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Inhibition of Cytochrome P-450 but Not Neutrophil Depletion Prevents the Pulmonary Toxicity Associated with 1-Nitronaphthalene

Steven Michael Green

INHIBITION OF CYTOCHROME P-450 BUT NOT NEUTROPHIL DEPLETION PREVENTS THE PULMONARY TOXICITY ASSOCIATED WITH 1-NITRONAPHTHALENE

by

Steven Michael Green

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INHIBITION OF CYTOCHROME P-450 BUT NOT NEUTROPHIL DEPLETION PREVENTS THE PULMONARY TOXICITY ASSOCIATED WITH 1-NITRONAPHTHALENE

Steven Michael Green, M.S.

Western Michigan University, 1993

1-Nitronaphthalene (1-NN) is an environmental contaminant that has been shown to cause pulmonary toxicity in rodents. To examine the contribution of cytochrome P-450 to the metabolism and subsequent toxicity of 1-NN, rats were pretreated with chloramphenicol, a suicide substrate of P-450. To determine if inflammation plays a role in 1-NN toxicity, rats were pretreated with cyclophosphamide to deplete neutrophils. In both experiments, morphological analysis of the lower airways was performed with light and electron microscopy. A second group of animals was examined for biochemical indicators of inflammation and cell damage. The parameters examined were cell content, protein content and lactate dehydrogenase activity in bronchoalveolar fluid, and tissue myeloperoxidase activity. The results showed that pretreatment with chloramphenicol prevented the cytotoxicity associated with 1-NN, but not the inflammatory response. Pretreatment with cyclophosphamide did not prevent 1-NN cytotoxicity, but did prevent neutrophil-dependent aspects of inflammation.

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Without hesitation, this thesis is dedicated to the memory of my father, who did not survive to see the end of my college career. I love you dad.

Steven Michael Green

Order Number 1353634

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Green, Steven Michael, M.S.

Western Michigan University, 1993

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

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STATEMENT OF THE PROBLEM

1-Nitronaphthalene (1-NN) is an environmental contaminant found in diesel exhaust, cigarette smoke and industrial processes (IARC Monographs). While there is no data available on human exposure to 1-NN, rodent studies have shown that exposure causes preferential damage to the distal bronchiolar epithelium (Johnson, Riley, & Cornish, 1984). Pulmonary damage is postulated to occur due to metabolic activation of 1-NN by cytochrome P-450 isozymes, however, attempts to prove this have produced conflicting results *in vivo* (Johnson, et al., 1984) and *in vitro* (Rasmussen, 1986). The elucidation of 1-NN metabolism is further complicated by the presence of other pulmonary enzymes that are capable of metabolizing foreign compounds. These include prostaglandin h synthase (PHS), flavin monooxygenase (FMO), and epoxide hydrolase.

Additionally, inflammation is a common consequence of cytotoxic tissue injury. This response can lead to tissue damage through the oxidative and enzymatic products of neutrophil and macrophage activation (Henson & Johnston, 1987). There are no published results defining the role of inflammation on the pathogenesis of 1-NN induced lesions. This study examines the effect of cytochrome P-450 inhibition or neutrophil depletion on the pulmonary toxicity of 1-NN using morphologic and biochemical indicators of damage.

INTRODUCTION

1-Nitronaphthalene

1-Nitronaphthalene is found in diesel exhaust, cigarette smoke and industrial dyes (IARC Monographs). There have been several types of studies done to determine the metabolic fate of 1-NN. Rasmussen (1986) showed that covalent binding to macromolecules occurs when slices, isolated cells, and microsomes from the lungs of mice are incubated with 1-NN. This study further showed that a metabolite rather than the parent compound was responsible for toxicity using heat-inactivated incubations, and that the reaction was oxygen dependent. This was proposed to be a cytochrome P-450 dependent mechanism by the use of traditional inhibitors such as SKF 525a and nitrogen gas. These results indicated that 1-NN was metabolized *in vitro* to oxidative metabolites by cytochrome P-450.

El-Bayoumy & Hecht (1982) showed that pretreatment of rat liver supernatant with Arochlor 1254, a powerful inducer of group IA isozymes, prior to treatment with 1-NN caused the production of oxidative metabolites consisting of nitronaphthols and dihydrodiols. Conversely, Johnson & Cornish (1978) showed that 1-naphthylamine, a reductive product of 1-NN metabolism was excreted in the urine 24 hours after a 100 mg/kg i.p injection of 1-NN. Another study by Johnson et al., (1984) showed that pretreatment of rats with SKF 525a prevented liver toxicity, but not lung toxicity *in vivo*. This conflicts with the *in vitro* results obtained by Rasmussen (1986) which show that SKF-525a inhibited the production of reactive metabolites from 1-NN. The results of these experiments indicate that both oxidative and reductive pathways may contribute to 1-NN metabolism and toxicity, although the studies performed up to

now do not indicate which metabolites are injurious *in vivo* and in what order they are produced.

1-Nitronaphthalene has not exhibited carcinogenic activity in either rats or mice fed oral doses of 1-NN (National Cancer Institute, 1978). However, positive results were obtained from the Ames test using error-prone S. Typhimuriam. Strains of bacteria that did not posses nitroreductase activity failed to produce positive results (McCoy, Rosenkranz, Petrullo, Rosenkranz & Mermelstein, 1981).

The Clara Cell

The Clara cell is the initial site of 1-NN toxicity after a 100mg/kg dose administered intraperitonealy (Sauer, 1991). This cell, originally described by Clara in 1937 is a multi-functional resident of the distal and terminal airways of mammals. Some aspects of Clara cell function include secretion of a phospholipid from electron-dense granules into the serous lining of the airways (Stinson & Loosli, 1978), metabolism of foreign compounds (Boyd, 1977), and possibly differentiation into other cell types to replace dead cells (Evans, Cabral-Anderson, & Freeman, 1978).

Histochemical results have shown that the majority of pulmonary xenobiotic-metabolizing enzymes are located within Clara cells (Baron & Voigt, 1990). These cells are rich in microsomal enzymes such as cytochrome P-450, prostaglandin h synthase, epoxide hydrolase and flavin monooxygenase; and in cytosolic enzymes such as UDP-glucoronyltransferase and glutathione-s-transferase (Baron & Voigt, 1990). This is in contrast to liver which has a relatively homogenous enzyme distribution.

Clara cells and ciliated cells compose the epithelium of the distal and terminal bronchioles; compared to the larger airways which are composed mainly of ciliated and goblet cells (Breeze & Wheeldon, 1977). This concentration of P-450 containing

cells correlates with the lower airways being a common site of damage due to cytotoxic drugs requiring activation (Boyd, Statham, & Longo, 1980). This has been illustrated with several chemicals that are converted to toxic metabolites within the lung, such as 4-ipomeanol (Boyd, 1976), 3-methylfuran (Boyd, 1978), and carbon tetrachloride (Boyd, Statham, & Longo, 1980).

Several P-450 isozymes are found in the lung. One isozyme, IIB1, has a pulmonary concentration that is nearly double the hepatic concentration (de Waziers, Cugnenc, Yang, Leroux, & Beaune, 1990). This isozyme is expressed primarily in the Clara cell (Baron & Voigt, 1990). Johnson et al., (1984) reported that pretreatment of rats with phenobarbital, a powerful inducer of hepatic IIB1, shifted the focus of 1-NN toxicity from the lung to the liver. This lead to the theory that pulmonary IIB1 was responsible for the first pass metabolism of 1-NN.

Metabolic Activation and Metabolism

Ideally, when a compound is metabolized, the products become less reactive and more water soluble; which aids in excretion by the kidneys. Conversely, a compound can be metabolically activated, resulting in the metabolites becoming more reactive then the parent compound (Guengerich & Liebler, 1987). In the case of toxic chemicals, activation produces a metabolite that damages the cell through destruction or modification of an essential component. Some examples include lipid peroxidation, alkylation and crosslinking of DNA, depletion of essential cofactors, such as NADPH and ATP (Nelson & Pearson, 1990), or modification of proteins to the point where the structure-function relationship is lost (Nelson & Pearson, 1990). This is of special importance in the case of enzymes and structural proteins such as membrane channels and receptors.

As reviewed by Sipes & Gandolfi (1986), two distinct classes of reactions (termed phases) have been identified in the metabolism of endogenous and exogenous molecules. Phase I reactions are associated with reactions that include oxidation, reduction, hydroxylation and dealkylation of the parent compound. Enzymatic hydrolysis of amides or esters and the reduction of epoxides to *trans*-dihydrodiols are also considered to be phase I reactions. Some enzymes that are associated with phase I are the cytochrome P-450 enzyme family, prostaglandin h synthase, flavin monooxygenase and epoxide hydrolase. Phase I enzymes are located in the microsomal portion of the cell, while phase II enzymes (see below) tend to be located in the cytoplasm.

Prostaglandin h synthase is a bifunctional enzyme with oxygenase and peroxidative activities (Eling et al., 1990). The oxygenase property, cyclooxygenase, catalyzes the conversion of arachidonic acid to the cyclic endoperoxide prostaglandin G2. The peroxidase property reduces prostaglandin G2 to the corresponding alcohol prostaglandin H2 (Eling et al., 1990). Sometimes, this reaction can cause the concomitant oxidation of a second, unrelated substrate and is termed cooxidation. Identical metabolites can be produced from cooxidation by PHS or oxidative metabolism by P-450 (Eling et al., 1990). Both P-450 and PHS can act upon a wide variety of compounds while FMO is usually associated with the N-oxidation of amines (Sipes & Gandolfi, 1986).

Phase II reactions cause the addition of a polar side group to the parent compound, and are termed conjugation reactions. Some of the additives include glucuronic acid, glutathione, sulfate, and amino acids (Sipes, 1986). These reactions are catalyzed by specific transferase enzymes with the exception of glutathione, which can react with compounds enzymaticaly and non-enzymaticaly (Sipes & Gandolfi,

1986). Conjugation produces bulkier, polar molecules that are more readily excreted by the kidneys than the parent compound (Sipes & Gandolfi, 1986). Addition of these groups can also increase the molecular weight of a molecule to the point where it can be excreted in the bile as well as the urine (Levine, 1978).

Boyd (1980) described three mechanisms of lung injury by toxic chemicals. The first consists of compounds whose metabolites are activated and bind within the lung. The second consists of compounds that are activated in the liver but are stable enough to enter the circulation and travel to the lung; which is the next capillary bed. Group two metabolites cause damage that results in symptoms similar to Adult Respiratory Distress Syndrome, which is characterized by endothelial damage and increased vascular permeability. The third group consists of compounds whose metabolites are not necessarily toxic themselves, but cause activation of oxygen to a reactive species with concomitant depletion of NADPH. This group is illustrated by paraquat, diquat and nitrofurantoin, which cause damage to endothelium and type I epithelial cells.

Cytochrome P-450

Cytochrome P-450 is a super-family of more then 20 heme-containing isozymes. P-450 isozymes are ubiquitous throughout the body, with the highest concentration found in the liver (de Waziers et al., 1990). Other organs such as the lungs, skin, intestine and kidneys contain significant amounts of P-450 isozymes; although constitutive expression differs between hepatic and extrahepatic tissues (de Waizers et al., 1990). The rat liver contains small amounts of isozymes IA2, IIB1, IIE1 and IIC6 with IIC11 being the major constitutively expressed isozyme (de Waziers et al., 1990). The rat lung contains isozyme IIB1 as its major isozyme with

smaller amounts of IA1 and IIIA2 present (Baron & Voigt, 1990). The amount of IIB1 expressed in the lung is over 50% greater than the liver and is concentrated in the bronchiolar Clara cells (Baron & Voigt, 1990).

