td>↓</td>
<td>Gunawardhana et al., 1993</td>
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<tr>
<td></td>
<td>Methyl palmitate</td>
<td>↓</td>
<td>Gunawardhana et al., 1993</td>
</tr>
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</table>

1,2-DCB = 1,2-Dichlorobenzene
CCl4 = Carbon tetrachloride
LPS = Lipopolysaccharide
TNF = Tumor necrosis factor
SOD = Superoxide dismutase
Table 1.6

Effect of modifying phagocyte activity in chemical induced lung injury

<table>
<thead>
<tr>
<th>Toxicant</th>
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<th>Toxicity</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Ozone</td>
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<td>Bleomycin</td>
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<td></td>
<td>BCG</td>
<td>↑</td>
<td>Chyczewska et al., 1993</td>
</tr>
<tr>
<td>Hyperoxia</td>
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<td>↓</td>
<td>Tang et al., 1994</td>
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has shown that seven days of oral dosing with all-trans-retinol (vitamin A) greatly enhances the hepatotoxicity of several hepatotoxicants (for reviews see, Hooser et al., 1993; Sauer et al., 1995a). The mechanism, at least in the case of carbon tetrachloride, for this potentiation appears to involve the enhanced release of reactive oxygen species (ROS) from retinol-primed Kupffer cells. However, in the lung it has been reported by Habib et al., (1993) that retinol can attenuate many of the inflammatory effects and oxidative damage caused by bleomycin. In this section, the ability of retinol to modulate the hepatotoxicity of carbon tetrachloride and pulmonary toxicity of bleomycin will be discussed.

**All-trans-retinol (Vitamin A):** The term vitamin A is a generic descriptor for a group of compounds, other than carotenoids, that possess the biological activity of all-trans-retinol or are closely related structurally. These compounds are derived from a mono-cyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion. Due to their close structural similarity to retinol, they are called retinoids. The retinoid family not only includes naturally occurring retinoids (Table 1.7), but also synthetic retinoids. Vitamin A and its naturally occurring analogs are an integral part of basic human health, and are vital to many biological processes. Natural retinoids occur in three basic forms; all-trans-retinol (vitamin A₁ alcohol, axerophthal®), all-trans-retinal (retinaldehyde, vitamin A₁ aldehyde, retinene®), and all-trans-retinoic acid (vitamin A₁ acid, tretinoin®). In solution these compounds can undergo geometric
Table 1.7

Naturally occurring retinoids and their structures

<table>
<thead>
<tr>
<th>Retinoid</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>all-trans-retinol (axerophthal®)</td>
<td><img src="image1.png" alt="Structure" /></td>
</tr>
<tr>
<td>all-trans-retinal (retinene®)</td>
<td><img src="image2.png" alt="Structure" /></td>
</tr>
<tr>
<td>all-trans-retinoic acid (tretinoin®)</td>
<td><img src="image3.png" alt="Structure" /></td>
</tr>
<tr>
<td>11-cis-retinal</td>
<td><img src="image4.png" alt="Structure" /></td>
</tr>
</tbody>
</table>
isomerization (cis-trans) in the presence of light and heat. Six isomers have been identified for each natural form of vitamin A; 13-cis, 11-cis, 9-cis, 7-cis, 9,13-cis, and 11, 13-cis. This vitamin is also sensitive to oxidation in ambient air, but if stored properly can be stable for several months. However, following esterification retinoids are extremely stable. Concentrated solutions of retinol and its esters are yellow to reddish in color. These compounds are insoluble in water or glycerol, but are readily soluble in most organic solvents. Most forms of vitamin A can be crystallized, but have low melting points (62 to 64 °C for retinol). Because of the unsaturated nature of vitamin A it has a strong absorption spectra ($\lambda_{\text{max}} = 325\text{nm}$ for retinol), as well as fluorescence properties (excitation = 325nm, emission = 480nm for retinol and retinyl esters). These properties aid in the analytical detection of these compounds.

Vitamin A is an essential fat soluble vitamin, present naturally in eggs, milk, butter, liver, and fish liver oils. In animal products it is found mostly in the form of retinyl palmitate. β-Carotene, the major "pro-" form of vitamin A, is found primarily in green and yellow leafy plants (Lotan, 1980). Pro-vitamin A is a generic indicator for all carotenoids that show the biological activity of vitamin A. Carotinoids are pigments found in both plants and animals with over 500 forms naturally occurring. However, only about 60 forms have pro-vitamin A activity and only 6 forms are commonly found in foods normally consumed. Interestingly, both retinoid and carotenoids in plants and animals occur almost exclusively in the all-trans form. They have been used as an
adjuvant to cancer chemotherapy, presumably because of their immunostimulatory activities. Furthermore, these compounds are available in over the counter vitamin preparations containing various amounts of vitamin A.

Two systems have been used to standardize the amount of vitamin A contained in food products and medications. These includes both the international unit (IU) and retinol equivalents (RE) system. One RE is defined as the biological activity equivalent to 1 mg retinol. Therefore, 6 mg β-carotene are required for 1 RE. One IU is defined as the amount of retinol required to produce normal growth in a standard rat or 0.3 mg retinol (0.344 mg retinyl acetate, 0.6 mg β-carotene). The National Research Council (1989) has set the adult US Recommended Dietary Allowance (USRDA) of vitamin A at 800 to 1,000 RE/day. Sustained intake of a diet with levels of vitamin A less than 100 RE/kg/d can result in hypovitaminosis A. Signs of hypovitaminosis A include loss of appetite, decreased growth, drying and keratinization of membranes, night blindness, ataxia, increased susceptibility to infection, and death.

Despite the known benefits of vitamin A, and its well-defined role in initiation of various biological processes, there is a distinct health risk associated with exposure to large quantities of vitamin A and its derivatives. Hypervitaminosis A can occur from either acute or chronic exposure. The effects of acute vitamin A toxicity occur within hours to days following a very large vitamin A intake, whereas the effects of chronic toxicity occur within weeks to years after consuming small quantities of vitamin A that
are not acutely toxic. Hypervitaminosis A is prevented by hepatic storage and sequestering of vitamin A, ineffective absorption of pro-vitamin A by the gut, and by irreversible oxidation of vitamin A to retinoic acid.

A wide variety of adverse effects accompany vitamin A toxicity. Biesalski (1989) has classified the typical symptoms of both acute and chronic hypervitaminosis A into four general categories: 1) changes in the central nervous system including increased cerebral pressure, headaches, dizziness, and loss of appetite; 2) liver disorders including hepatomegaly, cirrhosis, and increased collagen formation; 3) changes in bone such as pain, swelling, aching, and hypercalcemia; and 4) effects on skin and mucous membranes including dryness, peeling, brittle finger nails, and alopecia. There are numerous reported cases of vitamin A overdose causing toxicity, but only two documented cases whereby hypervitaminosis A resulted in death (Bush and Dahms, 1984; Leitner et al., 1975). However, a standard minimally toxic threshold dose for development of either acute or chronic vitamin A toxicity has not been defined.

Animal studies have confirmed that excess vitamin A causes liver injury, but very little mechanistic information is available on the hepatotoxicity of vitamin A. Leelaprute et al. (1973) reported that administration of retinol to rats at 50,000 IU/day for 16 days resulted in hepatotoxicity. The hepatotoxicity consisted of course granules of calcium confined to the sinusoids, as well as calcification and necrosis of Kupffer cells. The in vivo mechanism by which vitamin A causes hepatotoxicity is unknown. Unlike many chemical hepatotoxicants, vitamin A is not known to be bioactivated to a reactive
intermediate (i.e. radical) often associated as a cause of chemical induced liver injury. *In vitro*, retinol and its more water soluble metabolites (retinyl and retinoic acid) have been shown to disrupt membranes of hepatocellular organelles, such as mitochondria and lysosomes (Dingle and Lucy, 1961; Dingle, 1961; Lucy et al., 1963). This disruption can result in release of lysosomal enzymes, mitochondrial dysfunction, and eventually cell death. However, *in vitro* concentration required to cause subcellular organelle injury by this mechanism is extremely high (0.5 to 2 mg of vitamin A/mg of tissue). *In vivo*, the mechanism by which vitamin A causes membrane damage is likely related to its ability to alter phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity. Administration of 20,000 IU of vitamin A for seven days to rats significantly increased hepatic mitochondrial PLA<sub>2</sub> activity, and lysis of the hepatocyte membrane (Chandra, 1988). Phospholipase A<sub>2</sub> catalyzes the removal of fatty acids from the 2-position of phosphatidylcholine, and in this process forms lysolecithin, which has a detergent like action on membranes. Thus, an increase in PLA<sub>2</sub> activity by high doses of vitamin A administration may contribute to hepatocellular toxicity via this form of membrane destabilization. However, the literature suggests that vitamin A also causes liver damage by interacting with other agents, such as ethanol or hepatitis B viral infection (Leo et al., 1982; Hatoff et al., 1982).

**Carbon tetrachloride and all-trans-retinol:** ElSisi et al., (1993a) has shown that seven days of oral dosing with retinol greatly increases the hepatotoxicity of CCl<sub>4</sub> in rats. The mechanism for this potentiation appears to involve the enhanced release of reactive
oxygen species (ROS) from retinol-primed Kupffer cells and/or neutrophils. These ROS promote lipid peroxidation and increase the degree of parenchymal cell necrosis (ElSisi et al., 1993c). In support of this mechanism were the findings that retinol pretreated rats administered CCl₄ exhaled increased amounts of ethane (a marker of lipid peroxidation) in the absence of increased bioactivation of CCl₄ (ElSisi et al., 1993b). When methyl palmitate or gadolinium chloride (GdCl₃) was administered to inhibit Kupffer cell function, both the potentiation of CCl₄ hepatic injury and the increased exhalation of ethane were blocked. These results suggest that Kupffer cells release factors that promote lipid peroxidation and hepatocellular injury. The fact that superoxide dismutase and catalase (conjugated with polyethylene to prolong their half-lives) also blocked retinol potentiation of liver injury, implies that ROS play a major role in retinol potentiation of CCl₄ hepatotoxicity (ElSisi et al., 1993b). A graphical summary of this data is shown in figure 1.2.

Characterization of isolated Kupffer cells from animals pretreated with retinol showed that these cells possessed enhanced phagocytosis and the ability to produce reactive oxygen species (Mobley et al., 1991; Sipes et al., 1991; ElSisi et al., 1993c). A subpopulation of Kupffer cells isolated from retinol-treated rats were highly primed to release increased quantities of superoxide after stimulation with zymosan whereas other Kupffer cells isolated from the same rat released amounts of superoxide comparable to control upon stimulation (Mobley et al., 1991). Although zymosan stimulates superoxide production by Kupffer cells in retinol treated animals, it is the continued exposure to
Figure 1.2. Effect of antioxidants and Kupffer cell inhibitors on all-trans-retinol potentiation of carbon tetrachloride induced hepatotoxicity. Male Sprague-Dawley rats received retinol (RET; 75 mg/kg/day, p.o., for 7 days) followed by CCl₄ (0.2 mL/kg, i.p.) on day eight. Two hr after carbon tetrachloride (CCl₄) administration, animals were given polyethylene conjugated superoxide dismutase (SOD) and polyethylene conjugated catalase (CAT). Gadolinium chloride (GdCl₃) and methyl palmitate (MP) were given 24 hr before the administration of CCl₄. Over a 24 hr period following CCl₄ administration exhaled ethane was measured as a marker of lipid peroxidation. At the end of that period the rats were killed and plasma ALT activity was measured. °Significantly different from CCl₄ treated group. ND - data not determined.
retinol that "primes" Kupffer cells to produce the greatest amount of superoxide upon stimulation.

Although CCl$_4$ is the most studied chemical in the retinol model of potentiated hepatotoxicity, several other chemicals have been studied. In the rat, retinol pretreatment has been shown to potentiate the hepatotoxicity of acetaminophen, endotoxin, bromotrichloromethane, allyl alcohol, 1,1-dichoroethylene, among others.

Bleomycin and all-trans-retinol: Unlike the effects on hepatotoxicity, there is significantly less information available on the ability of retinol to modulate the pneumotoxicity of compounds. It has been reported by Habib et al., (1993) that retinol can attenuate many of the inflammatory effects caused by bleomycin in the rat lung. Bleomycin is an antineoplastic agent used in the treatment of lymphoma and seminoma. Bleomycin is believed to complex with ferrous iron and catalyze the formation of hydroxyl radical in the lung were it causes toxicity. Exposure to larger doses of this antineoplastic results in oxidative damage to the lung parenchyma and may progress into pulmonary fibrosis.

In the studies performed by Habib et al., (1993), the ability of retinol to alter the pneumotoxicity of bleomycin was performed. Briefly, male Sprague-Dawley rats were pretreated with retinol (50,000 IU/kg/day) for nine days prior to bleomycin. The rats were then subjected to an intratracheal injection of bleomycin sulfate (6 units/kg) and killed 7 and 14 days later. Rats pretreated with retinol demonstrated statistically significant
reductions in lung wet weight/body weight ratios, in the PMN cell count of BALF, and in alveolar macrophage superoxide anion production seven days after bleomycin administration as compared to animals which received retinol vehicle and bleomycin. The authors concluded that such a decrease in alveolar macrophage superoxide production was responsible for the attenuation of bleomycin pulmonary injury. Furthermore, the authors speculated that the mechanism behind the decreases in alveolar macrophage function was due to retinol’s antioxidant abilities.
STATEMENT OF THE PROBLEM

ElSisi et al., (1993a) have reported that acute hypervitaminosis A in rats dramatically increases the hepatotoxicity of a number of chemicals. The mechanism for this potentiation appears to be mediated by the enhanced release of reactive oxygen species (ROS) from retinol-primed Kupffer cells (resident liver macrophages). In the lung it has been reported by Habib et al., (1993) that retinol can protect the lung from many of the inflammatory effects caused by bleomycin. Whether or not retinol pretreatment can modulate chemical-induced injury of other pneumotoxicant is unknown. The studies in this dissertation were design to test the hypothesis that all-trans-retinol increases hepatic injury through a pro-inflammatory mechanism, while in the lung injury is attenuated though an anti-inflammatory mechanism. In other words, retinol potentiates chemically induced liver injury by increasing the participation of Kupffer cells and other phagocytes in the associated inflammatory response. However, in the lung the actions of retinol are opposite from that observed in the liver. It attenuates chemically induced injury by inhibiting the associated inflammatory response.