The cycle of P-450 action involves transfer of two reducing equivalents from NADPH to molecular oxygen by the enzyme NADPH-cytochrome P-450 reductase (Gungerich & Liebler, 1987). One atom of oxygen becomes reduced to water while the other is conjugated to the compound. The exception to this is nitroreduction, where reducing equivalents are transferred directly to the substrate. This reaction occurs in hypoxic conditions and is inhibited by oxygen, which competes for the reducing equivalents (Gungerich & Liebler, 1987).

As reviewed by Murray & Reidy (1990), exposure to xenobiotics can lead to induction or inhibition of P-450 isozymes. The specificity of inducers and inhibitors is highly variable. Some will interact with select isozymes, while others will affect several. Induction results in the production of isozymes at a greater rate then normal, or the production of an isozyme that is not normally expressed.

There are two types of cytochrome P-450 inhibitors, reversible and irreversible. Reversible inhibitors act by forming a complex with the enzyme that later dissociates. Examples include SKF 525a, and piperonyl butoxide. Irreversible inhibitors-also known as mechanism based inhibitors or suicide substrates-covalently bind to the enzyme, permanently inactivating it. Some examples of suicide substrates are chloramphenicol and 1-aminobenzotriazole.

Neutrophils and Oxidative Stress

Oxidative stress is defined as injury that occurs to tissue by reactive oxygen species. Neutrophils are the cell most commonly associated with reactive oxygen

production; although endothelial cells, macrophages and eosinophils also produce reactive species (Henson & Johnston, 1987). Neutrophils have received most of the attention since they are the most common granular leukocyte in the blood, and are the first cell type to arrive in high numbers after an inflammatory stimulus. Aside from the production of reactive oxygen species, neutrophils also possess enzymes that can damage tissue (Weiss, 1989).

As reviewed by Weiss (1989) the primary oxidative reaction associated with neutrophils is the myeloperoxidase/hypochlorus acid reaction. Stimulated neutrophils undergo a respiratory burst that results in activation of NADPH oxidase; a membrane bound enzyme that reduces molecular oxygen to superoxide. Hydrogen peroxide is produced from superoxide by the enzyme superoxide dismutase or the spontaneous interaction between two superoxide molecules. The copious amounts of hydrogen peroxide produced serve as a substrate for myeloperoxidase, an enzyme stored in azeurophilic granules that is released into phagolysozomes or to the exterior of the cell. This enzyme catalyzes the conversion of hydrogen peroxide to water with the concomitant oxidation of chloride ions to hypochlorus acid, a strong oxidizing agent well known for its bactericidal action.

Until recently, hypochlorus acid was not believed to cause intracellular damage because it is unable to cross membranes, however, recent studies (Test, Lampert, Ossanna, Thoene, & Weiss, 1984) have shown that hypochlorus acid can react with endogenous amines to form both hydrophilic and hydrophobic chloramines. Intracellular damage can be caused by hydrophobic chloramines which are lipophillic, longer lived, and able to cause damage by oxidation or chlorination of a substrate (Test et al., 1984). While many investigators believe that hypochlorus acid is the oxidative product primarily responsible for neutrophil toxicity, other compounds such

as singlet oxygen (Kanosky, Wright, Miles-Richardson, & Tauber, 1984) and hydroxyl radical (Repine et al., 1989) are also believed to play a role.

Singlet oxygen is formed from the interaction of hydrogen peroxide and hypochlorus acid (Kanosky et al., 1984). Hydroxyl radical is a product of the interaction between hydrogen peroxide and either iron or copper ions, illustrated in the Fenton or Haber-Weiss reactions (Bast, Haenen, & Doleman, 1991). While both are highly reactive and destructive molecules, it is difficult to attribute tissue damage to either, due to the difficulty of detecting them *in vivo.* Superoxide and hydrogen peroxide have also been implicated as the primary injurious agents in inflammatory damage, although superoxide tends to react with itself rather then other molecules (Weiss, 1989). Both superoxide and hydrogen peroxide are rapidly inactivated by large constitutive amounts of superoxide dismutase and catalase (Bast, Haenen, & Doleman, 1991).

Neutrophils also contain many hydrolytic enzymes that cause tissue damage. These include elastase, collagenase, gelatinase, cathepsin G and others (Weiss, 1989). The most destructive of these enzymes is elastase. This enzyme can act upon many molecules including elastic connective tissue, types III and IV collagen, plasma proteins, complement factors, fibronectin and proteoglycans (Janoff, 1985). This is especially injurious to the lung, which relies on elastic tissue for its compliance (Janoff, 1985). Studies have shown that intratracheal instillation of elastase causes emphysema-like diseases in experimental animals (Senior et al., 1977). Since the lung is the site of a continuous barrage of compounds capable of eliciting an inflammatory response, it is not surprising that the body is adept at preventing the accumulation of elastase. The major defense against elastase in the lung is α-1 proteinase inhibitor (α-1pi) which irreversibly binds to and inactivates elastase (Carrell, 1986). This protease

is found in equal concentrations in the extracellular matrix of the lung and blood plasma, and is the only significant protease activity in the normal lung (Gadek, Fells, Zimmerman, Rennard, & Crystal, 1981). This is illustrated in the case of persons with congenital deficiencies in α-1pi. These subjects exhibit a severe early onset emphysema, a disease associated with the loss of lung compliance by elastic tissue destruction (Janoff, 1985). In inflammatory processes, edema provides an influx of plasma proteases that include α-1pi and α-2 macrogloubin, another protease effective against elastase (Gadek et al., 1981).

Considering the abundance of antiproteases available, it is surprising that elastase can cause significant lung damage. This has been explained by the synergistic interactions between hypochlorus acid and elastase. Release of hypochlorus acid into the extracellular matrix causes the oxidation and deactivation of tissue α-1pi, which allows elastase to operate freely in the extracellular space (Weiss, & Regiani, 1984). Hypochlorus acid is also responsible for the activation of collagenase (Weiss, Peppin, Ortiz, Ragsdale, & Test, 1985) and gelatinase (Peppin, & Weiss, 1986). These enzymes are stored in granules and released as inactive proenzymes (Wiess, 1989). Interaction with HCA causes cleavage of the proenzymes to their active forms (Weiss, 1989). This was shown in subjects whose neutrophils do not produce hypochlorus acid. These persons secrete normal amounts of inactive enzymes from neutrophils, but no active enzyme can be detected (Weiss et al., 1985).

Taken individually, these processes seem simple and well defined. In an *in vivo* system this is not the case. Inflammation is provoked by a stimulus, which in the case of 1-NN could either be a direct reaction to the compound, its metabolites, or the result of toxic injury to the cells. Regardless of the stimulus, once initiated, resident cells such as mast cells, macrophages, and endothelial cells release cytokines and

chemotactic molecules that cause increased vascular permeability, hypotension, and expression of leukocyte adhesion molecules on endothelial cells (Williams & Hellewell, 1992). This results in the acute inflammatory response. Whether or not acute inflammation proceeds into chronic inflammation depends on the duration of the stimulus.

Purpose of the Study

The purpose of the following study was to determine if inhibition of cytochrome P-450 or neutrophil depletion could attenuate pulmonary damage caused by 1-NN. This was done using chloramphenicol, a suicide substrate for cytochrome P-450 that has been shown to be effective in the lung *in vivo* (Naslund & Halpert, 1984) and cyclophosphamide, a chemotherapeutic agent commonly used to deplete granulocytes prior to bone marrow transplants.

MATERIALS AND METHODS

Experimental Design

This study was divided into two parts. The first was an electron and light microscopic evaluation of rat lungs after various treatments. The second was an analysis of biochemical indicators of inflammation and cell damage in rats receiving identical treatments to the first. This was performed by analysis of bronchoalveolar lavage (BAL) fluid and tissue homogenates.

Procedures

Animals and Treatments

Male CD rats (Charles River Labs, Portage, Michigan) weighing between 150-250 grams were used in all experiments. Animals were housed according to University guidelines, with rat chow #5001(Purina Mills, St. Louis, MO) and tap water provided *ad libitum.*

Table 1 shows the treatment received by each group and the number of animals used in morphological analysis, while Table 2 shows the number of animals used for bronchoalveolar lavage analysis. In all experiments, the start time, which corresponds to 1-NN administration, is termed time 0. All drugs were administered as single i.p injections. 1-NN was solubilized in a few drops of diethyl ether, dissolved in food grade peanut oil (Planters), and given as a 100 mg/kg dose at a concentration of 50 mg/ml. Chloramphenicol was dissolved in propylene glycol and administered as a 100 mg/kg dose at minus 1 hour at a concentration of 50 mg/ml. Cyclophosphamide was

Table 1

Necropsy Schedule for Morphological Analysis

Number of Animals			
	Treatment Time		
Group	Treatment	+6 hour	+12 hour
1	1-NN	2	3
2	1-NN and CPA	3	3
3	1-NN and CPM	3	2
4	CPA only	3	3
5	CPM only	3	3
6	Untreated Control		5

1-NN = 1-nitronaphthalene, CPA = chloramphenicol, CPM = cyclophosphamide

dissolved in distilled water and administered as a single 150 mg/kg dose at minus 72 hours at a concentration of 30 mg/ml. Subjects were removed from food at time 0.

Lung Perfusions

At either plus 6 or plus 12 hours, the animals were sacrificed and their lungs were perfused according to the following procedure: After a lethal injection of 50 mg/kg sodium pentobarbital i.p, the trachea was exposed and cannulated with a 2 mm diameter polyethylene tube connected to a small animal respirator. The lungs were

Table 2

Necropsy Schedule for Bronchoalveolar Lavage Analysis

	Number of Animals per Group at +12 Hours					
Group	1	2	3	4	5	6
	7*	5	5	5	5	7*

*An n of 3 was used in these groups for myeloperoxidase analysis.

inflated to 20 mm of water pressure and the chest was opened to expose the thoracic cavity. The aorta was cut to gather a blood sample using EDTA as an anticoagulant, and the left ventricle of the heart was slit to allow perfusate to escape. A 23 gauge Minicath® (Deseret Medical, Sandy Utah) was inserted into the right ventricle and the lungs were perfused with 10 ml of saline containing 10 units of ammonium heparin/ml. This was followed by 20 ml of 3% gluteraldehyde in 0.1 M cacodylic acid buffer, which consisted of 0.1M cacodylic acid, pH 7.4, with 10% sucrose. This formulation was used in all electron microscope experiments. After perfusion with fixative, the left lung and the inferior lobe of the right lung were removed and immersed in fresh fixative for forty five minutes. Subsequently, the left lung was sectioned into three longitudinal slices and placed into aqueous 10% formalin overnight in preparation for light microscopic analysis. The inferior lobe of the right lung was sliced into several 0.5x3 mm pieces and processed for electron microscopic analysis.