The objective of this work was to evaluate the ability of all-trans-retinol to modulate chemically induced lung and liver injury, as well as show that phagocytes participate in the progression of chemically induced tissue injury. This dissertation is divided into chapters as outlined below to address the problems stated in the hypothesis.
Chapter 2. **Effect of all-trans-retinol pretreatment on chemically induced lung injury in the male Sprague-Dawley rat.**

Habib et al., (1993) have reported that acute hypervitaminosis A in rats dramatically attenuates the pneumotoxicity of i.t. administered bleomycin. Whether or not acute hypervitaminosis A can modulate chemical-induced injury of systemic lung toxicants is unknown. Therefore, the effects of all-trans-retinol pretreatment on pulmonary injury caused by 1-nitronaphthalene, 2-nitronaphthalene, and paraquat were evaluated. In this chapter the data from several experiments characterizing the ability of retinol to protect the lung from several systemic lung toxicants are presented.

Chapter 3. **Effect of all-trans-retinol pretreatment on chemically induced liver injury in the male Sprague-Dawley rat.**

ElSisi et al., (1993a) have reported that acute hypervitaminosis A in rats dramatically increases the hepatotoxicity of a number of chemicals. And, although the lung is the primary target of both 1-NN and 2-NN, these compounds can also cause hepatotoxicity. Therefore, in this chapter the data from several experiments characterizing the ability of retinol to potentiate hepatic injury of 1-NN and 2-NN are presented. Furthermore, data showing the ability of gadolinium chloride to block the potentiated hepatotoxicity of these compounds are reported. Data showing the toxicity induced by 1-
NN and 2-NN in primary isolated hepatocytes from phenobarbital, retinol, and control treated rats are also included in this section.

Chapter 4. **Effect of all-trans-retinol pretreatment on the function of pulmonary alveolar macrophage isolated from male Sprague-Dawley rats.**

Seven days of oral dosing with retinol greatly enhances the release of reactive oxygen species (ROS) from Kupffer cells. Furthermore, it has recently been reported by Habib et al., (1993) that retinol can reduce the production of superoxide anion by alveolar macrophages isolated from rats intoxicated with bleomycin. In the models of lung injury presented here alveolar macrophages may play an important role in recruiting inflammatory cells and releasing cytotoxic factors (ROS and cytokines). Therefore, an important objective was to understand what effect retinol had on alveolar macrophages function. In this chapter, data showing the ability of retinol to modulate alveolar macrophage function are presented. Markers of macrophage function include superoxide anion and nitric oxide production, as well as the release of TNF, LTB₄, and PGE₂.
CHAPTER 2

EFFECT OF ALL-trans-RETINOL PRETREATMENT ON CHEMICALLY INDUCED LUNG INJURY IN THE MALE SPRAGUE-DAWLEY RAT

Since acute hypervitaminosis A has been shown to potentiate the hepatotoxicity of several chemicals, it was hypothesized that retinol pretreatment would potentiate the lung injury caused by pulmonary toxicants. To test this hypothesis the effect of retinol on the pulmonary injury caused by known lung toxicants was investigated. Compounds chosen for this study were 1-nitronaphthalene, 2-nitronaphthalene, and paraquat. These all cause pulmonary injury following systemic administration.

1-Nitronaphthalene (1-NN): 1-Nitronaphthalene (Table 2.1) is a nitroaromatic environmental contaminant that has been detected in urban ambient airborne particulates in both the United States and Europe (Ramdahl and Urdal, 1982; Brorstrom-Lunden and Lindskog, 1985). A major source for 1-NN is the emissions of light- and heavy-duty diesel engines, which have been reported to be as high as 0.7 mg of 1-NN/kg of particulate (Draper, 1986). The toxicity of 1-NN has been extensively reviewed in IARC Monographs (1990). Environmental exposure to 1-NN would be through inhalation, and 1-NN has been shown to be a potent systemic lung toxicant. When given systemically, 1-NN causes both acute bronchiolar epithelial injury in the lung and parenchymal and bile
duct epithelial cell injury in the liver of rats (Johnson et al., 1984). However, the lung appears to be the primary target organ. In the lung, the development of 1-NN-induced toxicity can be divided into two distinct stages (Figure 2.1). The initial stage of injury is characterized by ultrastructural lesions (swelling of smooth endoplasmic reticulum and mitochondria) in nonciliated bronchiolar epithelial (Clara) cells of the distal-most bronchioles. These lesions occur within 1 hr of treatment. The second stage of pulmonary toxicity, which occurs approximately 6 hr after treatment, is marked by infiltration of inflammatory cells into the interstitial areas around damaged bronchioles, the onset of a unique respiratory distress syndrome, and progressive degeneration of the bronchiolar epithelium. In the lung, the in situ formation of reactive intermediates of 1-NN in Clara cells is presumably responsible for the early damage associated with the bronchiolar epithelium, while later lung injuries apparently are caused by the associated inflammatory responses (Sauer et al., 1996).

In vitro studies (Price et al., 1995; Rasmussen, 1986; Rasmussen et al., 1986) using lung microsomes, lung slices, and isolated lung cells have shown that 1-NN is metabolized by cytochrome P-450 enzymes via an oxidative pathway that results in macromolecular binding. Autoradiographic studies (Rasmussen, 1986) have indicated that the majority of binding occurs in the bronchiolar epithelium. In addition, Verschoyle and Dinsdale (1990), using inhibitors of specific cytochrome P-450 isoenzymes, have shown 1-NN toxicity to be correlated with cytochrome P-450 IIB1 activity in the lung. This isoenzyme of cytochrome P-450 is primarily localized in the Clara cell. Possible
metabolic pathways for the biotransformation of 1-NN are illustrated in Figure 2.2. Furthermore, the effect of cytochrome P-450 induction and inhibition on the toxicity of 1-NN is shown in Table 2.2.

2-Nitronaphthalene (2-NN): 2-Nitronaphthalene (Table 2.1) is a nitroaromatic environmental contaminant structurally similar to 1-NN that is also produced during the combustion of diesel fuel (Draper, 1986). The toxicity of 2-NN has been extensively reviewed in IARC Monographs (1990). Although not as well characterized as 1-NN, 2-NN is also a systemic lung toxicant. In rats, systemic administration of 2-NN results in lung toxicity characterized by necrosis of the bronchiolar epithelium (Dinsdale and Verschoyle, 1987). Very little information is available in the literature on pulmonary pathology or bioactivation of 2-NN. Furthermore, no information is available on the mechanism by which 2-NN causes pneumotoxicity.

Paraquat (PQ): Paraquat (1,1'-dimethyl-4,4'-dipyridylium dichloride), also known as methyl viologen, is an effective contact herbicide that is pneumotoxic in rodents and humans (Table 2.1). The first reported lung toxicity in humans appeared in 1966 (Bullivant, 1966). Because of its structural similarity to endogenous polyamines (spermidine, putrescine), PQ is actively accumulated by the lung which explains why the lung is its primary target organ (Smith, 1982). Experimental animal studies using a single dose of PQ showed extensive lung toxicity associated with damage to type I, type II,
endothelial, and bronchiolar epithelial cells 24 to 48 hr after administration (Sykes et al., 1977; Dearden et al., 1982). This destructive phase of PQ-induced pulmonary toxicity is associated with alveolar and interstitial edema, as well as an acute inflammatory response leading to the collapse of lung parenchyma. The initial destructive phase is followed by a proliferative phase which can progress to intraalveolar and interstitial fibrosis.

The metabolic biotransformation of paraquat is unique to the other compounds discussed in this section. Paraquat is a compound which causes the formation of reactive oxygen species during its cyclic enzymatic reduction/oxidation (Figure 2.3). Ilett et al., (1974) found no evidence that PQ or its metabolites became bound to lung tissue in vivo, as do toxicants such as bromobenzene, 4-ipomeanol, 3-methylfuran, and α-naphthylthiourea. Instead, evidence for PQ-dependent hydrogen peroxide formation was measured in lung and liver microsomal preparations from both rabbits and rats (Ilett et al., 1974). The formation of superoxide anion during the enzymatic reduction and oxidation of PQ has been shown to result in the production of hydrogen peroxide, as well as significant pulmonary lipid peroxidation. The lipid peroxidation is likely responsible for the early cellular damage and the initiation of the associated alveolar pneumonitis (Schweich et al., 1994).

Objective: The objective of these studies was to evaluate the effects of retinol pretreatment on the pulmonary injury caused by 1-NN, 2-NN, and PQ using bronchioalveolar lavage fluid (BALF) markers of cytotoxicity, as well as light and
scanning electron microscopy. These studies will provide a basic understanding of how retinol modulates the toxicity of systemically administered chemicals in the lungs of male Sprague-Dawley rats.
Table 2.1

Structures of 1-nitronaphthalene, 2-nitronaphthalene, and paraquat

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
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<tbody>
<tr>
<td>1-Nitronaphthalene</td>
<td><img src="image" alt="Structure of 1-NN" /></td>
</tr>
<tr>
<td>2-Nitronaphthalene</td>
<td><img src="image" alt="Structure of 2-NN" /></td>
</tr>
<tr>
<td>Paraquat (PQ)</td>
<td><img src="image" alt="Structure of PQ" /></td>
</tr>
</tbody>
</table>
Table 2.2

Effect of cytochrome P-450 induction and inhibition on 1-nitronaphthalene induced toxicity in the rat

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Effect on P-450</th>
<th>Liver toxicity</th>
<th>Lung toxicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>↑ hepatic P-450</td>
<td>↑</td>
<td>↓</td>
<td>Johnson et al., 1986</td>
</tr>
<tr>
<td>ABT</td>
<td>↓ hepatic P-450</td>
<td>↓</td>
<td>↓</td>
<td>Sauer and Sipes, 1996</td>
</tr>
<tr>
<td></td>
<td>↓ pulmonary P-450</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKF 525A</td>
<td>↓ hepatic P-450</td>
<td>↓</td>
<td>no effect</td>
<td>Johnson et al., 1986</td>
</tr>
<tr>
<td>Aroclor 1254</td>
<td>↑ hepatic P-450</td>
<td>↑</td>
<td>↓</td>
<td>Verschoyle et al., 1993</td>
</tr>
<tr>
<td>β-Naphthoflavone</td>
<td>↑ hepatic P-450</td>
<td>↑</td>
<td>↓</td>
<td>Verschoyle et al., 1993</td>
</tr>
<tr>
<td>p-Xylene</td>
<td>↓ pulmonary P-450</td>
<td>no effect</td>
<td>↓</td>
<td>Verschoyle et al., 1993</td>
</tr>
<tr>
<td>O,O,S-MeP(S)</td>
<td>↓ pulmonary P-450</td>
<td>no effect</td>
<td>↓</td>
<td>Verschoyle et al., 1993</td>
</tr>
</tbody>
</table>

ABT = l-aminobenzotriazole
O,O,S-MeP(S) = O,O,S-trimethylphosphorodithioate
↑ = Increase
↓ = Decrease
Figure 2.1. Progression of 1-nitronaphthalene induced lung injury. In the lung, the development of 1-NN induced toxicity can be divided into two distinct stages. The initial stage of injury is characterized by ultrastructural lesions (swelling of smooth endoplasmic reticulum and mitochondria) in nonciliated bronchiolar epithelial (Clara) cells of the distal-most bronchioles. These lesions occur within 1 hr of treatment. The second stage of pulmonary toxicity, which occurs approximately 6 hr after treatment, is marked by infiltration of inflammatory cells into the interstitial areas around damaged bronchioles, the onset of a unique respiratory distress syndrome, and progressive degeneration of the bronchiolar epithelium.
Figure 2.2. Metabolic bioactivation of 1-nitronaphthalene. 1-Nitronaphthalene is a compound which could cause the formation of reactive oxygen species during its cyclic enzymatic reduction/oxidation, or cause macromolecular binding following oxidation or reduction.
Figure 2.3. Metabolic bioactivation of paraquat. Paraquat is a compound which causes the formation of reactive oxygen species during its cyclic enzymatic reduction/oxidation. The formation of superoxide during the enzymatic reduction and oxidation of paraquat has been shown to result in the production of hydrogen peroxide, depletion of energy store, and pulmonary lipid peroxidation.
MATERIALS AND METHODS

Chemicals

All-trans-retinol (Aquasol A drops) was purchased from Bindley Western Drug Co. (Phoenix, AZ). 1-Nitronaphthalene, and 2-nitronaphthalene were purchased from Aldrich Chemical Co. (Milwaukee, WI). Paraquat (1,1'-dimethyl-4,4'-dipyridylium dichloride), lactate dehydrogenase diagnostic kit (procedure No. 228-UV), and gamma-glutamyl transpeptidase diagnostic kit (procedure No. 325) were purchased from Sigma Chemical Co. (St. Louis, MO). Diff-Quick stain reagent was purchased from Baxter Scientific Products (McGaw Park, IL). Bio-Rad protein assay was purchased from Bio-Rad Co. (Richmond, CA).

Animals

Male Sprague-Dawley rats (250 to 300 g) were purchased from Harlan (Indianapolis, IN). Animals were housed in pans and maintained on a 12-hr light-dark cycle at approximately 22°C and 40% humidity. Except for the pair-feeding study described below, they were allowed free access to food (Wayne Lab-Blox Co.) and water. Animals were allowed to acclimate for at least seven days prior to any treatment.
Experimental design

Rats were pretreated with 75 mg/kg/day (250,000 IU, 262 mmol) of retinol or vehicle (7% tween-20, 10% propylene glycol in water) by oral gavage once a day for seven days. Twenty four hr following the last retinol treatment, animals received a single i.p. injection of 1-NN (100 mg/kg in 2.0 mL/kg of peanut oil, i.p.), 2-NN (200 mg/kg in 2.0 mL/kg of peanut oil, i.p.), or PQ (25 mg/kg in 2.0 mL/kg saline, i.v.). Animals were observed for clinical signs of toxicity up to 48 hr after toxicant administration. At 3, 6, 12, 24, and 48 hr after 1-NN, 24 hr after 2-NN, and 48 hr following PQ treatment, rats were killed by carbon dioxide inhalation. Blood was collected from the posterior vena cava into heparinized tubes; lungs were lavaged; and sections of the lung processed for morphological analysis.

Biochemical evaluation of lung injury

At necropsy, subsequent to euthanasia by carbon dioxide inhalation, lungs were removed, weighed, and bronchioalveolar lavage was performed as described by Roth (1980). The lavage fluid was centrifuged for 10 minutes at 1200 g and supernatant removed from the sedimented cells. The cell pellet was resuspended in 0.5 mL of saline and evaluated for total cell number, as well as cell type using Diff-Quick stain reagent. In the cell free lavage fluid the activities of lactate dehydrogenase (LDH), and gamma-glutamyl transpeptidase (GGT), as well as protein lavage fluid content were measured.
Morphological evaluation of lung injury

Lungs from animals not subjected to bronchioalveolar lavage were examined by light and scanning electron microscopy (SEM). Lungs were fixed by tracheal inflation with 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3; 4°C) for 12 min. The periphery of the inferior lobe of the right lung was cut into 1 x 0.3 cm longitudinal blocks, and immersed in the 3% buffered glutaraldehyde for 24 hr. The samples were dehydrated using a standard ethanol series, infiltrated with hexamethyldisilazane and dried. They were mounted on aluminum stubs with conductive carbon glue, sputter coated with a 100 angstrom thick layer of gold, and examined in a Etec Autoscan scanning electron microscope at an accelerating voltage of 20 kV.

For histological evaluation, lobes of lung not used for SEM were further fixed by immersion in 10% neutral buffered formalin, processed, and embedded in paraffin. Sections, 5 micron in thickness, were stained with hematoxylin and eosin (H&E).

Statistical analysis

Results are expressed as the mean ± the standard error of the mean (SEM). Statistical comparisons were accomplished using a two-tailed ANOVA and the Newman-Keuls test. Data were considered significantly different when \( p < 0.05 \).
RESULTS

1-Nitronaphthalene

Clinical signs of toxicity: Administration of 1-NN to control animals, those which were pretreated with retinol vehicle, showed signs of respiratory distress syndrome (RDS) within 6 hr of treatment. These animals exhibited moderate dyspnea, as well as chromodacryorrhea (bloody tears). Rats pretreated with retinol and administered 1-NN did not exhibit respiratory distress syndrome (Table 2.2).

Bronchioalveolar lavage analysis: Rats administered retinol vehicle and 1-NN exhibited increases of protein content, as well as LDH and GGT activities in their lavage fluid over a 48 hr time period (Figure 2.4). Both retinol and vehicle pretreated animals possessed similar pulmonary lesions at time points earlier than 6 hr following 1-NN administration as determined by lavage fluid markers of pulmonary injury and lung wet/dry weight ratios. However, after 6 hr the toxicity caused by 1-NN differed dramatically in these groups. The vehicle pretreated rats showed a marked increase in lavage fluid cell content and number of neutrophils as compared to controls at 24 hr after 1-NN administration (Table 2.2). In the lavage fluid of rats pretreated with retinol, the 1-NN induced increases in protein content and activity of LDH were significantly decreased. In addition, the lavage fluid appeared free of inflammatory cells in these animals. Lavage fluid GGT activity, a specific marker for Clara cell damage (Day et al., 1990), was significantly
increased in retinol pretreated animals as compared to those pretreated with vehicle. All biochemical findings were confirmed with light microscopy (see below).

**Microscopic observations:** Microscopic examination of the lung showed characteristic 1-NN-induced toxicity as described by Johnson et al., (1984). Time-course experiments with 1-NN indicated that both retinol and retinol vehicle pretreated animals possessed similar pulmonary lesions at time points earlier than 6 hr following 1-NN administration. Pulmonary lesions consisted of general cellular swelling, as well as dilation of smooth endoplasmic reticulum and mitochondria of Clara cells in the distal bronchioles. By 24 hr, extensive lesions were observed in the lungs of rats given retinol vehicle and 1-NN. These lesions were restricted to the bronchioles and consisted of Clara cell and ciliated cell necrosis with exfoliation from the basement membrane (Figure 2.5 and 2.6). Severe interstitial and perivascular edema, as well as moderate pneumonitis were also observed. The pneumonitis consisted of predominantly neutrophils infiltrating into the interstitium around the damaged bronchioles. However, associated with this infiltrate were mononuclear cells, mast cells, and small lymphocytes. Rats pretreated with retinol and administered 1-NN demonstrated no gross changes in the lungs. Microscopic and ultramicroscopic observation of the lungs revealed that at 24 hr these animals had no interstitial edema or pneumonitis. However, severe swelling and ultrastructural alterations of Clara cells were evident after 1-NN administration. Retinol alone had no effect on the general morphology of the lung.
Retinol and food consumption: Retinol pretreated animals tend to lose weight during the seven day pretreatment period. This is not surprising since large doses of retinol are known to produce anorexia (Leelaprute et al., 1973). To determine if retinol pretreatment altered 1-NN-induced toxicity by a process independent of reduced food intake and body weight loss, vehicle-pretreated control rats were pair fed to the retinol pretreated rats for 7 days. By day 8, the pair fed controls lost 31 ± 3g, the retinol treated rats had lost 26 ± 7g, and the ad libitum control rats had gained 33 ± 4g. The average food intake for controls was 28 ± 4g of food/day, and retinol treated rats was 14 ± 2g of food/day. Administration of 1-NN 24 hr after the last dose of retinol, resulted in lavage fluid LDH activities (U/L) of 66 ± 9 and 48 ± 12 for the pair-fed control group and the ad libitum group respectively, but only 30 ± 3 for the retinol pretreated group. Thus, protection of lung injury by retinol is not merely the result of reduced food intake or reduction in body weight.

2-Nitronaphthalene

Clinical signs of toxicity: None of the animals treated with 2-NN showed signs of RDS, a prominent feature of 1-NN-induced pulmonary toxicity (Table 2.3). However, animals receiving retinol vehicle and 2-NN exhibited signs of lethargy. No signs of overt toxicity were observed in retinol pretreated rats administered 2-NN.
**Bronchioalveolar lavage analysis:** Rats administered retinol vehicle and 2-NN exhibited increased protein content, LDH and GGT activities in their lavage fluid at 24 hr. These rats also showed a marked increase in the infiltration of neutrophils into the lung as compared to controls (Table 2.2). When 2-NN was administered to rats pretreated with retinol, statistically significant decreases in lavage fluid protein content and LDH activity were observed compared to vehicle pretreated rats given and 2-NN (Figure 2.7). In addition, the lavage fluid of these rats was free of inflammatory cells. However, when compared to vehicle rats given 2-NN, the GGT activity in lavage fluid was significantly increased.

**Microscopic observations:** Microscopic examination of the lungs at 24 hr showed that the majority of pulmonary lesions were restricted to the bronchioles in animals pretreated with retinol vehicle and given 2-NN. These lesions were similar to those observed in 1-NN treated rats. This damage consisted of Clara and ciliated cell necrosis with only minor exfoliation of these cells from the basement membrane. Mild interstitial edema and moderate pneumonitis were also observed, with inflammatory cells infiltrating into the interstitium around the damaged bronchioles. The degree of epithelial cell exfoliation and inflammatory cell infiltration was not as severe as in 1-NN treated animals. Rats pretreated with retinol and administered 2-NN demonstrated no gross changes in the lung. However, at 24 hr swelling and vacuolization of the Clara cells was observed following administration of 2-NN to these rats pretreated with retinol (Figure 2.8).
**Paraquat**

**Clinical signs of toxicity:** Rats given retinol vehicle and PQ showed signs of mild dyspnea and lethargy. This feature of toxicity was absent from the retinol pretreated animals that were administered PQ.

**Bronchoalveolar lavage analysis:** Rats administered retinol vehicle and PQ exhibited increased protein content and LDH activity in their lavage fluid at 48 hr. A marked increase in the infiltration of neutrophils was also observed (Table 2.2). Pretreatment of rats with retinol caused a statistically significant decrease in the lavage fluid protein content and LDH activity in response to PQ administration, as compared to animals given vehicle and PQ (Figure 2.9). In addition, the lavage fluid of these rats appeared free of inflammatory cells.

**Microscopic observations:** Microscopic examination of the lungs at 48 hr after PQ showed that the majority of pulmonary lesions was associated with the alveolar sacs in animals pretreated with retinol vehicle and given PQ (Figure 2.10). These lesions consisted of focal areas of damage to the alveolar parenchyma, infiltration of inflammatory cells, as well as alveolar and interstitial edema. However, animals that received retinol did not develop edema or an inflammatory cell infiltrate following
treatment with PQ. Paraquat was not hepatotoxic and did not cause alterations in plasma ALT activity in either vehicle pretreated or retinol pretreated rats (data not shown).
Table 2.3

Effect of all-trans-retinol pretreatment on chemical induced pneumonitis.

<table>
<thead>
<tr>
<th>Pretreatment Treatment</th>
<th>Number of animals in respiratory distress</th>
<th>Number of cells in lavage fluid (cells/μL)</th>
<th>Number of neutrophils in lavage fluid (cells/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (peanut oil)</td>
<td>0</td>
<td>91 ± 39</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>1-Nitronaphthalene</td>
<td>12</td>
<td>367 ± 152&lt;sup&gt;a&lt;/sup&gt;</td>
<td>173 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-Nitronaphthalene</td>
<td>0</td>
<td>200 ± 21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>110 ± 30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paraquat</td>
<td>0</td>
<td>500 ± 50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>312 ± 95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>all-trans-Retinol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle (peanut oil)</td>
<td>0</td>
<td>93 ± 21</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>1-Nitronaphthalene</td>
<td>0</td>
<td>101 ± 50&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-Nitronaphthalene</td>
<td>0</td>
<td>98 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paraquat</td>
<td>0</td>
<td>110 ± 30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Number of animals in respiratory distress (labored breathing and gasping), as well as total number of cells and neutrophils in lavage fluid at 24 hr after administration of 1-nitronaphthalene (100 mg/kg, i.p.), of 2-nitronaphthalene (200 mg/kg, i.p.), or paraquat (25 mg/kg, i.v.) to vehicle and retinol pretreated rats (means ± SEM). n=12. <sup>a</sup>Significantly different from vehicle. <sup>b</sup>Significantly different from respective retinol vehicle group.
Figure 2.4. Effect of all-trans-retinol on 1-nitronaphthalene induced lung injury. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle followed 24 hr later by 1-NN (100 mg/kg, i.p.) or peanut oil. At various times after 1-NN administration rats were killed and markers of lung injury were evaluated as described in Materials and Methods. (A) BALF LDH activity, (B) BALF protein content, (C) BALF GGT activity, (D) dry lung to wet lung weight ratios. Bars represent means ± SEM. n=12. aSignificantly different from respective peanut oil control. bSignificantly different from respective retinol vehicle group (bronchioalveolar lavage fluid = BALF).
Figure 2.5. Effect of all-trans-retinol on 1-nitronaphthalene induced microscopic pathology of the lung. Male Sprague-Dawley rats received either retinol (75 mg/kg/d, p.o., for 7 days) or vehicle followed 24 hr later by 1-NN (100 mg/kg, i.p.). Twenty four hr after 1-NN administration rats were killed and lungs fixed by tracheal inflation as described in Materials and Methods. (A) Representative terminal bronchiole (TB) from a rat pretreated with retinol vehicle and administered 1-NN. Note the exfoliation of epithelium from basement membrane (arrows), as well as interstitial edema and infiltration of inflammatory cells (EI). (B) Representative terminal bronchiole (TB) from a rat pretreated with retinol and administered 1-NN. Note that the epithelium is intact (arrows) and the lack of interstitial edema and infiltration of inflammatory cells (33 X, original magnification). Stained with hematoxylin and eosin (H&E).
Figure 2.6. Effect of all-trans-retinol on 1-nitronaphthalene induced ultrastructural pathology of the lung. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle followed 24 hr later by 1-NN (100 mg/kg, i.p.). Twenty four hr after 1-NN administration rats were killed and lungs fixed by tracheal inflation as described in Materials and Methods. (A) Representative nonciliated (CC) and ciliated bronchiolar epithelium from a terminal bronchiole of a rat pretreated with retinol vehicle and administered 1-NN (1300 X, original magnification). (B) Representative nonciliated (CC) and ciliated bronchiolar epithelium from a terminal bronchiole of a rat pretreated with retinol and administered 1-NN (1600 X, original magnification).
Figure 2.7. Effect of all-trans-retinol on 2-nitronaphthalene induced lung injury. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o., for 7 days) or vehicle followed by 2-NN (200 mg/kg, i.p.) on day eight. Twenty four hr after 2-NN administration rats were killed and markers of lung injury were evaluated as described in Materials and Methods. (A) BALF LDH activity, (B) BALF protein content, (C) BALF GGT activity. Bars represent means ± SEM. n=12. aSignificantly different from respective vehicle control. bSignificantly different from respective retinol vehicle group (bronchioalveolar lavage fluid = BALF).
Figure 2.8. Effect of all-trans-retinol on 2-nitronaphthalene-induced microscopic pathology of the lung. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o., for 7 days) or vehicle followed 24 hr later by 2-NN (200 mg/kg, i.p.). Twenty four hr after 2-NN administration rats were killed and lungs fixed by tracheal inflation as described in Materials and Methods. (A) Representative terminal bronchiole (TB) from a rat pretreated with retinol vehicle and administered 2-NN. Note the minor exfoliation of epithelium (arrows), as well as interstitial edema and inflammation cells. (B) Representative terminal bronchiole (TB) from a rat pretreated with retinol and administered 2-NN. Note that the nonciliated epithelium is intact but swollen (arrows), as well as the lack of interstitial edema and infiltration of inflammatory cells (66 X, original magnification). Stained with hematoxylin and eosin (H&E).
Figure 2.9. Effect of all-trans-retinol on paraquat induced lung injury. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o., for 7 days) or vehicle followed by PQ (25 mg/kg, i.p.) on day eight. Forty eight after PQ administration rats were killed and markers of lung injury were evaluated as described in Materials and Methods. (A) BALF LDH activity, (B) BALF protein content. Bars represent means ± SEM. n=12. aSignificantly different from respective vehicle control. bSignificantly different from respective retinol vehicle group (bronchoalveolar lavage fluid = BALF).
Figure 2.10. Effect of all-trans-retinol on paraquat-induced microscopic pathology of the lung. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o., for 7 days) or vehicle followed by PQ (25 mg/kg, i.p.). Forty eight hr after PQ administration rats were killed and lungs fixed by tracheal inflation as described in Materials and Methods. (A) Representative alveolar sac from a rat pretreated with retinol vehicle and administered PQ. Note the severe damage to the alveolar parenchyma, infiltration of inflammatory cells, as well as structural of the alveolar sacs (B) Representative alveolar sac from a rat pretreated with retinol and administered PQ (66 X, original magnification). Note the lack of infiltration of inflammatory cells and alveolar remodeling. Stained with hematoxylin and eosin (H&E).
DISCUSSION