Microscopic Preparation

Electron Microscopy

After three washes in buffer, the samples were post-fixed in 1% osmium tetroxide in 0.1M cacodylic acid buffer for one hour followed by three washes in buffer. The tissue was dehydrated in a graded ethanol series, cleared in propylene oxide, and embedded in Polybed 812 (Polysciences, Warrington, PA) in flat embedment molds. One-half micron sections were cut on a Sorvall MT 5000 ultramicrotome and stained with toluidine blue. The following criteria was used to determine appropriate areas for electron microscopic examination: To be considered a distal or terminal bronchiole, the cell population consisted of Clara and ciliated cells with no goblet or basal cells present. The epithelium was simple in nature, and the basal lamina had irregular bundles of smooth muscle and connective tissue. This procedure insured that local changes in airway diameter or alteration of cell morphology did not cause the wrong type of airway to be subsequently examined. Blocks containing distal or terminal bronchioles were trimmed and thin sectioned on a Sorvall MT 5000 or LKB Nova ultramicrotome using glass knives and placed on 300 mesh copper or nickel grids. The samples were stained with 5% methanolic uranyl acetate and Reynolds lead citrate and examined on a Siemens 101 Elmiskop at 80Kv. Appropriate sections were photographed for later examination.

Light Microscopy

After fixing overnight in 10% formalin, the slices were dehydrated in a graded ethanol series followed by clearance in xylene and embedment in paraffin (Tissue-Tek, Fisher Scientific). The samples were cut on a Reichert-Jung Biocut 2030 microtome

and stained with Gills hematoxylin and phloxine eosin. The slides were viewed and photographed using a Nikon Microphot FXA and Kodak 160T film.

Bronchoalveolar Lavage

Lavage procedures were performed according to Roth (1981). Treatments were identical to the ones employed in the morphological analysis except that 12 hours was the only time point used. The number of subjects for this experiment is presented in Table 2. After a lethal injection of 50 mg/kg of sodium pentobarbital i.p., the abdomen was opened and the renal arteries or abdominal aorta was cut to exsanguinate the animal. In the case of animals receiving cyclophosphamide blood was collected over EDTA to confirm that leukocyte depletion had taken place. Results indicated that these animals had a leukocyte count similar to that observed in cyclophosphamide treated animals used for morphological analysis. The thorax was gently compressed to expel the remaining blood from the heart-lung block and the trachea was exposed and cannulated with a 2 mm diameter polyethylene tube. The lungs were lavaged twice with 23ml/kg of 0.9% saline using a glass syringe. The recovered lavages were pooled and centrifuged at 900 xg for 10 minutes at 4°C. The supernatants were drawn off and stored at -70° F for further analysis. The cells pellets were resuspended in 0.9% saline and stored on ice for cells counts. Tissue samples selected for measurement of myeloperoxidase activity were excised and homogenized with a glass homogenizer in 2 ml of hexadecyltrimethylammonium bromide buffer (HTBS, H-5882, Sigma Chemical Co), which consisted of 0.5 grams of HTBS in 100 ml of 50 mM phosphate buffer, pH 6.0. After centrifugation at 20,000 xg for thirty minutes at 4° C, the supernatants were drawn off and stored at -70° F for later evaluation.

Protein Analysis

Protein was measured using a Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951). A 100μL sample of cell-free BAL fluid was added to 400μL of water, and 2 ml of Lowry reagent D. This was vortexed, and 200μL of 1N phenol reagent was added. After 30 minutes the Absorbance was measured at 550nm using a Biorad microtiter plate reader. Bovine serum albumin (Sigma Chemical Co, St. Louis, MO) was used to generate a standard curve.

Cell Counting Procedure

After the BAL supernatant was removed, the cell pellet was resuspended in an identical amount of 0.9% saline. Cells were counted in the four 0.1μL counting chambers of an American Optical hemocytometer excluding red cells and fragments.

White blood cell counts were determined by the method described by Brown (1984). A sample of blood was collected using EDTA as an anticoagulant, drawn into a white blood cell counting pipette, and diluted to the mark with 3.0% acetic acid. After shaking for several minutes, roughly half of the fluid was drained, and the rest was placed into a hemocytometer and counted on the WBC counting squares of an American Optical Hemocytometer and expressed as cells/μL.

Lactate Dehydrogenase Assay

Lactate dehydrogenase activity was assayed using diagnostic assay kit DG-1340k from The Sigma Chemical Company (St. Louis, MO), which measures the loss of NAD+ in a sample. Horseradish Peroxidase was used as a positive control, a sample mixture made from boiled tissue was used as a negative control and the assay

mixture was standardized with Sigma LIN-TROL. All samples were run at one time to prevent any change in reagent activity. Change in Absorbance was measured with a Gilford spectrophotometer and defined as units/liter.

Myeloperoxidase Assay

Measurement of tissue myeloperoxidase activity was defined utilizing the method of Krawisz, Sharon, & Stenson, (1984). After homogenization with a glass homogenizer in 2 ml of HTBS buffer, and three freeze-thaws, the sample was centrifuged at 20,000 x g for 30 minutes in a microfuge at 4°C. The supernatant was removed and stored at -70° F. To determine myeloperoxidase activity, 50 µL of the supernatant was added to 2.95 ml of 0.169 mg/ml o-dianisidine dihydrochloride in 100ml of 6.0 mM phosphate buffer, pH 6.0, containing 0.0005% hydrogen peroxide. The sample and incubation mixture were shaken and the change in Absorbance was read at 460 nm using a Gilford spectrophotometer over three minutes, and expressed as units/milligram of protein.

Statistical Analysis

Statistical analysis was used to determine if significant differences occurred between the groups being examined. Data was run as two sets. The first consisted of the Chloramphenicol treated groups and was composed of the 1-NN control, chloramphenicol pretreated group, chloramphenicol control, and untreated control The second data set was composed of groups treated with cyclophosphamide and contained the 1-NN control, cyclophosphamide pretreated group, cyclophosphamide control, and untreated control. An analysis of variance was first done using the program supplied with Microsoft Excel®. A p of 0.05 was used to determine if a

significant difference existed across the set. If this occurred, the components were examined using the Newman-Keuls test of multiple comparisons with $p<0.05$.

RESULTS

General Observations

1-Nitronaphthalene has been shown to produce a pronounced respiratory distress in rodents (Johnson, et al., 1984). This observation held true beginning as early as 1 hour after 1-NN treatment, where exaggerated chest movements were displayed. Six hours after 1-NN treatment, the symptoms included a wet, raspy breathing. These animals were very lethargic, and displayed none of the evasive responses to being handled that control animals did. By +12 hours the symptoms included pronounced gasping and signs of a fluid discharge around the nostrils. At no time did the animals show significant signs of hypoxia, such as cyanosis of the mucus membranes, and no animals died due to drug treatments.

Morphological Evaluation

Untreated Control Rats

Terminal airway epithelium from untreated controls displayed morphology that was consistent with observations described by Breeze & Wheeldon (1977), as seen in Figure 1. The Clara cells appeared as columnar cells with rounded apical surfaces that were centrally located, oval in shape and indented. The chromatin was fine and evenly dispersed throughout the nucleus. Small mitochondria were scattered throughout the cytoplasm along with electron dense granules that were located primarily in the apical region of the cell. The Clara cells also contained an abundant amount of agranular and granular endoplasmic reticulum as compared with other epithelial cells.

The surrounding ciliated cells appeared to have normal morphology as described by Breeze & Wheeldon (1977). The cells had a cuboidal appearance roughly half the size of the columnar Clara cells. The ciliated epithelium had an overall electron-dense cytoplasm, although small mitochondria and endoplasmic reticulum could be seen throughout. The nuclei were centrally located with fine, evenly dispersed chromatin. Abundant cilia was present and attached at apical basal bodies. Lateral tight junctions between the Clara cells and ciliated cells were readily observable. The basal lamina consisted of tightly packed bundles of collagen and irregular bundles of smooth muscle and elastic connective tissue.

Toluidine blue sections showed a simple epithelium with columnar Clara and cuboidal ciliated cells present, as seen in Figure 2. This epithelium was attached to the basement membrane, and there did not appear to be any debris in the airway. Blood vessels proximal to the airway did not show an edematous region around the vessel or an apparent efflux of cells, and the alveolar air spaces had a normal appearance.

1-Nitronaphthalene Treated Rats

In all of the following groups, most characteristics evaluated displayed minimal changes between animals examined at +6 hours compared to +12 hours. When significant differences are noted they will be followed by a notation of the time.

There was a marked difference between morphological profiles of untreated controls and 1-NN treated animals as seen in Figure 3. Electron micrographs showed patchy sloughing of epithelial cells from the majority of the distal airways. The sloughed cells that were still present in the airways consisted of single cells that were usually Clara cells, or groups of 2-5 cells that were combinations of Clara and ciliated cells joined at lateral tight junctions. Cells that were free in the airways tended to

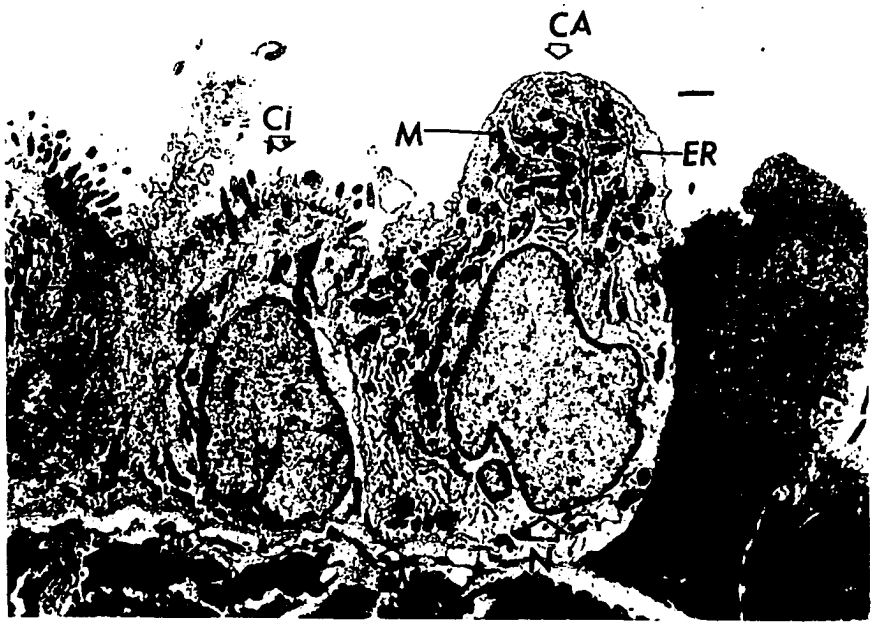


Figure 1. Electron Micrograph of Distal Airway Epithelium From an Untreated Rat.

In this micrograph, observe the columnar Clara (CA) cells and cuboidal ciliated cells (CL) attached to the basement membrane (BM). Also, notice the fine chromatin in the nuclei (N), the well defined mitochondria (M) and endoplasmic reticulum (ER). The bar is 1 micron.