In these studies, the effects of retinol pretreatment on the lung of 1-NN, 2-NN, and PQ were evaluated in male Sprague-Dawley rats using both microscopic and lavage fluid markers of toxicity. The results reported here demonstrate that treatment of rats with large doses of retinol dramatically protects against chemically-induced lung injury. Furthermore, retinol apparently does not affect the initial injury caused by the pneumotoxicant, but instead limits the progression of injury.

At 24 hr after either 1-NN or 2-NN administration, retinol protected from the majority of pulmonary injuries. The bronchioles from the lungs of both 1-NN and 2-NN treated animals displayed epithelial necrosis and exfoliation, interstitial edema, and pneumonitis. It should, however, be noted that 1-NN is significantly more pneumotoxic than 2-NN in the rat. Retinol pretreatment blocked the inflammatory response, edematous changes, exfoliation of bronchiolar epithelium, and several of the lavage fluid markers of pulmonary cytotoxicity caused by both 1-NN and 2-NN. However, at both the light and electron microscopic levels, the Clara cells from these animals showed significant morphological alterations consisting of vacuolization and cellular swelling. Furthermore, lavage fluid GGT activity, a specific marker for Clara cell damage (Day et al., 1990), was significantly increased in the retinol pretreated animals given 1-NN or 2-NN. The increased lavage fluid GGT activity is suggestive of increased Clara cell damage.
However, the lack of exfoliation and/or edema may be responsible for the enhanced GGT activity present in lavage fluid.

An important finding is that retinol did not block the initial degenerative lesions, occurring up to six hr after 1-NN treatment. 1-Nitronaphthalene induced changes in the nonciliated bronchiolar epithelium as indicated by lung morphology and lavage fluid marker of injury were equivalent in the retinol and vehicle groups. Because both the time course for the development and pattern of initial injury resembled that of 1-NN at its earliest stages of toxicity, it appears that retinol inhibits the progression of injury (associated inflammatory response) in the lung, but has little effect on its initial injury (nonciliated bronchiolar epithelial cell injury). Interestingly, the anti-inflammatory activity afforded by retinol could be overcome with higher doses of 1-NN (<150 mg/kg). Rats pretreated with retinol and given either 150 or 200 mg/kg of 1-NN exhibited increased lavage fluid cellular infiltrate and LDH activity, as well as respiratory distress syndrome.

Retinol pretreatment also significantly decreased the amount of pulmonary injury caused by PQ as measured by lavage fluid enzymes and histopathology at 48 hr. Lavage fluid protein content and LDH activity were significantly decreased in animals that received retinol and PQ compared to animals which received vehicle and PQ. In retinol pretreated animals the infiltration of inflammatory cells into the lung following PQ administration was greatly reduced. At the light microscopic level, the lungs of PQ treated animals displayed focal areas of parenchymal cell damage, edema, pneumonitis,
and structural remodeling associated with the alveolar sacs. Following retinol pretreatment there was a lack of edema and pneumonitis associated with alveolar sacs after PQ administration. However, these rats displayed focal areas of alveolar parenchymal cell damage. Unlike 1-NN and 2-NN, PQ does not require cytochrome P-450 for bioactivation. Paraquat undergoes cyclic enzymatic reduction/oxidation forming reactive oxygen species which ultimately results in pulmonary injury. Thus, retinol blocks the toxicity of chemicals that are bioactivated by different mechanisms and enzyme systems. This adds further credence to the hypothesis that retinol is acting as an anti-inflammatory agent in the lung and not altering bioactivation.

From the data presented here, it appears that retinol reduces pulmonary damage though an anti-inflammatory mechanism which effects the progression, not the initiation of injury. However, there remain several other possibilities by which retinol pretreatment protects the lungs from chemically induced injury. Retinol pretreatment causes a variety of changes in the rat beside the alteration of immune cell function. For example, retinol causes weight loss, altered metabolism, induction of cytochrome P-450, hepatomegaly, hypercalcemia, as well as other changes (Pentiuk et al., 1991; Rosengren et al., 1994; Biesalski, 1989). It is likely that retinol does not work though a single mechanism to alter tissue injury, and therefore, other mechanisms of alteration must be considered. The alteration of metabolism by retinol would fit with much of the data presented. However, it is equally as apparent that the altered metabolism could not be totally responsible for the protective effects observed in the lung. There are three line of evidence which lead to this
conclusion: 1) Retinol pretreatment attenuates pulmonary injury caused by several compounds shown to act through different mechanism (i.e. 1-NN, 2-NN, PQ, and bleomycin), 2) Pretreatment with retinol increases the amount of drug metabolizing enzymes in the lung (i.e. cytochrome P-450 2B1), 3) The initial stages of pulmonary injury is seen in retinol pretreated animals following chemical induced injury (Clara and type II cell damage).

A significant role for inflammatory phagocytes has been shown for a number of lung toxicants. For example, the acute toxicity of ozone has been shown to mediated by both ozone itself acting as an oxidant and its associated inflammatory response. Neutrophil depleting antibodies have been shown to limit a significant amount of the damage caused by ozone in the lung (Pino et al., 1992a). Interestingly, it likely that infiltrating inflammatory cells do not contribute to the initial damage to epithelial cells, but are responsible for the pulmonary edema and later cellular damage associated with the progression of lung injury (Pino et al., 1992b; Donaldson et al., 1991). Both alveolar macrophages and neutrophils have been implicated in the pneumotoxicity of the antineoplastic agent bleomycin. Alveolar macrophages release increased amounts of cytokines and bioactive lipids during bleomycin induced lung injury (Scheule et al., 1992), including MIP-1, a potent neutrophil chemoattractant (Smith et al., 1995). Interestingly, stimulation of alveolar macrophage activity significantly enhances the pulmonary fibrosis caused by bleomycin (Chyczewska et al., 1993). In several studies it has been shown that alveolar macrophages produce increased amounts of both reactive
oxygen and nitrogen species following bleomycin intoxication (Habib et al., 1993; Huot et al., 1992). Likewise, reactive oxygen species released by recruited neutrophils also plays a significant role during bleomycin induced lung toxicity (Tarnell et al., 1992).

The key observation from these experiments was that in the lung retinol significantly attenuates chemical induced lung injury. The mechanism by which retinol alters chemically induced lung injury appears to be by reducing the progression of injury via an attenuation of the inflammatory response rather than the initial injury. In the lung, inflammation is attenuated and the pulmonary tissue is protected from toxicity. In Figure 2.11, a mechanistic model is proposed for retinol alteration of chemical induced pulmonary injury in which inflammatory cells play a key role.
Figure 2.11. Proposed mechanism of all-trans-retinol attenuation of chemical induced lung injury. In this proposed model of retinol alteration of chemical induced pulmonary injury, the initial toxicity caused by the bioactivation of the chemical (initial epithelial cell injury) is similar in both retinol vehicle and retinol pretreated animals. At later time points, further progression of injury (infiltration of inflammatory cells) is seen in animals pretreated with retinol vehicle, but not in animal which received retinol.
CHAPTER 3

EFFECT OF ALL-trans-RETINOL ON CHEMICALLY INDUCED LIVER INJURY IN THE MALE SPRAGUE-DAWLEY RAT.

It has been shown that seven days of oral dosing with all-trans-retinol (vitamin A) greatly enhances the hepatotoxicity of several compounds in rats (Sipes et al., 1989; El-Sisi et al., 1993a). The mechanism for this potentiation appears to be mediated by the enhanced release of reactive oxygen species (ROS) from retinol-primed Kupffer cells. These ROS promote lipid peroxidation and increase the degree of parenchymal cell necrosis (El-Sisi et al., 1993c). Although the lung is the primary target of both 1-NN and 2-NN, these compounds can also cause hepatotoxicity. Therefore, it was of interest to determine if retinol modulates the liver injury produced by 1-NN and 2-NN.

1-Nitronaphthalene (1-NN): In male Sprague-Dawley derived rats, a single intraperitoneal injection of 1-NN results in liver injury consisting of both centrilobular and periportal hepatocyte necrosis (Johnson et al., 1984; Verschoyle and Dinsdale 1990). The hepatic toxicity induced by 1-NN, both centrilobular and periportal, has been shown to be enhanced by phenobarbital pretreatment and diminished with the pretreatment of rats with SKF 525-A (Johnson et al., 1984). Verschoyle et al., (1993) have recently reported that 1-NN also causes necrosis of bile duct epithelial cells (BEC) in rats.
Although there is an abundance of literature on the pathology of 1-NN, very little information is available on its metabolism. However, El-Bayoumy and Hecht (1982) have shown, using rat liver supernatant (S-9 fraction) under aerobic conditions, that 1-NN undergoes ring oxidation, resulting in the production of dihydrodiols and nitronaphthol metabolites. In a similar experiment, the formation of 1-naphthylamine was detected under anaerobic conditions (Poirier and Weisburger, 1974). In an in vivo study, Johnson and Cornish (1978) reported the presence of 1-naphthylamine in the urine of male Sprague-Dawley rats administered 1-NN. Furthermore, it appears that 1-NN is bioactivated by cytochrome P-450 1A1 and 1A2 in the non-induced rat liver (Verschoyle et al., 1993).

2-Nitronaphthalene (2-NN): In male Sprague-Dawley derived rats, systemic administration of 2-NN results in hepatotoxicity which consists of centrilobular hepatocellular necrosis (Johnson et al., 1984). Damage to the periportal hepatocytes or BEC by 2-NN has not been reported. 2-Nitronaphthalene has been shown to be reduced in rats to 2-naphthylamine (Johnson and Cornish, 1978). However, very little information is available on the mechanism by which 2-NN causes hepatotoxicity or its metabolism.

Objective: The objective of these studies was to evaluate the effects of retinol pretreatment on the hepatic injury caused by 1-NN and 2-NN using plasma markers of cytotoxicity, as well as light microscopy. This objective should provide important
information to determine if the modulation of 1-NN and 2-NN induced toxicity by retinol is chemical specific or tissue specific.
MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250 to 300 g) were purchased from Harlan (Indianapolis, IN). Animals were housed in pan cages and maintained on a 12-hr light-dark cycle at approximately 22 °C and 40% humidity. Except for the pair-feeding study described below, they were allowed free access to food (Wayne Lab-Blox Co.) and water. Animals were allowed to acclimate for at least seven days prior to any treatment.

Chemicals

All-trans-retinol (Aquasol A drops) was purchased from Bindley Western Drug Co. (Phoenix, AZ). Gadolinium chloride (GdCl₃), 1-nitronaphthalene, and 2-nitronaphthalene were purchased from Aldrich Chemical Co. (Milwaukee, WI). Alanine aminotransferase diagnostic kit (procedure No. 59-UV), gamma-glutamyl transpeptidase diagnostic kit (procedure No. 545), and total bilirubin diagnostic kit (procedure No. 605) were purchased from Sigma Chemical Co. (St. Louis, MO). Williams medium E (WME) was purchased from Gibco-BRL (Grand Island, NY). Collagenase was purchased from Boehringer Mannheim (Indianapolis, IN).
Experimental design

Rats were pretreated with 75 mg/kg/day (250,000 IU, 262 mmol) of retinol or vehicle (7% tween-20, 10% propylene glycol in water) by oral gavage once a day for seven days. Twenty four hr following the last retinol treatment, animals received a single injection of 1-NN (100 mg/kg in 2.0 mL/kg of peanut oil, i.p.) or 2-NN (200 mg/kg in 2.0 mL/kg of peanut oil, i.p.). At 3, 6, 12, 24, and 48 hr after 1-NN administration, and 24 hr after 2-NN treatment rats were killed by carbon dioxide inhalation and blood was collected from the posterior vena cava into a heparinized tube. For inhibition of Kupffer cells by gadolinium chloride (GdCl₃), rats received retinol for 7 days and were then given GdCl₃ (10 mg/kg in 2.0 mL/kg acidic saline, i.v.) via the tail vein 24 hr before toxicant administration. At twenty-four hr after toxicant administration rats were killed by carbon dioxide inhalation and blood and livers was collected as above. Plasma biochemical and liver morphological analysis was performed to evaluate hepatotoxicity.