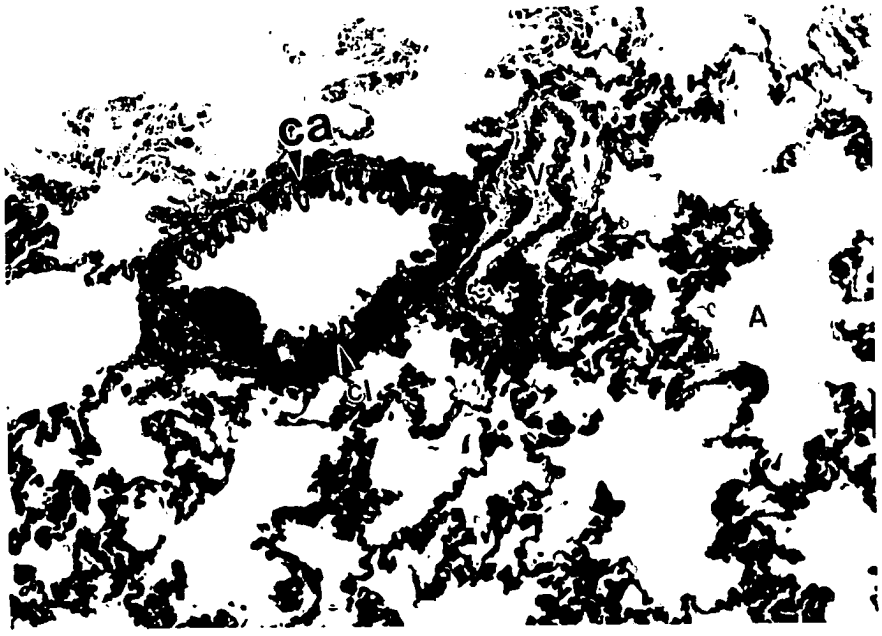


Figure 2. Photomicrograph of Distal Airway Epithelium From an Untreated Rat.

In this micrograph, observe the Clara (CA) and ciliated cells (CL) on the basement membrane. Also observe the alveolar air spaces (A), and proximal blood vessel (V) with no swelling or fluid accumulation visible.

retain the integrity of their plasma membranes evaluated by the absence of cytoplasmic components outside the cell. The most prominent feature of the adherent epithelium was that the once columnar Clara cells now had a low cuboidal shape. These cells showed hydropic changes in the form of dilation of the endoplasmic reticulum indicated by the presence of hydropic vacuoles and vesicles. The average Clara cell had an occasional hydropic vacuole, with many more vesicles seen throughout the cell. Many of the mitochondria were enlarged. There was a reduction in the number of granules present compared to untreated controls. The nuclei displayed clumping of chromatin along the periphery of the nuclear membrane six hours after 1-NN treatment, and by twelve hours many of the cells had a dilated nuclear membrane.

The ciliated cells also presented an altered profile six hours after treatment with 1-NN; which increased in severity after twelve hours. The cells showed hydropic changes in the form of vacuoles, but this was not as severe as the adjacent Clara cells. At both time points, many ciliated cells had a loss of cilia with only the basal bodies remaining. A number of ciliated cells were also sloughed from the basement membrane. While some of these cells were free, the majority were attached to Clara cells at lateral tight junctions.

The basal lamina exhibited an expanded appearance compared to untreated controls. There was an accumulation of fluid in the interstitum, as evidenced by the separation of the collagen bundles. There was also an increase in the cellularity of the interstitum that did not seem to increase significantly between +6 and +12 hours. Electron micrographs showed these cells to be neutrophilic and agranular leukocytes.

Toluidine blue sections showed airways containing a patchy sloughing of epithelial cells. The cells appeared highly vacuolated and free cells were present in the lumen. Epithelial damage was present in all bronchioles observed regardless of size or

cell type present, as seen in Figure 4. The basal lamina had an accumulation of fluid, and blood vessels proximal to the airways were also edematous with a cellular accumulation around them. The air spaces were enlarged compared to untreated controls.

Chloramphenicol With 1-NN Treated Rats

Animals treated with 100 mg/kg chloramphenicol one hour prior to treatment with 100mg/kg 1-NN i.p. showed a reduction in intracellular damage compared to animals treated with 1-NN only, as seen in Figure 5. The most prominent feature was that the Clara cells retained their columnar shape. The cells were adherent to the basement membrane and there was no change in the morphology of the ciliated cells. Some cells displayed a hydropic profile consisting of a slight accumulation of vacuoles and vesicles in the cytoplasm. While this feature was not common, it was not present in the untreated controls nor animals treated with chloramphenicol only, and was therefore attributed to be an effect of 1-NN. The basal lamina displayed an interstitial fluid accumulation in half of the micrographs, but was not as prominent as in animals treated with 1-NN only.

Toluidine blue sections showed bronchioles that appeared similar to untreated controls with the Clara cells retaining their columnar appearance, as seen in Figure 6. Tissue surrounding blood vessels proximal to the airways displayed an interstitial edema that closely resembled animals treated with 1-NN. A similar accumulation of neutrophils and lymphocytes was present in the interstitum. The alveolar air spaces were enlarged as compared with untreated controls. At +12 hours many of the blood vessels were surrounded with an opaque exudate in contrast to the clear transudate seen in the six hour samples.

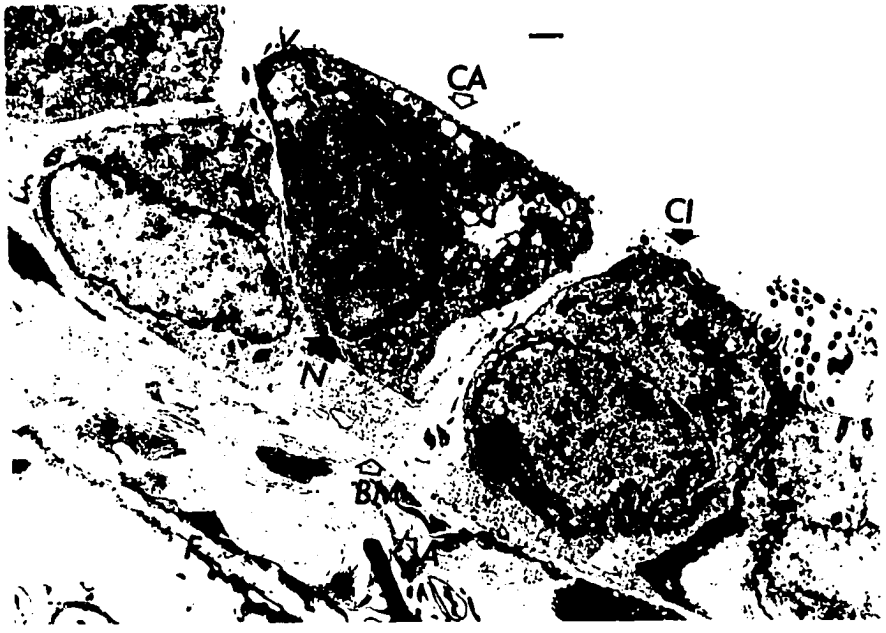


Figure 3. Electron Micrograph of Distal Airway Epithelium From a Rat 12 Hours After Treatment With 1-Nitronaphthalene.

In this figure, note the Clara cells (CA) sloughing from the basement membrane (BM). The cells have condensed chromatin in the nucleus (N) and hydropic vacuole formation (V). The ciliated cells (CL) show less extensive damage, although in some cases. only the basal bodies (arrowheads) of the cilia are visible. Also, note the fluid accumulation in the interstitial space (F). An artifact (A) is also present in this micrograph. The bar is 1 micron.

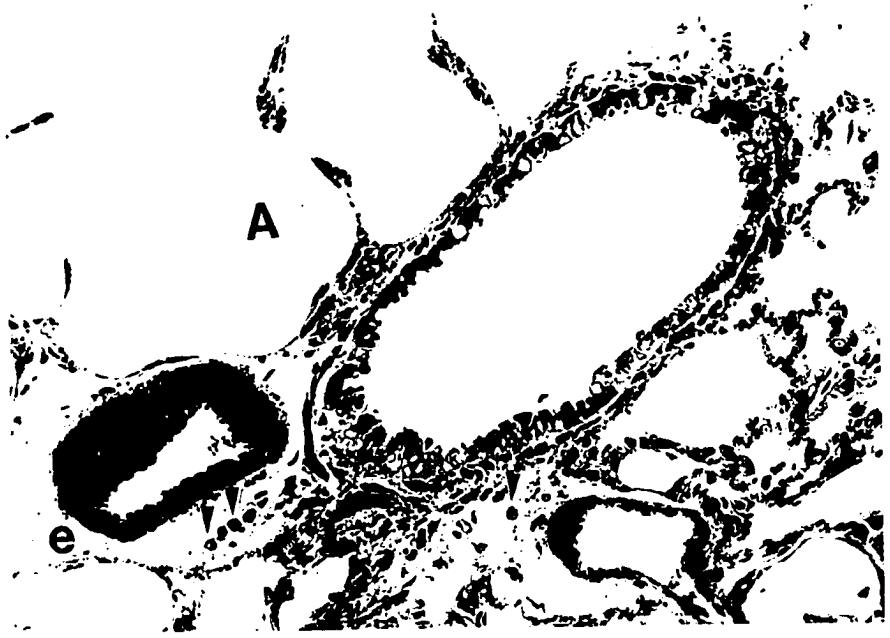


Figure 4. Photomicrograph of Distal Airway Epithelium From a Rat 12 Hours After Treatment With 1-Nitronaphthalene.

In this micrograph, observe the flattened appearance of the airway epithelium, and the perivascular edema (E) with cellular infiltration (arrowheads). Swelling of the alveolar air spaces is also visible (A).

Chloramphenicol Control Rats

Animals treated with 100mg/kg chloramphenicol showed no changes as compared with untreated controls except for the following: There appeared to be a prominent blebbing from the apical portion of many Clara cells, as seen in Figure 7. The bleb consisted of a cytoplasmic outpouching filled with a fine electron dense material. There were no characteristic Clara cell granules present in the blebs. This morphology was not present in animals pretreated with chloramphenicol prior to 1-NN or untreated controls. The material present in the blebs was described by Breeze & Wheeldon (1977) as disintegrating smooth endoplasmic reticulum.

Ciliated cells showed no changes in morphology as compared with untreated controls.

Toluidine blue sections showed the normally round apical surfaces of many of the Clara cells appearing as irregularly shaped, elongated protrusions as seen in Figure 8. Tissue surrounding blood vessels proximal to the distal bronchioles showed little to no edema or neutrophil emigration. The alveolar air spaces did not show enlargement compared to untreated controls.

Cyclophosphamide With 1-NN Treated Rats

Except for the following nuclear changes, animals treated with cyclophosphamide and 1-NN did not show any significant changes compared to animals treated with 1-NN only, as seen in Figure 9. Many Clara cells displayed a cuboidal appearance in contrast to the columnar shape seen in untreated controls. Epithelial sloughing was present and involved both ciliated and Clara cells. The nuclei were pyknotic with chromatin that was much more electron dense than at

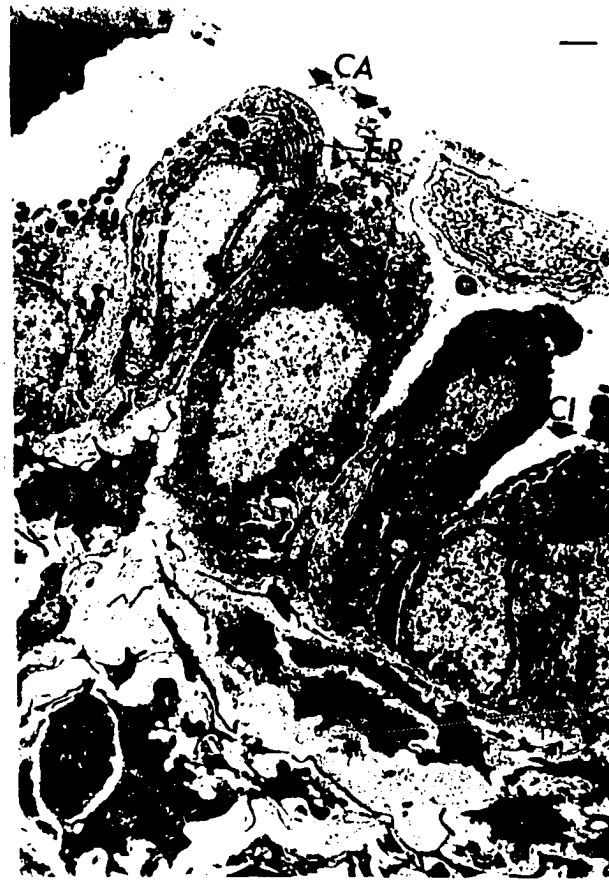


Figure 5. Electron Micrograph of Distal Airway Epithelium From a Rat 12 Hours After 1-Nitronaphthalene Treatment With Chloramphenicol Pretreatment.

In this figure, note the columnar appearance of the Clara cells (CA) and the well defined endoplasmic reticulum (ER). There is a lack of toxicity in the Clara cells and ciliated cells (CL) compared to 1-NN controls in Figure 3. Note that all of the cells are attached to the basement membrane (BM). Also, note cellular accumulation in the interstitum (arrowheads). The bar is one micron.

Figure 6. Photomicrograph of Distal Airway Epithelium From a Rat 12 Hours After 1-Nitronaphthalene Treatment With Chloramphenicol Pretreatment.

In this micrograph, observe the airway epithelium which closely resembles the untreated control. Also, observe the perivascular edema (E), cellular infiltration (I) and enlargement of the alveolar air spaces (A).

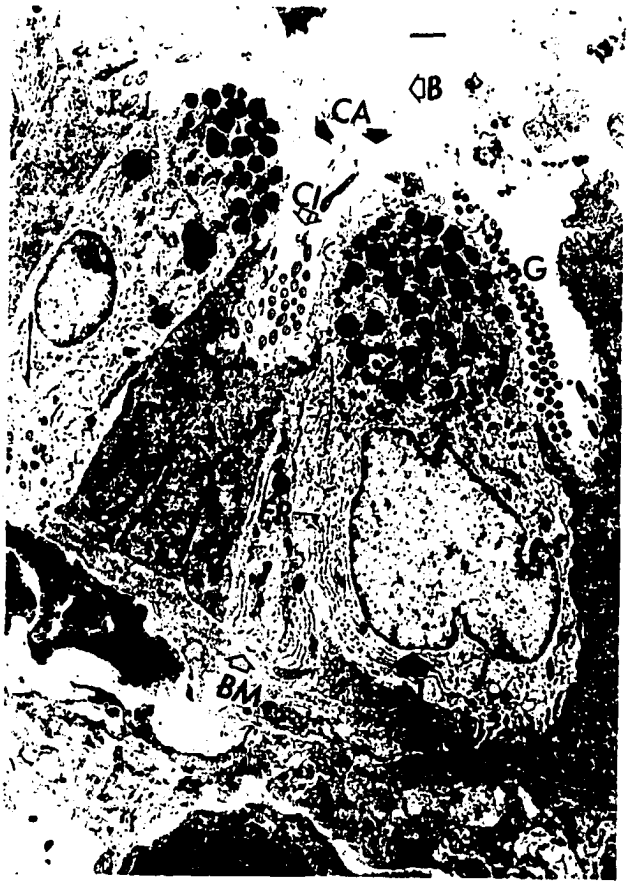


Figure 7. Electron Micrograph of Distal Airway Epithelium From a Rat Treated With Chloramphenicol.

In this micrograph, notice the Clara (CA) and ciliated (CL) cells do not show a morphological difference compared to untreated controls. These cells have well defined granules (G), endoplasmic reticulum (ER) and nuclei (N). All of the cells are attached to the basement membrane (BM). Also, note the bleb (B) from the Clara cell which was specific to this group. The bar is one micron.

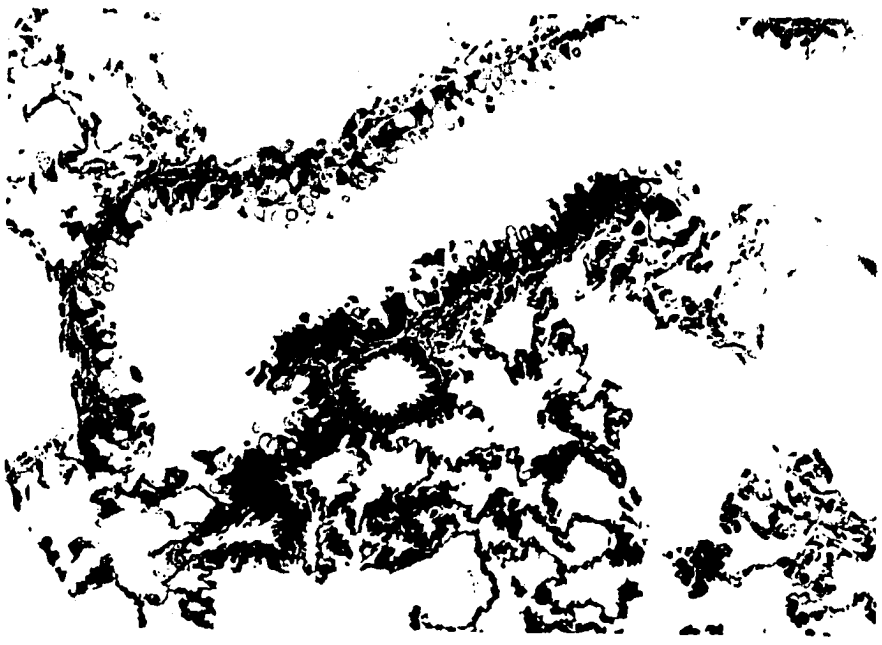


Figure 8. Photomicrograph of Distal Airway Epithelium From a Rat Treated With Chloramphenicol.

In this micrograph, observe the airway epithelium, which resembles the untreated control. Also, observe the lack of perivascular edema, cell infiltration and alveolar enlargement compared to the group pretreated with chloramphenicol prior to 1-NN.

corresponding time points in animals treated with 1-NN only. Many of the nuclei were accumulating fluid and some were beginning to show early signs of karyorrhexis. Many Clara cells had dilated nuclear membranes. Mitochondria were swollen and there was a loss of granule contents. Hydropic vacuole and vesicle formation observed was consistent with 1-NN treated animals.

Ciliated cells showed damage similar to that seen in animals treated with 1-NN only. There was a loss of cilia, and the cells in the twelve hour groups showed slight hydropic changes. Many of the ciliated cells were sloughed from the basement membrane; although most of these cells were still attached to Clara cells at lateral tight junctions.

Toluidine blue sections showed a morphology similar to 1-NN controls as seen in Figure 10. The Clara cells were flattened and sloughed from the basement membrane. Blood vessels proximal to the airways showed edema with an accumulation of cells in the interstitum.

Cyclophosphamide Control Rats

There were no significant morphological changes observed between Cyclophosphamide controls and untreated control rats at the electron level, as seen in Figure 11.

Toluidine blue sections showed normal airway epithelium, as seen in Figure 12. The tissue surrounding the blood vessels displayed no edema, and the alveolar air spaces did not appear enlarged compared to untreated controls.

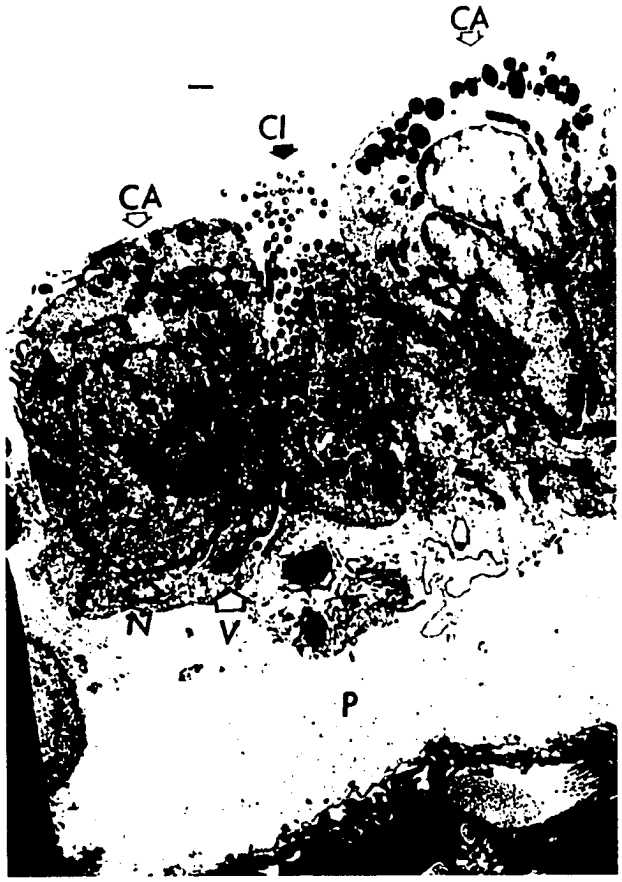


Figure 9. Electron Micrograph of Distal Airway Epithelium From a Rat 6 Hours After 1-Nitronaphthalene Treatment With Cyclophosphamide Pretreatment.

In this micrograph, notice the Clara (CA) and ciliated (CL) cells sloughed from the basement membrane (BM) and the are of protein-containing fluid (P) between the two. Note the hydropic vacuole (V) formation, and the fluid accumulation in the nuclei (N). There was also an accumulation of fluid (F) in the interstitum. The bar is 1 micron.

Figure 10. **Photomicrograph of Distal Airway Epithelium From a Rat 12 Hours After 1-Nitronaphthalene Treatment With Cyclophosphamide Pretreatment.**

In this micrograph, observe the sloughed airway epithelium (E), perivascular edema (D), cell infiltration (C), and alveolar enlargement (A).

Figure 11. Electron Micrograph of Distal Airway Epithelium From a Rat Treated With Cyclophosphamide.

In this figure, note the appearance of the Clara (CA) and ciliated (CL) cells, which closely resemble the untreated controls. The cells have well defined endoplasmic reticulum (ER), electron dense granules (G), and are attached to the basement membrane (BM). Also, note the fine, evenly distributed chromatin in the nuclei (N). The bar is one micron.

Figure 12. Photomicrograph of Distal Airway Epithelium From a Rat Treated With Cyclophosphamide.

In this micrograph, observe the close appearance of the airway epithelium to the untreated control. There is no perivascular edema, alveolar enlargement or cellular infiltration.

Biochemical Analysis

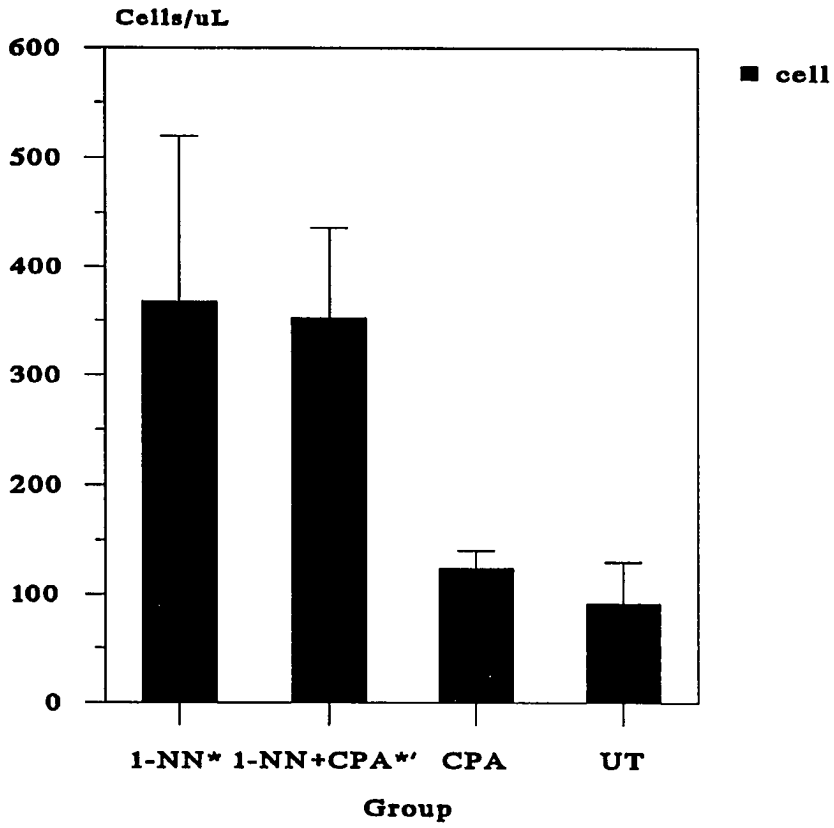
Cell Counts

In Figures 13-20, bars represent the average value for the parameter being examined, while error bars represent the standard deviation.