Morphological and biochemical evaluation of liver injury

The plasma enzyme activities of alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (GGT), as well as total bilirubin were measured using Sigma diagnostic kits. Livers were fixed by immersion in 10% neutral buffered formalin. Samples were further processed, embedded in paraffin, and 5 micron sections were stained with hematoxylin and eosin (H&E). Livers were examined by light microscopy.
Toxicity of 1- and 2-nitronaphthalene in primary hepatocytes

Rats were pretreated with phenobarbital (80 mg/kg/d for 4 days), retinol (75 mg/kg/day for 7 day) or vehicle (7% tween-20, 10% propylene glycol in water). One day after the last treatment, hepatocytes were isolated using a two-step in situ perfusion with collagenase as described by McQueen (1989). Approximately 5 x 10^5 cells were seeded into 6 well dishes in Williams media E (WME) supplemented with 10% fetal bovine serum. After 2 hr, cultures were washed, placed in serum free WME, and exposed to either 1-NN or 2-NN dissolved in DMSO. The final concentration of DMSO in the culture was 1.0%. After 18 hr, media was removed and cells scraped from the plate. Cytotoxicity was determined by the amount of lactate dehydrogenase (LDH) released in the media compared to the amount of LDH present in the scraped cells (% LDH release). Lactate dehydrogenase was determined by a Sigma diagnostic kit spectrophotometrically at 340 nm using a Dynotech MR 5000 microplate reader (Dynatech laboratories, Chantilly, VA).

Statistical analysis

Results are expressed as the mean ± standard error of the mean (mean ± SEM). Statistical comparisons were accomplished using a two-tailed ANOVA and the Newman-Keuls test. Data were considered significantly different when p < 0.05.
RESULTS

1-Nitronaphthalene

Plasma Chemistry Analysis: 1-Nitronaphthalene treatment caused only minor increases in plasma ALT activity at 24 hr; however, pretreatment with retinol significantly increased plasma ALT activity. Furthermore, retinol pretreatment significantly increased the amount of plasma GGT activity, an indicator of bile duct epithelial cell integrity (Szaz, 1969), and total bilirubin after 1-NN treatment (Figure 3.1). Pretreatment with GdCl₃ significantly decreased the potentiation of 1-NN induced hepatic necrosis (assessed by ALT activity) observed in retinol pretreated rats. However, pretreatment with gadolinium chloride (GdCl₃) had no protective effect on retinol elevation of plasma GGT activity. Indeed, GdCl₃ administration significantly increased plasma GGT activity in both control and retinol pretreated animals. These biochemical findings of liver injury were confirmed with light microscopy.

Microscopic Observations: Liver sections of 1-NN treated animals displayed mild multifocal necrosis, typically limited to a few hepatocytes in the centrilobular and periportal regions. In the periportal regions, damage consisted of both hepatocyte and BEC necrosis (Figure 3.2). A similar but more extensive pattern of both periportal and centrilobular damage was observed in the retinol pretreated animals given 1-NN. Enhanced inflammatory cell infiltration, as well as increased hepatocyte and BEC damage
were observed. In the periportal regions damage consisted of moderate, multifocal periportal hepatocyte necrosis with infiltration of moderate numbers of neutrophils, as well as a smaller number of macrophages and lymphocytes. In addition, severe widespread necrosis of bile duct epithelial cells was observed in the retinol pretreated animals. Liver sections from 1-NN treated animals pretreated with both retinol and GdCl₃ showed no overt differences in the periportal regions from retinol pretreated animals given 1-NN. However, there was protection from 1-NN-induced parenchymal necrosis in the centrilobular region in the livers of these animals.

Retinol alone had no effect on the morphology of the liver. However, a slight swelling of Ito (fat storing) cells in the hepatic sinusoids was observed.

**Retinol and Food Consumption:** To determine if decreased food intake and body weight loss associated with retinol pretreatment altered 1-NN-induced toxicity, vehicle-pretreated control rats were pair fed to the retinol pretreated rats for 7 days. By day 8, the pair fed controls lost $31 \pm 3g$, the retinol treated rats had lost $26 \pm 7g$, and the *ad libitum* control rats had gained $33 \pm 4g$. The average food intake for controls was $28 \pm 4g$ of food/day, and retinol treated rats was $14 \pm 2g$ of food/day. Furthermore, in the same animals administration of 1-NN resulted in plasma ALT activities (U/L) of $41 \pm 5$ and $31 \pm 5$ for the pair-fed control group and the *ad libitum* group, respectively, but $151 \pm 21$ for the retinol pretreated group.
2-Nitronaphthalene

**Plasma Chemistry Analysis:** 2-Nitronaphthalene at the dose used caused no significant increases in plasma ALT activity at 24 hr. Following pretreatment with retinol there was a significant potentiation of liver damage as measured by histopathology and plasma ALT activity. However unlike 1-NN, 2-NN did not cause BEC damage and no increase in plasma GGT was observed. Administration of GdCl₃ to retinol pretreated rats significantly decreased the amount of 2-NN induced hepatocyte necrosis as determined plasma ALT activity (Figure 3.3).

**Microscopic Observations:** Morphological evaluation of liver sections confirmed that in control rats 2-NN did not cause liver damage at 24 hr. However, following retinol pretreatment evidence of liver injury was apparent. Histologically, liver injury consisted of severe centrilobular hepatocyte necrosis with the infiltration of a moderate numbers of macrophages, neutrophils, and lymphocytes (Figure 3.4). Pretreatment with GdCl₃ significantly decreased the potentiated centrilobular necrosis in retinol pretreated rats as determined by histopathology. Accompanying this protection was a decrease in the number of inflammatory cells present at the site of injury.
Toxicity of 1- and 2-nitronaphthalene in primary hepatocytes

Further evidence that the altered toxicity is unrelated to retinol induced changes in liver metabolism was obtained from studies using monolayers of primary isolated hepatocytes. Pretreatment with either retinol or phenobarbital had no significant effect on hepatocyte yield or viability compared to controls. 1-Nitronaphthalene and 2-NN induced release of LDH was unaffected in hepatocytes isolated from retinol pretreated rats as compared to control. However, hepatocytes isolated from phenobarbital pretreated rats showed significantly increased susceptibility to both 1-NN and 2-NN (Figure 3.5). The LC_{50} for the cultures were 35.7 ± 18.4 μM for vehicle, 43.2 ± 8.5 μM for retinol, and 20.6 ± 8.5 μM for phenobarbital following 18 hr of 1-NN exposure. The LC_{50} for the cultures were 39.2 ± 1.8 μM for vehicle, 37.7 ± 1.7 μM for retinol, and 22.0 ± 0.5 μM for phenobarbital following 18 hr of 2-NN exposure.
Figure 3.1. Effect of gadolinium chloride pretreatment on all-trans-retinol potentiated 1-nitronaphthalene induced liver injury. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o.) for 7 days and on day 7 rats were given GdCl₃ (10 mg/kg, i.v.) via the tail vein. One day after GdCl₃ administration rats received 1-NN (100 mg/kg, i.p.) or peanut oil. Twenty four hr after 1-NN administration rats were killed and markers of liver injury were evaluated as described in Materials and Methods. (A) Plasma ALT activity, (B) Plasma GGT activity, (C) Plasma total bilirubin. Bars represent means ± SEM. n=12. aSignificantly different from respective peanut oil control. bSignificantly different from respective retinol vehicle group. cSignificantly different from respective retinol and 1-NN group. (ND = not determined).
Figure 3.2. Effect of all-trans-retinol on 1-nitronaphthalene induced microscopic pathology of the liver. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle followed 24 hr later by 1-NN (100 mg/kg, i.p.). Twenty four hr after 1-NN administration rats were killed and liver sections fixed by immersion as described in Materials and Methods. (A) Representative central vein (CV) and periportal (PP) region with bile ducts (arrows) from a rat pretreated with retinol vehicle and administered 1-NN. (13.2 X, original magnification). (B) Representative central vein (CV) and periportal (PP) with bile ducts (arrows) region from a rat pretreated with retinol and administered 1-NN. (13.2 X, original magnification).
Figure 3.3. Effect of gadolinium chloride pretreatment on all-trans-retinol potentiated 2-nitronaphthalene induced liver injury. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o., for 7 days) or vehicle, on the seventh day given GdCl₃ (10 mg/kg, i.v.), followed 24 hr later by 2-NN (200 mg/kg, i.p.). Twenty four hr after 2-NN administration rats were killed and markers of liver injury were evaluated as described in Materials and Methods. Plasma ALT activity. Bars represent means ± SEM. n=12. aSignificantly different from vehicle control. bSignificantly different from respective retinol vehicle group.
Figure 3.4. Effect of all-trans-retinol on 2-nitronaphthalene induced microscopic pathology of the liver. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o.) for 7 days and on day 8 animals received 2-NN (200 mg/kg, i.p.). Twenty four hr after 2-NN administration rats were killed and liver sections fixed by immersion as described in Materials and Methods. (A) Representative central vein (CV) and periportal (PP) region from a rat treated with retinol vehicle and 2-NN (33 X, original magnification). (B) Representative central vein (CV) and periportal (PP) region from a rat treated with retinol and 2-NN. Note the centrilobular hepatocellular necrosis and inflammation (33 X, original magnification).
Figure 3.5. Effect of *in vivo* all-trans-retinol and phenobarbital pretreatment on 1- and 2-nitronaphthalene induced toxicity in primary cultured hepatocytes. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days), vehicle or phenobarbital (80 mg/kg/d, i.p., for 4 days). Twenty four hr after the last dose hepatocytes were isolated, exposed to 1-NN or 2-NN (0 to 100 μM), and the cytotoxicity evaluated by lactate dehydrogenase (LDH) release as described in Materials and Methods. (A) 1-Nitronaphthalene, (B) 2-Nitronaphthalene. Points represent means ± SEM. n=3. *Significantly different from vehicle control group.*
DISCUSSION

As previously reported, administration of large doses of retinol to rats can potentiate the hepatotoxicity caused by a variety of xenobiotics (ElSisi et al., 1993a; Sipes et al., 1989). However, as reported in the previous chapter, retinol pretreatment decreases pulmonary toxicity of 1-NN and 2-NN, chemicals that can also cause liver injury. Therefore, it was important to determine if this protection caused by retinol was chemical specific or tissue specific. A portion of these studies were dedicated to observing the effects of retinol on 1-NN and 2-NN induced hepatotoxicity.

1-Nitronaphthalene administration to retinol vehicle pretreated rats caused slight increases in plasma ALT, bilirubin and GGT activity at 24 hr. Pretreatment with retinol significantly increased plasma biochemical markers of liver injury caused by 1-NN. The livers of 1-NN treated animals possessed both hepatocyte and bile duct epithelial cell (BEC) lesions which were significantly potentiated by retinol pretreatment. The pattern of periportal liver damage was similar in pattern and degree to that found in animals treated with α-naphthylisothiocyanate (ANIT), a model intrahepatic choleostatic agent which causes BEC necrosis and is structurally similar to 1-NN (McLean and Ree, 1958). Associated with 1-NN induced BEC necrosis was a moderate inflammatory response. This consisted predominantly of neutrophil infiltration with small numbers of
macrophages and lymphocytes. The inflammatory response was significantly increased in animals that received retinol and 1-NN.

Evidence that activated Kupffer cells are involved in the potentiation of 1-NN-induced toxicity in the liver was provided by experiments using GdCl₃, a compound that selectively destroys phagocytic macrophages including Kupffer cells (Hustzik et al., 1980). ElSisi et al., (1993c) have shown a mechanism for retinol potentiation of chemically-induced hepatotoxicity by which retinol increases the activity of Kupffer cells and their ability to generate reactive oxygen species during liver injury. This mechanism was further supported by the fact that GdCl₃ was shown to protect against retinol potentiation of carbon tetrachloride-induced liver injury (Hill and Sipes, 1993). In the present study GdCl₃ produced different effects on the potentiation of 1-NN-induced hepatocyte and BEC injury. Gadolinium chloride pretreatment protected against the potentiated centilobular and periportal hepatocyte injury caused by 1-NN. However, pretreatment with GdCl₃ had no protective effect on the retinol potentiation of BEC damage. Indeed, GdCl₃ administration increased BEC injury in both control and retinol pretreated animals. Gadolinium chloride has been shown not to effect neutrophil function or migration in to the liver upon hepatic injury (Jaeschke et al., 1994). Therefore, if Kupffer cells do not play a key role in 1-NN-induced BEC damage it is not surprising that GdCl₃ did not decrease injury in retinol pretreated animals. However, the mechanism by which GdCl₃ enhances BEC damage has yet to be determined and further investigation is required.
2-Nitronaphthalene administration to retinol vehicle pretreated rats caused no change in plasma ALT or GGT activity at 24 hr. Pretreatment with retinol significantly potentiated 2-NN-induced liver injury as assessed by plasma ALT or histopathology. When examined by light microscopy, the livers of 2-NN treated animals possessed no morphological alterations. However, following retinol pretreatment 2-NN caused severe necrosis of the hepatocytes around the central vein. This centrilocular injury was much more extensive than that observed in retinol pretreated rats given 1-NN. However, unlike 1-NN induced liver injury, damage to the BEC was not observed. Gadolinium chloride pretreatment dramatically protected against retinol potentiated hepatocyte damage caused by 2-NN. Thus, it appears that retinol pretreatment increases 2-NN-induced hepatocyte injury through a mechanism essential to Kupffer cell function.