An influx of cells into BAL fluid is indicative of an inflammatory response. Figure 13 shows the average total cell counts for animals treated with chloramphenicol. There was a significant increase in cells found in BAL fluid between the 1-NN control and the chloramphenicol pretreated groups compared to the untreated control. There was also a significant increase between the chloramphenicol pretreated group and the chloramphenicol control. A differential analysis of cells recovered from BAL fluid did not produce conclusive results due to poor readability of the slides.

Figure 14 shows the average cell counts for groups treated with cyclophosphamide. There was a significant increase between the 1-NN control and the cyclophosphamide pretreated groups compared to the untreated control.

Table 3 shows the leukocyte counts from all groups. There was a significant decrease between animals treated with cyclophosphamide and the untreated control, regardless of treatment with 1-NN. Table 4 shows the differential white blood counts from all groups. This is presented as % neutrophils and % lymphocytes. Since the combination of eosinophils, basophils and monocytes totaled less then 6% of the total differential count, their numbers are not presented. There was a significant decrease in neutrophil content between groups treated with cyclophosphamide compared to the untreated control, regardless of treatment with 1-NN.



Legend. * Significant difference compared to untreated control
' Significant difference compared to treated control
UT = untreated ontrol, CPA = chloramphenicol

Figure 13. Average Cell Count From BAL Fluid of Chloramphenicol Pretreated Rats.

Protein Analysis

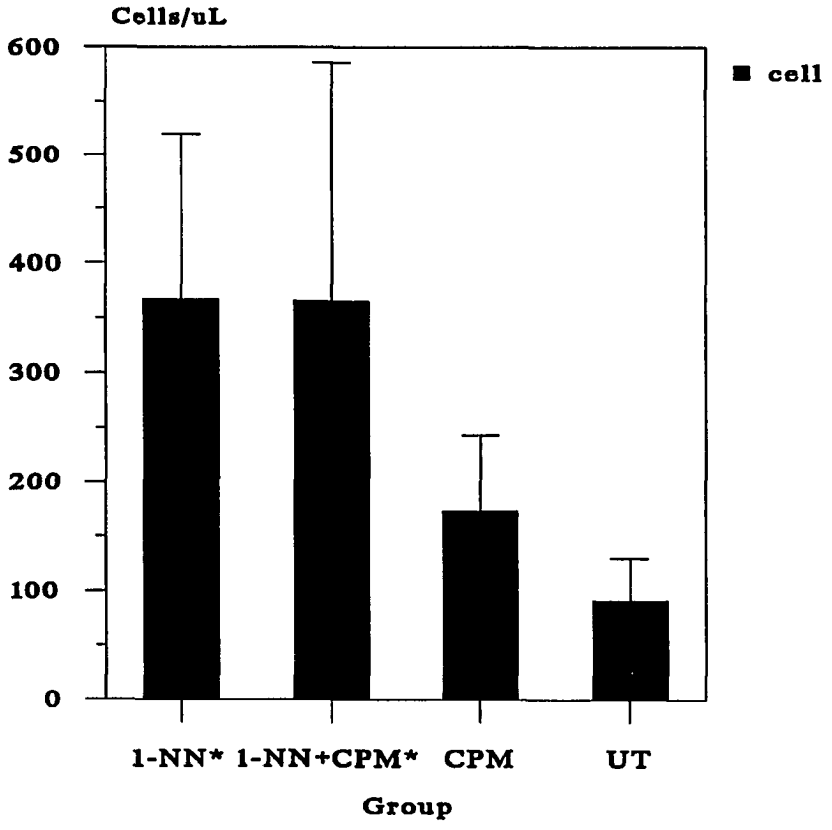
An accumulation of protein in BAL fluid is another indicator of inflammation. Figure 15 shows the average protein content from BAL fluid of animals treated with chloramphenicol. There was a significant increase between 1-NN controls and chloramphenicol pretreated groups compared to the untreated control. There was also a significant difference between the chloramphenicol pretreated group and the chloramphenicol control.

Figure 16 shows the protein content of BAL fluid from animals receiving cyclophosphamide. There was not a significant increase between any of the groups.

Myeloperoxidase Analysis

Myeloperoxidase is an enzyme secreted from neutrophils and macrophages in response to inflammatory stimuli. Tissue myeloperoxidase activity was measured to determine whether there was an efflux of neutrophils from the vasculature into the extracellular space. Figure 17 shows the average tissue myeloperoxidase activity from animals treated with chloramphenicol. There was a significant increase in myeloperoxidase activity in 1-NN controls and chloramphenicol pretreated groups compared to the untreated control. There was also a significant increase between the chloramphenicol pretreated group compared to the chloramphenicol control.

Figure 18 shows the average tissue myeloperoxidase activity from animals treated with cyclophosphamide. There was a significant difference between the 1-NN control and the untreated control. There was also a significant difference between the 1-NN control and the cyclophosphamide pretreated group.



Legend. * Significant difference compared to untreated control.
UT = untreated control, CPM = cyclophosphamide

Figure 14. Average Cell Count From BAL Fluid of Cyclophosphamide Pretreated Rats.

Table 3

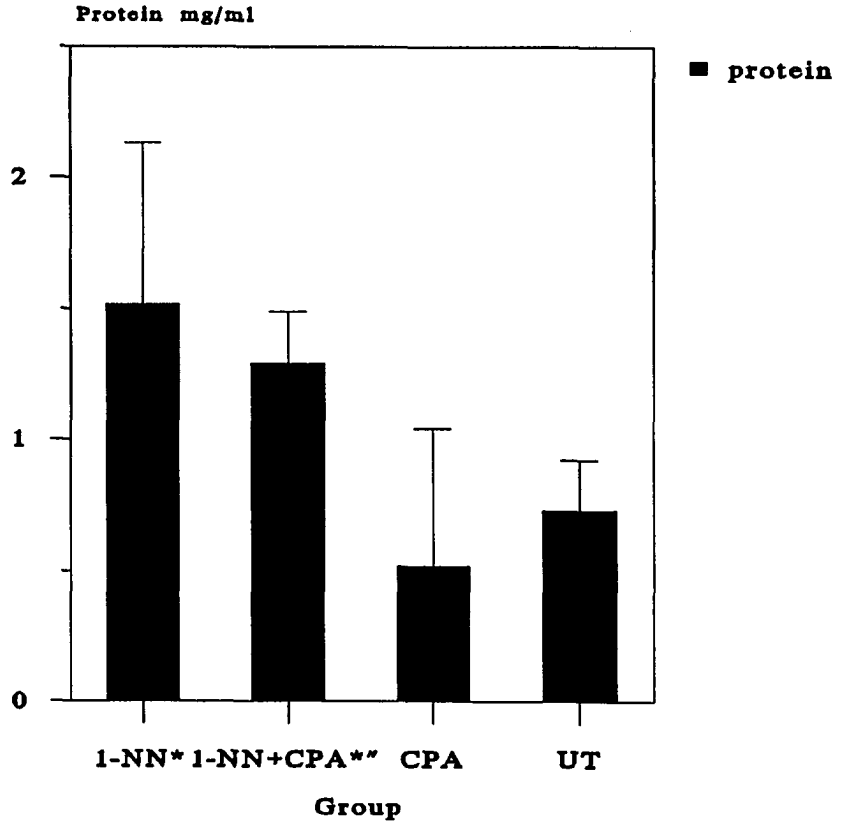
Average White Blood Cell Counts

		White Count*	
		Treatment Time	
Group	Treatment	+6 Hours	+12 Hours
1	1-NN	4,767 +/- 2,702	4,791 +/- 2,790
2	1-NN and CPA	5,125 +/- 1,530	5,443 +/- 1,366
3	1-NN and CPM	1,187 +/- 300	1,050 +/- 247
4	CPA	9,641 +/- 1,661	6,833 +/- 1,502
5	CPM	625 +/- 163	791 +/- 76
6	Untreated Control	7,865 +/- 2,061	7,865 +/- 2,061

* Represented as cells/μL +/- standard deviation.
CPA = chloramphenicol, CPM = cyclophosphamide

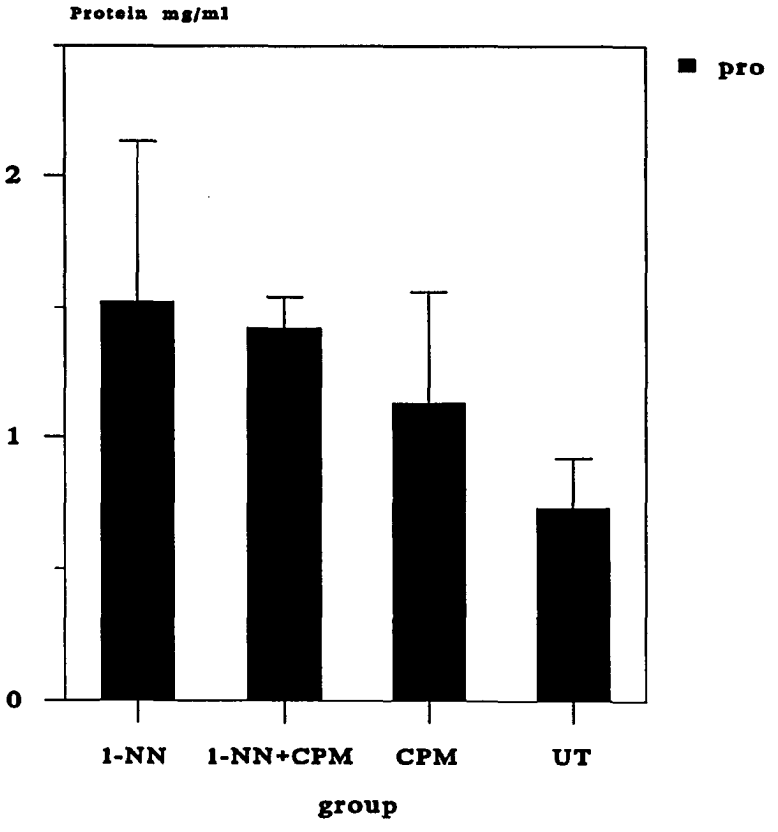
Lactate Dehydrogenase Analysis

Lactate Dehydrogenase is an intracellular enzyme that is commonly used as a marker of cellular damage or membrane leakiness. The average lactate dehydrogenase activity from BAL fluid in groups treated with chloramphenicol is seen in Figure 19 while the average lactate dehydrogenase activity in groups treated with cyclophosphamide is seen in Figure 20. There was not a significant change between any of the groups.



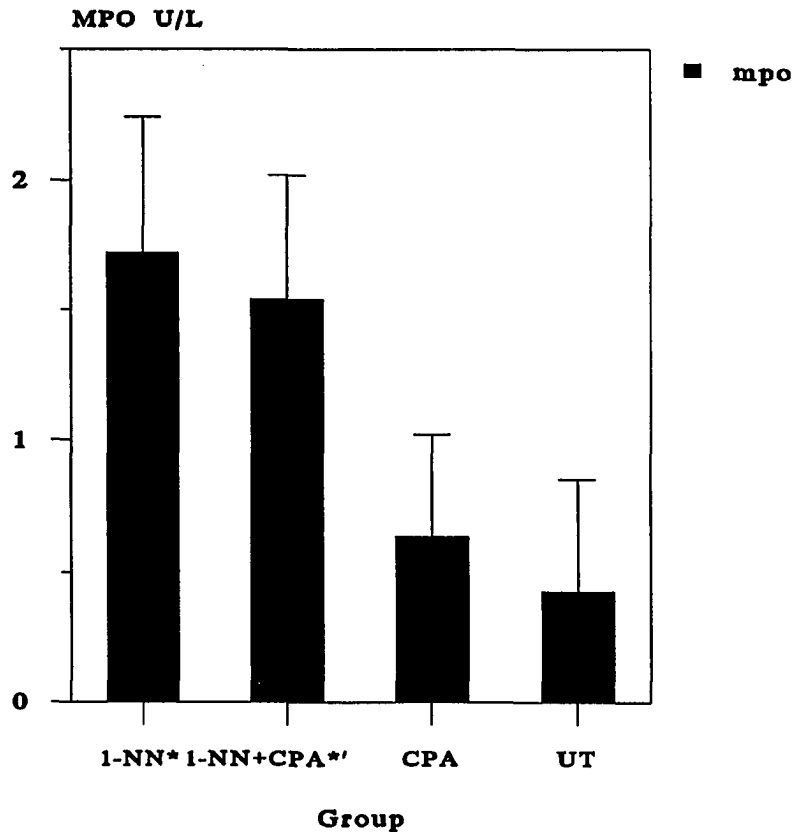
Legend. * Significant difference compared to untreated control.
" Significant difference compared to chloramphenicol control
UT = untreated control, CPA = chloramphenicol

Figure 15. Average Protein Content of BAL Fluid From Chloramphenicol Pretreated Rats.



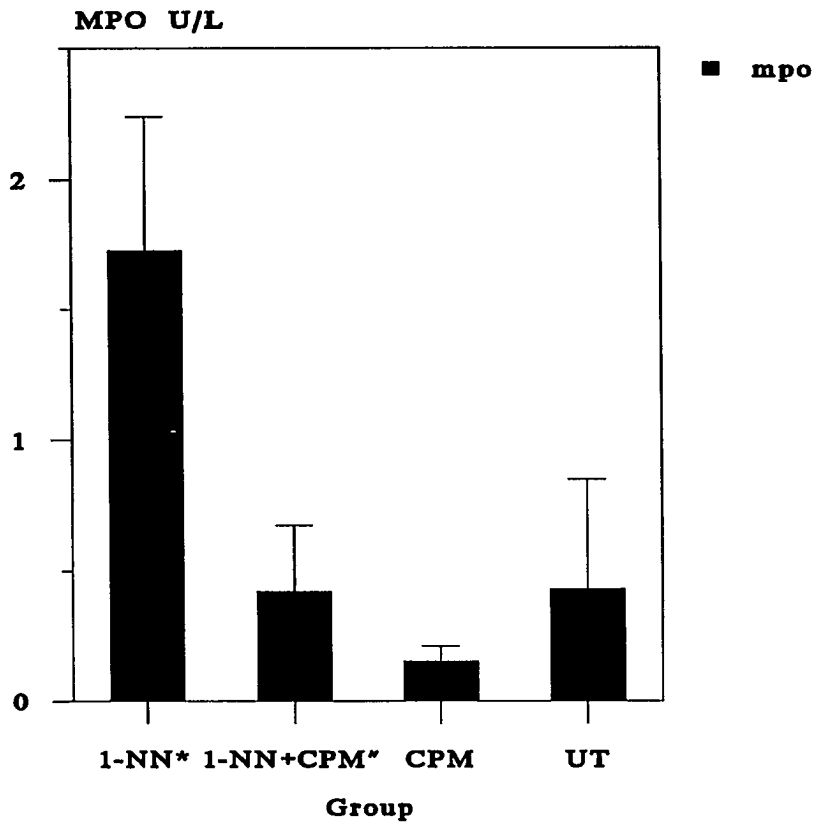
Legend. CPM = cyclophosphamide, UT = Untreated Control

Figure 16. Average Protein Content From BAL Fluid From Cyclophosphamide Pretreated Rats.



Legend. * Significant difference compared to untreated control.
' Significant difference compared to treated control.
UT = untreated control, CPA = chloramphenicol
MPO = myeloperoxidase

Figure 17. Average Tissue Myeloperoxidase Activity From Chloramphenicol Pretreated Rats.



Legend. * Significant difference compared to untreated control.
" Significant difference compared to 1-NN control.
UT = untreated control, CPM = cyclophosphamide
MPO = myeloperoxidase

Figure 18. Average Tissue Myeloperoxidase Activity From Cyclophosphamide Pretreated Rats.

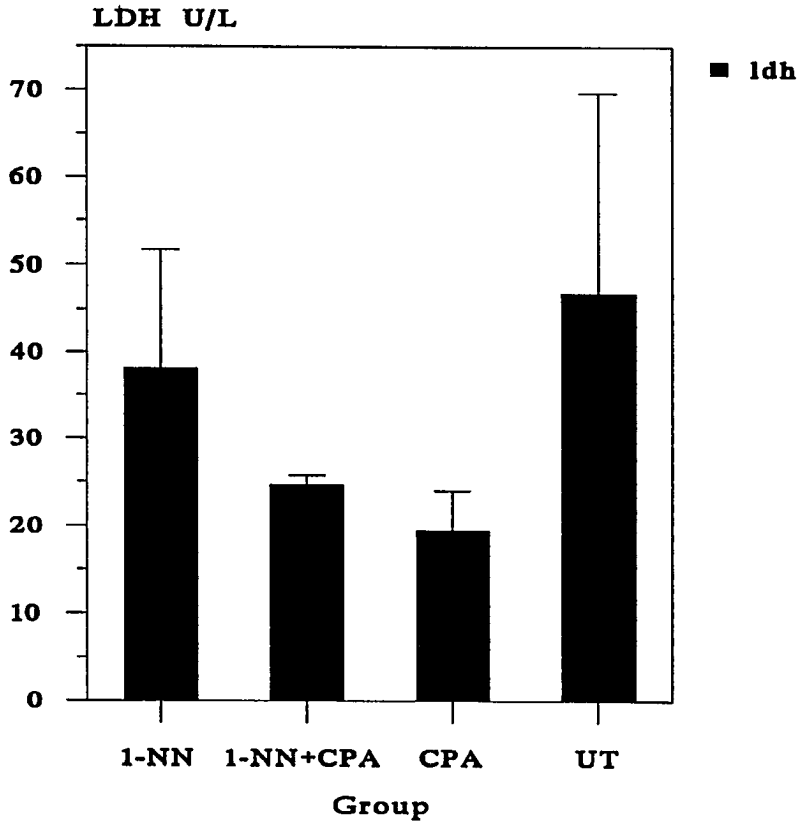
Table 4

Average Differential White Blood Cell Counts

	% of Cells*			
	Treatment Time			
Group	+6 Hours		+12 Hours	
	%Neutro	%Lymph	%Neutro	%Lymph
1	27	70	30 +/- 2	66 +/- 1
2	33 +/- 12	64 +/- 12	34 +/- 7	63 +/- 9
3	6 +/- 0.7	88 +/- 0.1	7 +/- 2	88 +/- 2
4	49 +/- 8	46 +/- 8	47 +/- 14	48 +/- 15
5	5 +/- 2	88 +/- 1	5 +/- 1	89 +/- 0.9
6	27 +/- 7	70 +/- 7	27 +/- 7	70 +/- 7

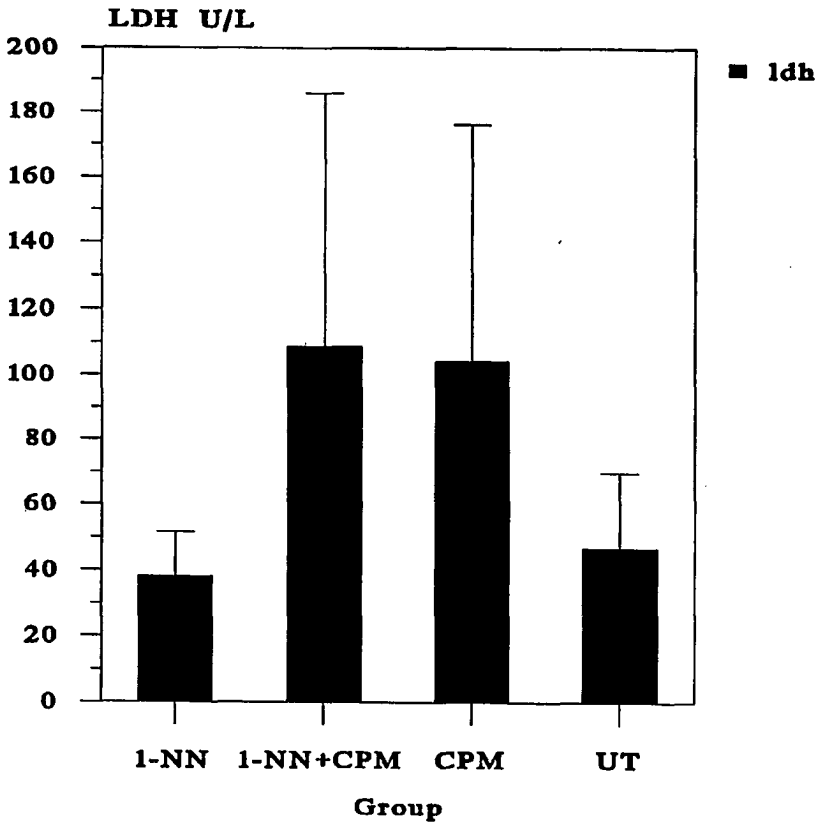
*Represented as average % of this cell type +/- the standard deviation.

Neutro = Neutrophil, Lymph = Lymphocyte



Legend. UT = untreated control, CPA = chloramphenicol
LDH = lactate dehydrogenase

Figure 19. Average Lactate Dehydrogenase Activity From BAL Fluid of Chloramphenicol Pretreated Rats.



Legend. UT = untreated control, CPM = cyclophosphamide
LDH = lactate dehydrogenase

Figure 20. Average Lactate Dehydrogenase Activity From BAL Fluid of Cyclophosphamide Pretreated Rats.

DISCUSSION

The purpose of this study was to determine if inhibition of cytochrome P-450 or neutrophil depletion could modulate the pneumotoxicity caused by 1-NN. Assuming that 1-NN metabolites are cytotoxic, inhibition of their production would be expected to decrease the extent of intracellular damage associated with 1-NN toxicity; while depletion of neutrophils would remove the majority of cytotoxic products of inflammatory cell infiltration.