Interestingly, the basic hypothesis for retinol potentiation of hepatic injury has required a “minimal” injury to be induced by the hepatotoxicant itself in order for potentiation to occur. In the rat, the potentiation of CCl₄, acetaminophen, and allyl alcohol by retinol all require a dose of the toxicant which caused some measurable toxicity (i.e. small elevation in ALT). It has been thought that the injury to hepatocytes triggers events which result in the activation of retinol primed Kupffer cells and the potentiated response. However, 2-NN alone does not cause injury at the doses given, at least as assessed by plasma ALT. 2-Nitronaphthalene may not follow the same mechanism to initialize or trigger the potentiated response (Kupffer cell activation?). Indeed, the mechanism of potentiation may be independent of measurable toxicity, but
instead depend upon factors released from hepatocytes (i.e. pro-inflammatory cytokines) that lead to the progression of injury.

A significant role for inflammatory phagocytes has been shown for a number of liver toxicants. For example, phagocytic cells play a critical role in the necrosis caused by acetaminophen. Laskin and Pilaro (1986) showed that an analgesic dose of acetaminophen activates mononuclear phagocytes (Kupffer cells) and causes their infiltration into the centrilobular regions of the liver even in the absence of necrosis. In further studies, Laskin et al., (1986) demonstrated that factors released by hepatocytes exposed to acetaminophen caused Kupffer cells to migrate into the centrilobular regions of the liver and become activated. Furthermore, Bailie et al., (1995a) have reported that Kupffer cells modulate much of the hepatotoxicity caused by a large dose of acetaminophen. In these studies, rats and mice were protected from acetaminophen-induced liver damage by pretreatment with GdCl₃. More recent studies evaluating the role of pro-inflammatory cytokines in the hepatotoxicity of acetaminophen have shown that a number of macrophage derived factors including TNF-α, and IL-1 are produced in the liver following intoxication (Blazka et al., 1995).

Edwards et al., (1993) have reported that the Kupffer cells are involved in the hepatotoxicity caused by a high dose of CCl₄. In these studies, rats were protected from CCl₄-induced liver damage by pretreatment with GdCl₃. These authors concluded that Kupffer cells participate in CCl₄ toxicity most likely by attracting neutrophils. Finally, a significant role for inflammatory phagocytes has been shown by several investigators
with the model intrahepatic choleostatic agent α-naphthylisothiocyanate (ANIT). In experiments utilizing a neutrophil depleting antibody, Dahm et al., (1991) have shown that neutrophils are required for the elicitation of toxicity. Interestingly, inhibition of Kupffer cells with GdCl₃ has no protective effect on ANIT induced liver injury (Bailie et al., 1995b). Although neutrophils play an important role in the hepatotoxicity caused by ANIT, Kupffer cells apparently do not orchestrate these processes.

In Figure 3.6, a mechanistic model is proposed for retinol alteration of chemically induced liver injury in which inflammatory cells play a key role. This model is based on the data reported here and by reports in published literature. Important to the model is the fact that retinol pretreatment can prime Kupffer cells. When activated, they respond with a greater release of mediators and toxic products (TNF-α, superoxide anion, etc.). From the results of these experiments, it is concluded that retinol appears to potentiate 1-NN and 2-NN induced liver injury by increasing the associated inflammatory responses. This infiltration of inflammatory cells into the liver is associated with increased hepatocellular necrosis. Furthermore, the enhanced damage appears to be orchestrated by Kupffer cells. Such a mechanism is not operative for the retinol potentiation of 1-NN induced BEC injury, since inhibition of Kupffer cell function did not decrease the severity of injury. Thus it appears that, at least for 1-NN, two separate mechanisms of potentiation may be occurring in the liver with retinol pretreatment.
Figure 3.6. Proposed mechanism of all-trans-retinol alteration of chemical induced liver injury. In this proposed model of retinol potentiation of chemical induced liver injury, the initial toxicity caused by bioactivation (initial hepatocyte and bile duct epithelial cell injury) is similar in both retinol vehicle and retinol pretreated animals. At later time points, progression of hepatic injury (Kupffer cell activity and infiltration of inflammatory cells) is increased animals pretreated with retinol. Thus, it appears that retinol is altering chemical induced hepatotoxicity by increasing the associated inflammatory responses and not altering the initial injury caused by the bioactivation of the hepatotoxicant.
CHAPTER 4

EFFECT OF ALL-trans-RETINOL PRETREATMENT ON THE FUNCTION OF PULMONARY ALVEOLAR MACROPHAGES ISOLATED FROM MALE SPRAGUE-DAWLEY RATS.

In Chapter 2 it is reported that seven days of oral dosing with retinol greatly decreases the pulmonary toxicity of 1-NN, 2-NN, and PQ. It has been reported by Habib et al., (1993) that retinol can attenuate many of the inflammatory effects caused by bleomycin in the lung. Rats pretreated with retinol exhibited statistically significant reductions in pulmonary alveolar macrophage (PAM) superoxide anion production after bleomycin administration. The authors concluded that such a decrease in PAM superoxide anion production was responsible for the attenuation of bleomycin pulmonary injury. In our model of lung injury PAM may play an important role in recruiting inflammatory cells and releasing cytotoxic factors (ROS and cytokines).

Following activation, several cytokines and eicosanoids are released by PAM, including interleukin-1 (IL-1), leukotriene B₄ (LTB₄), prostaglandin E₂ (PGE₂), transforming growth factor-beta, platelet activating factor, gamma interferon, and tumor necrosis factor-alpha (TNF-α). These bioactive compounds can alter parenchymal cell function, as well as participate in pulmonary injury. Because of a previously reported
interaction between retinol and bleomycin, as well as decreased inflammatory response associated with chemical induced lung injury, we hypothesized that retinol would inactivate PAM. Therefore, the objective of this chapter was to understand the effects retinol pretreatment had on PAM function.
MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250 to 300 g) were purchased from Harlan (Indianapolis, IN). Animals were housed in pans and maintained on a 12-hr light-dark cycle at approximately 22 °C and 40% humidity. Rats were allowed free access to food (Wayne Lab-Blox Co.) and water. Animals were allowed to acclimate for at least seven days prior to any treatment.

Chemicals

All-trans-retinol (Aquasol A drops) was purchased from Bindley Western Drug Co. (Phoenix, AZ). Leukotriene B₄ (LTB₄) and prostaglandin E₂ (PGE₂) were determined by a commercially available ELISA from Cayman Chemicals (Ann Arbor, MI). Nitroblue tetrazolium (NBT), RPMI 1640 media, actinomycin D, and lipopolysaccharide (Salmonella Minnesota) were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental design

Rats were treated with 75 mg/kg/day (250,000 IU, 262 mmol) of retinol or vehicle (7% tween-20, 10% propylene glycol in water) by oral gavage once a day for seven days. Twenty four hr following the last retinol treatment, animals anesthetized with
pentobarbital (50 mg/kg, i.p. in saline:ethanol [90:10]), blood was collected from the posterior vena cava into a heparinized tube, and alveolar macrophages isolated by bronchioalveolar lavage.

Isolation and cell culture of alveolar macrophages

Following blood collection, alveolar macrophages were isolated by lavage according to the method of Lantz et al., (1994). Ten-milliliter aliquots of 37 °C Hanks balanced salt solution (no calcium or magnesium) were used to fill the lung. A total of 50 mL of lavage fluid (5-6 washings) were pooled into a 50 mL polypropylene centrifuge tube and centrifuged 500g for 15 minutes. Pelleted cells were resuspended in RPMI 1640 media. Cell counts showed normal yields of 2 to 5 x 10⁶ cells per rat with viability of >90% as determined by trypan blue exclusion. Cells were plated in 48 well culture plates (Costar, Cambridge, MA) at a density of 100,000 cells per well and allowed to adhere for 2 hours. Media was then decanted and replaced with RPMI 1640 with 5% heat-inactivated (56 °C, 30 minutes) fetal bovine serum and 1% penicillin/streptomycin (1 mL/well). Lipopolysaccaride (LPS, 1 μg/mL, Salmonella Minnesota) was added to some cultures at this time. Cells remained in culture for 24 hr and were assayed for tumor necrosis factor (TNF), leukotriene B₄ (LTB₄), prostaglandin E₂ (PGE₂), nitric oxide and superoxide anion production.
**Tumor necrosis factor assay**

Following 24 hr of incubation with LPS or vehicle, culture media was removed, aliquoted into four 250 µl samples and stored at -70 °C. TNF activity was measured in culture media by quantifying cytolytic activity against L929 target cell line (Ruff and Gifford 1980). Briefly, 2.0 X 10^4 L929 target cells in 0.1 mL of RPMI 1640 medium supplemented with 10 % heat inactivated fetal bovine serum and 50 µg gentamicin/mL were aliquoted into wells of a 96-well microtiter plate and grown to confluency. The medium was decanted and samples of alveolar macrophage supernatant were added to the wells in the presence of 1 mg actinomysin D/mL. After 18 hr of incubation, the medium was removed and the cells were simultaneously fixed and stained with 50 µL of 0.5% crystal violet in 50% methanol for 15 minutes, washed with water, and air dried. Cytolysis was calculated from the reduction in mean absorbance at 570 nm relative to control wells incubated with medium only using a Dynotech MR 5000 microplate reader (Dynatech laboratories, Chantilly, VA). Potency is expressed as units/mL based on the highest dilution producing 50% cell lysis. Data are expressed as units/hr of incubation with LPS/10^6 cells based on the potency. Each assay was standardized by the inclusion of a 50 units/mL TNF reference standard.
**Leukotriene B₄ and Prostaglandin E₂ assay**

Leukotriene B₄ and prostaglandin E₂ were determined in the culture media after stimulation of alveolar macrophages with LPS. Commercially available ELISA kits from Cayman Chemicals (Ann Arbor, MI) were used for each. Data are expressed as units/hr of incubation with LPS/10⁶ cells.

**Nitric oxide assay**

Nitric oxide was determined indirectly in supernatants of alveolar macrophages by measuring nitrite. A calorimetric assay in which nitrite is reacted with sulfanilamide and N-1-naphthylethylenediamine (NEDA) was utilized as described by Archer et al., (1993). The reaction forms a pink chromophore which is measured spectrophotometrically. Briefly, 90 µL of supernatant was mixed with 90 µL of sulfanilamide and allowed to incubate at room temperature for 5 minutes. Following incubation 90 µL NEDA was added and samples were read at 570 nm using a Dynotech MR 5000 microplate reader (Dynatech laboratories, Chantilly, VA). The amount of nitrite produced by the alveolar macrophage was calculated with a standard curve of nitrite concentrations (0 to 15 nmoles). Data are expressed as units/hr of incubation with LPS/10⁶ cells.
**Superoxide anion assay**

The production of superoxide anion was measured by the production of formazan from nitroblue tetrazolium (NBT) as described by Pick (1986). Briefly, media was removed from cells and replaced with NBT (2 mg/mL of NBT buffer) in a solution of 140 mM NaCl, 1.5 mM CaCl₂, 5.0 mM glucose, and 10.0 mM Hepes buffer (pH 7.3) containing either PMA (1 µg/mL) or its vehicle (ethanol). One hour later, the NBT solution was removed and plates were washed with saline and then methanol (2 times each). Solubilization of formazan was accomplished using by adding 140 µL of 1.0 M KOH and 120 µL DMSO. The amount of formazan produced was determined spectrophotometrically and its mean absorbance read at 570 nm using Dynotech MR 5000 microplate reader (Dynatech laboratories, Chantilly, VA). The amount of formazan produced during the 1 hr incubation was calculated from a standard curve of formazan concentrations. Oxygen radical production was calculated assuming a two-electron transfer for full reduction of nitroblue tetrazolium to formazan. Data are expressed as units/hr of incubation with NBT/10⁶ cells.

**Statistical analysis**

Results are expressed as the mean ± standard error of the mean (mean ± SEM). Statistical comparisons were accomplished using a two-tailed ANOVA and the Newman-Keuls test,
or the Mann-Whitney U test (TNF analysis). Data were considered significantly different when $p < 0.05$. The $n$ value represents the number of rats and cell preparations were performed in triplicate.
RESULTS

In order to assess the effect of retinol on PAM function, PAM were isolated from rats pretreated with retinol or vehicle for 7 days. Lavage cell numbers and differential counts for macrophages were not significantly different from those isolated from vehicle retinol pretreated rats. However, pretreatment of rats with retinol caused a slight increase in both PAM size and volume as assessed morphologically. It also significantly stimulated the production and release of TNF, and LTB₄ by PAM (Figure 4.1 and 4.2). Retinol had no significant effect on the release of PGE₂, superoxide, and nitric oxide (Figure 4.3, 4.4, and 4.5). Stimulation with LPS caused a significant increase in the production and release of nitric oxide, TNF, PGE₂, and LTB₄ in both vehicle and retinol treated rats. However, retinol treated rats produced significantly more TNF and LTB₄ following LPS stimulation. No alteration in either nitric oxide or superoxide anion production following LPS stimulation was observed. LPS had no significant effect on the production of superoxide anion by PAM. Alveolar macrophages stimulated with PMA produced significant amounts of superoxide anion. However, retinol pretreatment had no significant effect on superoxide anion production by PAM.
Figure 4.1. Effect of all-trans-retinol pretreatment on tumor necrosis factor production by isolated alveolar macrophages. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle. Twenty four hr after the last dose alveolar macrophages were isolated by lavage, exposed to LPS (1 μg/mL), and TNF activity was measured in culture media as described in Materials and Methods. aSignificantly different from respective LPS vehicle group. bSignificantly different from respective retinol vehicle group. (N.D. = not detectable).
Figure 4.2. Effect of all-trans-retinol pretreatment on leukotriene B₄ production by isolated alveolar macrophages. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle. Twenty four hr after the last dose alveolar macrophages were isolated by lavage, exposed to LPS (1 µg/mL), and LTB₄ content was measured in culture media as described in Materials and Methods. aSignificantly different from respective LPS vehicle group. bSignificantly different from respective retinol vehicle group.
Figure 4.3. Effect of all-trans-retinol pretreatment on prostaglandin E₂ production by isolated alveolar macrophages. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle. Twenty-four hr after the last dose alveolar macrophages were isolated by lavage, exposed to LPS (1 μg/mL), and PGE₂ content was measured in culture media as described in Materials and Methods. aSignificantly different from respective LPS vehicle group. bSignificantly different from respective retinol vehicle group.
Figure 4.4. Effect of all-trans-retinol pretreatment on nitric oxide production by isolated alveolar macrophages. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle. Twenty four hr after the last dose alveolar macrophages were isolated by lavage, exposed to LPS (1 μg/mL), and nitrite was measured in culture media as described in Materials and Methods. *Significantly different from respective LPS vehicle group. *Significantly different from respective retinol vehicle group.
Figure 4.5. Effect of all-trans-retinol pretreatment on superoxide anion production by isolated alveolar macrophages. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle. Twenty four hr after the last dose alveolar macrophages were isolated by lavage, exposed to PMA (1 μg/mL), and conversion of NBT to formazan was measured in isolated cells as described in Materials and Methods. aSignificantly different from respective PMA vehicle group. bSignificantly different from respective retinol vehicle group.
DISCUSSION

It has been reported by Rhodes and Oliver (1980) that retinoids can modulate the function of macrophages. For example, retinol pretreatment causes altered responsiveness to stimulation as well as the production of superoxide anion. Alveolar macrophages can recruit inflammatory cells, as well as release products which are cytotoxic to surrounding cells, thereby participating in tissue injury. In the lung, retinol has been reported to modulate the function of alveolar macrophages, increasing tumoricidal processes as well as decreasing inflammatory activities and superoxide anion production (Habib et al., 1993; Kishino et al., 1985; Tachibana et al., 1984). Because retinol decreased the inflammatory response associated with chemical induced lung injury, it was hypothesized that retinol would inactivate PAM. However, retinol activated PAM stimulating the production and release of both TNF, and LTB₄.

The stimulation of PAM function by retinol is inconsistent with the findings of Habib et al., (1993). These authors found that retinol pretreatment dramatically protects rats from the pulmonary toxicity and inflammatory cell infiltration caused by bleomycin, similar to findings reported here. However, Habib et al., (1993) also reported that retinol decreased alveolar macrophage superoxide production. The authors speculated that the mechanism behind these decreases was due to the antioxidant actions of retinol. The results of the experiments reported here indicate that retinol does not decrease the production of superoxide anion by PAM. No significant effect on superoxide anion
production was observed. However, it is important to note that Habib et al., (1993) were evaluating PAM taken from rats administered both retinol and bleomycin. In the experiments performed here PAM were isolated from animals administered only retinol or its vehicle.

Thus, it appears that although retinol acts as an anti-inflammatory agent in the lung, it activates several functions in PAM. Following LPS treatment PAM isolated from retinol treated animals produced significantly more LTB$_4$ and TNF as compared to those from vehicle pretreated rats. Since PAM are capable of mounting an inflammatory response and participate in chemical induced injury, one would expect that retinol would potentiate the toxicity of pneumotoxic agents. However, this is not the case and retinol protects against chemical induced lung injury. Leukotriene B$_4$ in humans and animals is a known chemoattractant for neutrophils, but recently it has been reported that rat neutrophils have only a modest response to LTB$_4$ when compared to those of other species (Sugawara et al., 1995). Thus, it appears that LTB$_4$ is not as critical in the recruitment of neutrophils in the rat. However, no difference has been reported in the ability of the rat to respond to TNF as compared to other animals. It therefore appears that the role of PAM in retinol protection of the lung is not as clear-cut as is the role for the Kupffer cell in the potentiation of liver injury.

The mechanism by which retinol stimulates PAM is likely related to its ability to increase phospholipase A$_2$ (PLA$_2$) activity. Administration of as little as 20,000 IU of retinal (the aldehyde form of retinol) for seven days significantly increases PLA$_2$ activity
in the rat liver (Chandra, 1988). Furthermore, retinol has been shown to alter platelet function via activation of PLA₂ (Nakano et al., 1988). Phospholipase A₂ catalyzes the hydrolysis of the ester linkage in phosphatidyl choline. This results in the production of arachidonic acid, the precursor to all eicosanoids. It has recently been shown that PLA₂ activity can indirectly increase the synthesis of IL-1 and TNF in human monocytes (Bomalaski et al., 1995). Interestingly, a number of cytokine signal transduction pathways are mediated through PLA₂ which results in the increased production of eicosanoids. Thus, a significant increase in PLA₂ in PAM could be responsible for the increased production of LTB₄ and TNF. However, the production of PGE₂, an eicosanoid whose production is closely linked to that of LTB₄, is not increased in the PAM following retinol pretreatment. In macrophages, PGE₂ serves to down-regulate the production of TNF, causing the cell to become quiescent (Decker, 1990). This result could be explained by the findings of Fiedler-Nagy et al., (1987) who reported that retinoids, including retinol, can inhibit the functions of cyclooxygenase and the production of prostinoids. This proposed mechanism of the functional alteration of PAM by retinol pretreatment is illustrated in figure 4.6.

In summary, these data indicate that the anti-inflammatory effects of retinol observed in the lung are not directed through the inhibition of PAM function. This anti-inflammatory modulation must therefore be mediated by the alteration of inflammatory processes distal to the PAM, such as the pro-inflammatory cytokine cascade or neutrophil function.
Figure 4.6. Proposed mechanism of the functional alteration of alveolar macrophages (PAM) by all-trans-retinol pretreatment. In this model retinol increases the activity of PAM by increasing the activity of phospholipase A$_2$ (PLA$_2$) and inhibiting the function of cyclooxygenase. This scheme of enzyme induction and inhibition causes increased tumor necrosis factor alpha (TNF-α) and leukotriene B$_4$ (LTB$_4$) production without the concomitant release of prostaglandin E$_2$ (PGE$_2$).
CHAPTER 5

SUMMARY: ALL-trans-RETINOL MODULATION OF CHEMICALLY-
INDUCED PULMONARY AND HEPATIC TOXICITY.

The key observation from these studies was that retinol potentiates hepatotoxicity while it concomitantly attenuates lung injury with respect to the same chemicals. The overall mechanism by which retinol alters chemically induced lung and liver injury appears to be by modulating the progression of injury via an alteration of the inflammatory response rather than the initial injury. In the lung inflammation is attenuated and pulmonary tissue is protected from toxicity, while in the liver inflammation is increased and chemically induced hepatic injury is potentiated.

Retinol is a required vitamin naturally found in foods and is necessary for normal cellular maturation and function. Although it has many physiological effects, of particular interest to these studies is the ability of retinol at high doses to modulate the function of immune cells, notably tissue macrophages and phagocytic cells involved in acute inflammatory responses (Moriguchi et al., 1985; Tachibana et al., 1984). Retinoids have been shown to modulate the functions of macrophages and neutrophils throughout the body (Camisa et al., 1982; Hatchigian et al., 1989; Hemila and Wikstrom, 1985; Fumarulo et al., 1991). Increased phagocytic activity, as well as increased production of reactive oxygen species have been observed following retinol pretreatment (Guzman et al., 1991; Lison et al., 1990).
Seven days of oral dosing with retinol has been shown to activate Kupffer cells, greatly enhancing the production of reactive oxygen species and cytokines, as well as phagocytic activity (ElSisi et al., 1993c; Abril et al., 1989). Blockade of Kupffer cell function with GdCl₃ significantly inhibited retinol potentiation of 1-NN and 2-NN hepatotoxicity. Unlike in retinol pretreated rats, GdCl₃ could not antagonize the increased liver injury caused by either 1-NN or 2-NN following phenobarbital pretreatment (Appendix B). Furthermore, in isolated hepatocytes phenobarbital significantly increase the susceptibility to both 1-NN and 2-NN. However, retinol pretreatment had no significant effect on the response of hepatocytes to either 1-NN or 2-NN. Thus, while phenobarbital alters 1-NN and 2-NN induced liver toxicity by increasing the rate of bioactivation, retinol apparently enhances Kupffer cell activity to potentiate hepatotoxicity.

Although Kupffer cells have been shown to possess an important role in retinol potentiation of chemicals in the liver, it is likely that retinol does not work though a single mechanism to alter hepatotoxicity. For example, other cells such as neutrophils and monocytes likely participate in the enhanced injury caused retinol pretreatment. Furthermore, retinol may affect hepatic bioactivation of xenobiotics. In Appendix A the effects of 7 days of retinol pretreatment on cytochrome P-450 2B1, the enzyme responsible for the bioactivation of 1-NN in the lung, is shown. Although the increase of 2B1 in the liver is minor, it represents another mechanism by which retinol could alter the hepatotoxicity of chemicals (Appendix A). The major mechanism by which 1-NN and 2-
NN are potentiated by retinol appears to be similar to what has been hypothesized in previously by ElSisi et al., (1993a) and a mechanistic view is illustrated in Figure 3.6.

Interestingly, retinol potentiation of 1-NN induced BEC injury does not conform to the Kupffer cell/retinol model of potentiation. Associated with the BEC necrosis caused by 1-NN was a moderate inflammatory response. This consisted predominantly of neutrophil infiltration with small numbers of macrophages and lymphocytes. The neutrophil infiltration and associated inflammatory response were significantly increased in animals that received retinol and 1-NN. However, pretreatment with GdCl₃ had no protective effect on retinol potentiation of BEC damage. Indeed, GdCl₃ administration significantly increased damage in both control and retinol pretreated animals. Gadolinium chloride has been shown not to effect neutrophil function or migration into the liver upon hepatic injury (Jaeschke et al., 1994). Therefore, if Kupffer cells do not play a key role in 1-NN-induced BEC damage it is not surprising that GdCl₃ did not decrease injury in retinol pretreated animals. However, the mechanism by which GdCl₃ enhances BEC damage has yet to be determined.

Opposite to the findings in the liver, retinol pretreatment significantly protects against 1-NN, 2-NN, and PQ induced pulmonary toxicity. During the early stages of 1-NN induced injury there is no morphological difference between the lungs of vehicle and retinol pretreated animals. From the biochemical markers of damage, the retinol pretreated animals possessed an even greater amount of Clara cell injury as compared to vehicle pretreated rats at both earlier and later time points. This increased injury to Clara
cells could be accounted for by the induction of cytochrome P-450 2B1, the enzyme responsible for the bioactivation of 1-NN in the lung (Appendix A). Although Clara cells from retinol pretreated rats are probably damaged to a greater degree than those of vehicle pretreated rats, the lung is protected from the overall injury.

The protection of the lung by retinol pretreatment seems to occur as a result of the down regulation of the inflammatory response and lack of neutrophil infiltration. However the mechanism of lung protection by retinol is not as defined as is the mechanism in the liver. For example, retinol has a stimulatory effect on PAM, significantly increasing the production of TNF and LTB4. Both of these bioactive products have been associated with the initiation and mounting of an inflammatory response. However, the hyperactivity of PAM may indeed be responsible for the decreased inflammation seen during chemical induced lung injury in retinol pretreated animals. Protection from oxygen toxicity has been demonstrated in rats given either recombinant TNF-α or interlukin-1 (IL-1) (White and Tsan, 1994). These authors showed that repeated doses of cytokines induce Mn-containing superoxide dismutase (Mn-SOD) in the pulmonary parenchyma. The induction of Mn-SOD has the ability of protecting tissues from oxidative stress (White and Tsan, 1994). Oxidative stress, as well as the production of TNF has been shown to be an important signal for the elicitation of pro-inflammatory cytokines in the lung (Driscoll, 1994). Thus, the inhibition of oxidative stress may alter the production of pro-inflammatory cytokines and decrease the ensuing inflammatory response.
With this information a united mechanism for retinol protection of the lung has been difficult to arrive at until recent data has been made available. Over the last year a collaboration between Dr. Sipes, myself, and Dr. Kevin Driscoll at Procter and Gamble has been formed. The objective of this collaboration has been to evaluate the effect of retinol on the expression of pro-inflammatory cytokines in the lung during chemically induced injury. Frozen lung tissues were sent to Dr. Driscoll, and analyzed for pro-inflammatory cytokine production in the lungs of retinol and vehicle pretreated rats given 1-NN or it vehicle. Interestingly, it was found that the 1-NN treated vehicle lungs contained significant amounts of message (mRNA) for macrophage inflammatory protein (MIP-2), as well as other pro-inflammatory cytokines. This message was absent from the lungs of rats pretreated with retinol and given 1-NN, nor was it found in the vehicle control groups. These findings represent a mechanism by which retinol could decrease the inflammatory response associated with 1-NN induced lung injury. This proposed mechanism is outlined in Figure 5.1.

The proposed mechanism of protection uses many of the components examined in the lung following retinol pretreatment. In the lung, retinol stimulates PAM and increases the production of cytokines and eicosinoids via the activation of PLA2. Prolonged exposure to PAM derived TNF leads to changes in the bronchiolar epithelium. The epithelium becomes more resistant to oxidative stress, down regulating the signal for pro-inflammatory cytokine production in the lung. Furthermore, chronic TNF exposure to the epithelium may alter the regulation of pro-inflammatory cytokine gene expression by
Figure 5.1. Proposed mechanism of all-trans-retinol protection from chemical induced lung injury. In this proposed model retinol alters pulmonary epithelial cell production of pro-inflammatory cytokines by primarily increasing alveolar macrophage cytokine release (TNF). The abnormal release of TNF leads to the induction of superoxide dismutase (Mn-SOD) and decreased intracellular oxidative stress (an important signal for the production of pro-inflammatory cytokines) in pulmonary epithelial cells. The decreased intracellular oxidative stress results in an attenuated pro-inflammatory cytokine response following tissue injury. This down-regulation of the pro-inflammatory response hinders the recruitment of neutrophils into the lung.
other yet undefined mechanisms. Following a chemical insult to the lung the production of pro-inflammatory cytokines is decreased from a "normal" response, and an altered inflammatory response ensues.