When drugs are used to assess the mechanism of action of a toxic compound, several criteria must be met. The drug should be specific for the process that is to be examined, and relatively non-toxic. If the drug has unwanted effects, they should not interfere with the scope of the investigation. It is preferable to know the mechanism of action so that the possibility of cross-reactivity can be eliminated. In the case of chloramphenicol, these criteria are met fairly well. This compound covalently binds in the active site of several cytochrome P-450 isozymes (Halpert, 1981) with no evidence of cross-reactivity with other enzymes or the heme moiety of P-450. This is an important consideration, since heme is present in many enzymes, and reactions with it can interfere with other systems. While chloramphenicol does cause blood dyscrasias, such as neutropenia and thrombocytopenia in a clinical setting, this is not likely to be a factor in an acute experiment.

Chloramphenicol functions as an anti-bacterial agent by inhibiting protein synthesis, which could pose questions regarding the turnover of P-450 and other enzymes. Chloramphenicol was shown to distinguish between isozymes of P-450 by the use of specific inducing agents (Halpert et al., 1985), which indicates that the initial

inhibitory action of this compound is selective, and not due to inhibition of *de novo* protein synthesis. Also, the normal half-life for P-450 IIB1 is 19 hours (Gasser, Hauri, & Meyer, 1982), which indicates that unless there is specific induction of the isozyme, less then 50% of the existing P-450 would be replaced by the 12 hour time point used in this experiment.

The previously mentioned criteria does not apply well to cyclophosphamide. The task of granulocyte depletion is not an easy one. There are two ways in which granulocytes can be depleted. The first is physical depletion of the cells, which is commonly done using cytotoxic agents that prevent the division of granulocytic precursors in the bone marrow. Cyclophosphamide along with many other chemotherapeutic agents fall into this category. The primary fault of these compounds is their toxicity, which in many cases is directed towards the lung. The second type of depletion is functional, where there is no physical removal of cells, but some aspect of their function is altered. Included in this group is an enormous variety of drugs including antimetabolites, steroidal anti-inflammatories and antibodies to endothelial adhesion molecules. The latter tends to be cost prohibitive, while the former tend to either be toxic or have a myriad of effects that would complicate a study.

Cyclophosphamide was chosen for several reasons: While it is toxic, cyclophosphamide has been thoroughly investigated (Moore, 1991) and is currently used in clinical settings to deplete granulocytes prior to bone marrow transplants. The toxic effects, which include lung fibrosis (Gould & Miller, 1975) have been determined and the time course for their pathogenesis is well known and is outside of the range of this experiment (Gould & Miller, 1975). Cyclophosphamide is known to be metabolized by PHS enzymes in murine lungs (Smith & Keherer, 1991) and its efficacy

is not affected by inducing agents (Jao, Jusko, & Cohen, 1972). This indicates that while cyclophosphamide is not an ideal drug, it is acceptable for this experiment.

Chloramphenicol was administered prior to treatment with 1-NN to determine if inhibition of P-450 would affect lung toxicity. Both light and electron microscopic evidence clearly shows that cellular damage caused by 1-NN is greatly reduced after pretreatment with chloramphenicol. Sloughing of bronchiolar epithelium was not observed, and the Clara cells retained their columnar shape; which was not seen in groups treated with 1-NN alone. However, chloramphenicol pretreatment did not prevent the inflammatory response associated with 1-NN. This was observed in light and electron micrographs, where fluid accumulation and inflammatory cell emigration was evident. Consistent with 1-NN controls, alveolar air spaces were enlarged, and all animals displayed signs of respiratory distress regardless of chloramphenicol pretreatment.

Biochemical results agreed with morphologic findings regarding the inflammatory response observed in chloramphenicol pretreated groups. In animals receiving 1-NN with or without chloramphenicol pretreatment, there was a significant increase in tissue myeloperoxidase activity, and a significant increase in protein and cell content of BAL fluid compared to untreated controls. There was also a significant increase between cell counts, protein content and tissue myeloperoxidase activity in groups receiving chloramphenicol prior to 1-NN compared to chloramphenicol controls. There was no difference in these parameters between untreated controls and chloramphenicol controls. This indicates that the inflammatory response was not caused by chloramphenicol treatment and was due to 1-NN.

Since the initial signal for the inflammatory response associated with a 1-NN induced lesion is unknown, it is difficult to speculate on why inflammation is present in

chloramphenicol pretreated animals. The most practical theory is that 1-NN does not arrive in the lungs totally unchanged. Since a material injected i.p. makes contact with peritoneal macrophages and the liver, both of which contain P-450 isozymes, it is likely that some 1-NN is metabolized prior to the involvement of Clara cells. The pulmonary endothelium also contains P-450 isozymes and is capable of stimulating an inflammatory response. A second theory is that some aspect of pulmonary P-450 function, either uninhibited IIB1 or another isozyme that is not affected by chloramphenicol, activates a basal amount of 1-NN that is sufficient to stimulate an inflammatory response without causing overt morphological changes.

Aside from an apparent increase in nuclear degeneration, animals pretreated with cyclophosphamide prior to 1-NN did not display observable morphological differences compared to 1-NN controls at either the light or electron level. Intracellular changes included hydropic vacuole formation, mitochondrial swelling and granule loss; while extracellular changes included edema, cellular infiltration and epithelial sloughing. In many cases, there appeared to be greater nuclear damage in animals receiving cyclophosphamide prior to 1-NN than with 1-NN alone. These changes consisted of pyknosis, with the chromatin exhibiting a very electron dense appearance, and fluid accumulation in many of the nuclei. There were no morphological differences between the nuclei of cyclophosphamide controls and untreated controls. There is no explanation for this result in the literature reviewed, but one possibility might be that since cyclophosphamide alkylates DNA, pretreatment prior to 1-NN may cause an increase in the precipitation of chromatin. Epithelial sloughing and loss of columnar shape were also visible in cyclophosphamide pretreated groups. Neutrophils could be involved in epithelial sloughing due to direct attack of the basement membrane by oxidative or enzymatic products. Since cyclophosphamide

pretreated groups displayed an 85% decrease in circulating lymphocytes and no significant change in tissue myeloperoxidase activity, this does not seem likely.

Biochemical analysis did not necessarily support the micrographic evidence regarding the inflammatory response. While there was a significant increase in cell content of the BAL fluid, there was not a significant increase in either tissue myeloperoxidase activity or BAL protein content in animals pretreated with cyclophosphamide prior to 1-NN. Both protein content and myeloperoxidase activity can be readily explained. Myeloperoxidase is a marker of neutrophil emigration, and protein accumulation in tissue during inflammation has been shown to be a neutrophil-dependent process (Wedmere & Williams, 1981). Considering the 82% decrease in blood neutrophil content, it is not surprising that these parameters did not increase.

The increase in BAL cell content in cyclophosphamide pretreated groups was unexpected. A differential analysis of these cells was attempted, but the results were inconclusive. One possibility was that the increase could be due to division of alveolar macrophages and a decrease in macrophage adhesion due the increase in chemotactic stimuli. Another could be migration of lymphocytes from bronchus associated lymphoid tissue into the airways.

Morphological evidence can be used to explain the continued presence of the inflammatory response. Both electron and light micrographs show that the lungs from cyclophosphamide pretreated animals contained mast cells and macrophages. Since these cells are capable of stimulating the primary inflammatory response (Metcalfe, Costa, & Burd, 1992; Adams & Hamilton, 1992) this could explain why cyclophosphamide had no apparent effect on inflammation. Another possibility lies in the pulmonary endothelium. There was no morphological evidence that cyclophosphamide damaged these cells, which are capable of stimulating an

inflammatory response. The morphological and biochemical results show that cyclophosphamide pretreatment does not prevent pulmonary cytotoxicity or an inflammatory response despite an 82% reduction in circulating leukocytes.

Surprisingly, there was not a significant increase in lactate dehydrogenase activity in any of the groups regardless of treatment. An accumulation of lactate dehydrogenase is indicative of cell lysis or an increase in membrane permeability. Considering the extent of epithelial damage observed, a significant increase in lactate dehydrogenase activity was expected. The explanation for this was found in the electron micrographs. While these pictures give little indication of membrane permeability, they do show that few if any of the cells have lost the integrity of their plasma membranes, evaluated by the absence of intracellular components in the airways. Also, the hydropic changes observed in these cells do not seem as severe compared to other studies which used agents that are known to attack membranes, such as carbon tetrachloride, which show severe hydropic changes.

CONCLUSION

The results presented in this study have shown that inhibition of P-450 isozymes by chloramphenicol decreases the pneumotoxicity associated with 1-NN exposure. However, chloramphenicol pretreatment did not prevent the inflammatory response associated with 1-NN exposure evaluated by significant increases in tissue myeloperoxidase activity, and BAL protein and cell content. While the stimulus for the inflammatory response is not known, it seems likely that an alternate pathway produces 1-NN metabolites in an amount that is sufficient to signal an inflammatory response without causing significant morphological changes.

Depletion of neutrophils with cyclophosphamide prior to 1-NN treatment did not prevent the cytotoxicity or inflammatory response associated with 1-NN. This indicates that intracellular damage is not due to involvement with reactive oxygen or enzymatic products from neutrophils, and that the primary inflammatory response, which is characterized by increased vascular permeability, is neutrophil-independent.

Appendix A

IACUC Approval Forms

INVESTIGATOR CERTIFICATION

Title of Project: 1-Nitronaphthalene alteration of lung toxicity by inhibition of Clara cell oxidative function or neutrophil activity.

If any of the above procedures are changed, I will submit a new protocol.

I understand that any failure to comply with the *Animal Welfare Act*, the provisions of the *DPHS Guide for the Care and Use of Laboratory Animals* and requirements set down by the IACUC may result in the suspension of my animal studies.

Steve Green — Biomedical Sciences — 10/11/91
Signature: Principal Investigator — Department — Date

REVIEW BY THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

_____ Disapproved _____ ✓ Approved _____ Approved with the provisions listed below

Provisions or Explanation: Monitor closely - if severe distress give 100% oxygen or terminate.
Apply MB fans in a head.

James A. Swenberg — Oct. 25. 91
IACUC Chairperson — Date

Researcher's Acceptance of Provisions:

_____ _____
Signature: Principal Investigator — Date

_____ _____
IACUC Chairperson Final Approval — Date

Approved IACUC Number 91-10-05

Revised February 12, 1991

WESTERN MICHIGAN UNIVERSITY
INVESTIGATOR IACUC CERTIFICATE

Title of Project: Alteration of 1-Nitronaphthalene Pneumotoxicity by Inhibition of Cytochrome P-450 or Neutrophil Depletion

The information included in this IACUC application is accurate to the best of my knowledge. All personnel listed recognize their responsibility in complying with university policies governing the care and use of animals.

I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. Technicians or students involved have been trained in proper procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project.

If this project is funded by an extramural source, I certify that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal to the funding agency noted above.

Any proposed revisions to or variations from the animal care and use data will be promptly forwarded to the IACUC for approval.

_____ Disapproved ___✓___ Approved _____ Approved with the provisions listed below

Provisions or Explanations:

_____

_____

_____

_____

_____

_____

Leonard Jelinsky
IACUC Chairperson

12-17-92
Date

Acceptance of Provisions

Signature: Principal Investigator/Instructor

12-17-92
Date

_____
IACUC Chairperson Final Approval

_____
Date

Approved IACUC Number 92-12-01

Rev. 3/92

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