Although the diet is the main source of vitamin A (retinol), it is available in over the counter vitamin preparations. Retinol and its synthetic derivatives are prescribed for acne and other skin disorders as topical and oral preparations. It is also used as an adjuvant to chemotherapy, as well as a chemo-preventative agent for several types of cancer because of its immuno-stimulatory activities. Thus, the widespread usage of this vitamin creates the potential for exposure to large doses and the possibility of interaction with other chemicals. How such large doses of retinol may interact with other chemicals in humans is unknown. However, the results do underscore that immunomodulation may be an important mechanism by which on chemical influences the toxicity of another.

**Future Directions**

Future direction of this project must expand upon the groundwork previously laid (Sauer et al., 1995b; Sauer and Sipes, 1995; ElSisi et al., 1993a; Sipes et al., 1989). In the lung, future research should focus on further characterization of the effects of retinol on inflammation during chemically induced toxicity. Initially, the progression of inflammatory cell infiltration using immunohistochemical techniques comparing retinol and vehicle pretreated animals should be followed. In the same animals *in vivo* evaluation
of pulmonary cytokines (MIP, TNF, IL-1, and IL-6) and arachidonic acid metabolites (LTB₄, and PGE₂) could be evaluated. From a toxicological standpoint, it will be important to elucidate the effect which retinol has on Clara cell susceptibility to toxicants (i.e. 1-NN or 2-NN). This could be accomplished by isolating Clara cells from retinol and vehicle pretreated rats. However methods would need to established and isolated cells characterized. The isolated Clara cells could also be analyzed for CC10, cytochrome P-450 activity, as well as glutathione content following retinol pretreatment. Furthermore, it will be important to evaluate the effects of retinol pretreatment on the toxicity caused by inhaled environmental toxicants, such ozone or sulfur dioxide, in the whole animal.

One of the most interesting, as well as difficult future objectives should be to separate the effects of retinol on liver and lung. It would be significant if the pro-inflammatory actions of retinol in the liver could be prevented, while the anti-inflammatory effect in the lung was retained. This could possibly be accomplished by using synthetic retinoids which are not stored in the liver, but still possesses systemic effects (i.e. isotretinoin, etretinate, or fenretinide). Finally, one of the most intriguing findings was the potentiation of 1-NN induced BEC damage by retinol. The potentiated BEC damage was not attenuated by GdCl₃ pretreatment; instead it was increased. In future studies not only the potentiation of BEC damage by retinol needs to further evaluated, but also the ability of GdCl₃ to increase 1-NN induced BEC injury.
APPENDIX A:

THE EFFECT OF ALL-trans-RETINOL PRETREATMENT ON BLOOD KINETICS AND TISSUE DISTRIBUTION OF 1-NITRONAPHTHALENE, AND CYTOCHROME P-450 2B1 LEVELS

For both 1-NN and 2-NN, bioactivation has been shown to important in the elicitation of lung and liver toxicity (Johnson et al., 1984; Rasmussen, 1986). Furthermore, these two compounds have been shown to be metabolized by the cytochromes P-450 (Verschoyle and Dinsdale 1990; Verschoyle et al., 1993). Verschoyle and Dinsdale (1990), using inhibitors of specific cytochrome P-450 (CYP) isoenzymes, have shown 1-NN toxicity to be correlated with CYP-2B1 activity in the lung. However, in the non-induced rat liver it appears that 1-NN is bioactivated by CYP-1A1 and 1A2 (Verschoyle et al., 1993).

Besides altering the associated inflammatory responses in the lung and liver, several possible mechanisms exist by which retinol could alter the toxicity of a xenobiotic. For example, high doses of retinol have been shown to alter drug metabolism and disposition (Pentiuk et al., 1991; Rosengren et al., 1994). This could result in an alteration in the toxicity of 1-NN in the lung or liver. Indeed, pretreatment of rats with phenobarbital has been shown to protect completely the lung from 1-NN-induced injury by reducing the amount of parent compound which reaches the lungs to be metabolically
activated (Dankovic and Cornish, 1982). Because metabolism play a central role in both the hepatic and pulmonary toxicity of 1-NN, we were concerned that the modulation of toxicity caused by retinol could be due to an alteration in metabolism. Therefore, the ability of retinol to alter the blood kinetics and tissue disposition of 1-NN was evaluated in the whole animal. The effect of seven days of retinol pretreatment on cytochrome P-450 2B1 in the lung and liver was also examined.

**Western Blot Analysis**

Hepatic and pulmonary microsomal CYP-2B1 protein levels were estimated by western blot analysis from rats pretreated with retinol (75 mg/kg/day) or vehicle for 7 days. The method used here has been described in detail by Stamer et al., (1994). In brief 0.5 µg of microsomal protein was loaded on to a 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and separated by electrophoresis. Following electrophoresis, separated proteins were transferred to nitrocellulose membrane. Blots were then blocked with 5% non-fat milk for 1 hr, and incubated at room temperature for 2 hr with anti-rat CYP-2B1 primary antibody. Blots were then washed and incubated with anti-rabbit HPR-conjugated secondary antibody for 2 hr at room temperature. Following washing immuno-bLOTS were stained luminol and visualized by chemiluminescence on Kodak X-OMAT film.
Figure A.1. Effect of all-trans-retinol pretreatment on pulmonary and hepatic microsomal CYP-2B1. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o.) or vehicle for 7 days. Twenty four hr after the last dose hepatic and pulmonary microsomes were isolated, and the CYP-2B1 protein levels evaluated by western blot analysis as described above. Bars represent means ± SEM. Lanes 1 & 2 are pulmonary microsomes harvested from retinol pretreated rats, lanes 3 & 4 are pulmonary microsomes harvested from vehicle pretreated rats, lanes 5 & 6 are hepatic microsomes harvested from retinol pretreated rats, lanes 7 & 8 are hepatic microsomes harvested from vehicle pretreated rats.
In vivo Plasma Kinetics and Tissue Disposition of 1-Nitronaphthalene

Rats were pretreated with retinol (75 mg/kg/day for seven days, p.o.) or vehicle (7% tween-20, 10% propylene glycol in water for seven days, p.o.). Twenty four hr after the last pretreatment animals were given 1-NN (100 mg/kg in 2 mL/kg peanut oil, i.p.) and killed by carbon dioxide inhalation 0.5, 1, 2, 4, 6, 12, and 24 hr later. Following sacrifice blood was collected from the posterior vena cava into heparinized tubes, and 1-NN content determined by HPLC. Before blood was analyzed for 1-NN, it was extracted with ethyl acetate (1 mL blood to 1 mL solvent) 2 times. The extract was evaporated under a nitrogen stream and resuspended in 200 μL of methanol containing 1 mg/mL of 4-(4-nitrobenzyl)pyridine (4-NBP; internal standard). Samples were analyzed using HPLC (Spectra Physics, SP 8700 Solvent Delivery System) equipped with a spectrophotometric detector (Waters System Products, Lambda Max 480). A 250 x 4.6 mm C-18 Vydac, 5μm column (Alltech, Deerfield, IL) was eluted with 100 mM sodium phosphate:methanol (60:40; pH 4.5) at a flow rate of 1.0 ml/min.

Tissue disposition was determined in rats pretreated with retinol (75 mg/kg/day for 7 days, p.o.), vehicle (7% tween-20, 10% propylene glycol in water for 7 days, p.o.), or phenobarbital (80 mg/kg/day for 4 days). Twenty four hr after the last pretreatment animals were given $^{14}$C-labeled 1-NN (40 μCi/kg, 100 mg/kg in 2 mL/kg peanut oil, i.p.) and placed in metabolism cages. The $^{14}$C-labeled 1-NN was synthesized from $^{14}$C-naphthalene by the Synthetic Core of the SWEHC at The University of Arizona. Animals
were killed by carbon dioxide inhalation 24 hr after 1-NN administration and blood was collected from the posterior vena cava into heparinized tubes. At necropsy lungs, livers, and kidneys were collected. Blood and tissue samples were analyzed for total radioactivity by sample oxidation using a Packard Oxidizer model 306D (Packard Instruments, Meirdan, CT).

Figure A.2. Effect of retinol on 1-nitronaphthalene (1-NN) blood kinetics. Male Sprague-Dawley rats received retinol (75 mg/kg/d, p.o., for 7 days) or vehicle followed 24 hr later by 1-NN (100 mg/kg, i.p.). At 0.5, 1, 2, 4, 6, 12 and 24 hr after 1-NN administration rats were killed, blood drawn, and measured by HPLC for 1-NN as described above. Points represent means ± SEM. (n=3).
Tissue content of $^{14}$C-1-nitronaphthalene equivalents in all-trans-retinol, vehicle, and phenobarbital pretreated Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Vehicle (% dose)</th>
<th>Retinol (% dose)</th>
<th>Phenobarbital (% dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>0.15 ± 0.07</td>
<td>0.02 ± 0.01$^a$</td>
<td>0.03 ± 0.02$^a$</td>
</tr>
<tr>
<td>Liver</td>
<td>1.17 ± 0.33</td>
<td>0.55 ± 0.24$^a$</td>
<td>0.56 ± 0.16$^a$</td>
</tr>
<tr>
<td>Lung</td>
<td>0.14 ± 0.03</td>
<td>0.11 ± 0.02</td>
<td>0.02 ± 0.01$^a$</td>
</tr>
</tbody>
</table>

Amount of total radiolabel in lung, liver, and kidney 24 hr after the administration of $^{14}$C 1-nitronaphthalene (40 μCi/kg, 100 mg/kg, i.p.), following retinol, vehicle, or phenobarbital pretreatment. (n=3). Numbers represent means % dose ± SEM. $^a$Significantly different from vehicle.
APPENDIX B:

PHENOBARBITAL ALTERATION OF 1- AND 2-NITRONAPHTHALENE INDUCED IN THE MALE SPRAGUE-DAWLEY RAT.

Bioactivation has been shown to be important in the elicitation of both 1-nitronaphthalene (1-NN) and 2-nitronaphthalene (2-NN) induced lung and liver toxicity. Therefore, the modulation of toxicity caused by retinol could be due to an altered metabolism of 1-NN or 2-NN. The objective of these studies were to evaluate the ability of phenobarbital to modulate the toxicities of 1-NN and 2-NN. When rats were pretreated with phenobarbital (80 mg/kg/day for 4 days) a similar, but discretely different, alteration in 1-NN and 2-NN toxicity was observed as compared to retinol pretreatment. Grossly, in both the phenobarbital and retinol animals, hepatotoxicity is increased and lung toxicity is attenuated. However, at both the microscopic and biochemical levels the lungs of phenobarbital pretreated rats given 1-NN or 2-NN had no damage associated with their Clara cells. The lungs of retinol pretreated rats given 1-NN revealed damage to the Clara cells. In the liver, again unlike retinol, phenobarbital potentiated injury caused by either 1-NN or 2-NN could not be antagonized by GdCl₃. From these data it is concluded that retinol alteration of 1-NN induced toxicity is different from the modulation caused by phenobarbital.
Figure B.1. Effect of phenobarbital on 1-nitronaphthalene induced lung injury. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o., for 7 days) or vehicle followed by 1-NN (100 mg/kg, i.p.) on day eight. Twenty four hr after 2-NN administration rats were killed and markers of lung injury were evaluated as described in Materials and Methods. (A) BALF LDH activity, (B) BALF protein content, (C) BALF GGT activity. Bars represent means ± SEM. n=9. aSignificantly different from respective vehicle control. bSignificantly different from respective phenobarbital vehicle group (bronchioalveolar lavage fluid = BALF).
Figure B.2. Effect of phenobarbital on 2-nitronaphthalene induced lung injury. Male Sprague-Dawley rats received either retinol (75 mg/kg/day, p.o., for 7 days) or vehicle followed by 2-NN (200 mg/kg, i.p.) on day eight. Twenty four hr after 2-NN administration rats were killed and markers of lung injury were evaluated as described in Materials and Methods. (A) BALF LDH activity, (B) BALF protein content, (C) BALF GGT activity. Bars represent means ± SEM. n=9. αSignificantly different from respective vehicle control. βSignificantly different from respective phenobarbital vehicle group (bronchioalveolar lavage fluid = BALF).
Figure B.3. Effect of gadolinium chloride pretreatment on phenobarbital potentiated 1-nitronaphthalene induced liver injury. Male Sprague-Dawley rats received phenobarbital (80 mg/kg/d, i.p.) for 4 days, and on day 4 rats were given GdCl₃ (10 mg/kg, i.v.) via the tail vein. One day after GdCl₃ administration rats received 1-NN (100 mg/kg, i.p.) or peanut oil. Twenty four hr after 1-NN administration rats were killed and markers of liver injury were evaluated as described in Materials and Methods. Plasma ALT activity. Bars represent means ± SEM. n=9. aSignificantly different from respective peanut oil control. bSignificantly different from respective phenobarbital vehicle group.
Figure B.4. Effect of gadolinium chloride pretreatment on phenobarbital potentiated 2-nitronaphthalene induced liver injury. Male Sprague-Dawley rats received phenobarbital (80 mg/kg/d, i.p.) for 4 days, and on day 4 rats were given GdCl₃ (10 mg/kg, i.v.) via the tail vein. One day after GdCl₃ administration rats received 2-NN (200 mg/kg, i.p.) or peanut oil. Twenty four hr after 2-NN administration rats were killed and markers of liver injury were evaluated as described in Materials and Methods. Bars represent means ± SEM. n=9. aSignificantly different from respective peanut oil control. bSignificantly different from respective phenobarbital vehicle group.
REFERENCES


