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1-NITRONAPHTHALENE CAUSES ULTRASTRUCTURAL CHANGES PRIMARILY
IN NONCILIATED BRONCHIOLAR EPITHELIUM FOLLOWED
BY CILIATED EPITHELIUM IN THE RAT.

by

John-Michael Sauer

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biological Sciences

Western Michigan University
Kalamazoo, Michigan
June 1991

1-NITRONAPHTHALENE CAUSES ULTRASTRUCTURAL CHANGES PRIMARILY
IN NONCILATED BRONCHIOLAR EPITHELIUM FOLLOWED
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John-Michael Sauer, M.S.

Western Michigan University, 1991

This study describes morphological changes in the lung at several time intervals following a single injection of 1-Nitronaphthalene (100 mg/kg intraperitoneal) in male Sprague-Dawley rats using transmission and scanning electron microscopy. Dilatation of the smooth endoplasmic reticulum and mitochondrial distension in the nonciliated bronchiolar (Clara) cells was apparent as early as 1 hour after injection, while adjacent ciliated cell showed minimal changes. At 6 hours, when respiratory distress was apparent in the animals, Clara and ciliated cells exhibited chromatin clumping, loss of mitochondrial cristae and cytoplasmic vacuolization. Also at 6 hours, the interstitial area around the terminal bronchioles was infiltrated with mast cells and neutrophils. By 24 hours, terminal bronchioles were denuded of epithelial cells and both Clara and ciliated cells in larger bronchioles exhibited mitochondrial distension and cytoplasmic vacuolization. These findings support the theory that initial toxicity occurs in Clara cells through the *in situ* formation of reactive intermediates and extrabronchiolar injury results from inflammatory responses.

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John-Michael Sauer

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INTRODUCTION

Statement of the Problem

1-Nitronaphthalene (1-NN), a mutagenic component of diesel exhaust particulates, has been shown to produce acute liver and lung toxicity in rats (Johnson, Riley, & Cornish, 1984). The ultrastructural alterations caused by 1-NN toxicity in the liver have already been described and shown to be localized in the centrilobular hepatocyte (Lehmann, Johnson, & Beuving, 1989). Although 1-NN toxicity in the lung is known to be directed at the distal most bronchioles, the ultrastructural changes associated with 1-NN toxicity in the lung have not been evaluated, nor has the possibility that 1-NN causes cell specific toxicity in the bronchioles.

Purpose of the Study

The purpose of this study was to evaluate the pulmonary lesions caused by a single 100 mg/kg intraperitoneal injection of 1-NN over a 24-hour period in the rat, using light microscopy, scanning electron microscopy and transmission electron microscopy. The time sequence of cellular toxicity was important to establish whether the nonciliated bronchiolar epithelial cell, where high levels of cytochrome P-450 enzymes are located, was indeed the initial site of 1-NN toxicity.

Pulmonary Toxicity

Several compounds have been shown to be efficient pneumo-toxins in rodents, causing specific ultrastructural and biochemical changes in lung cells following systemic administration (Kehrer & Kacew, 1985). Paraquat, for instance, causes ultrastructural alteration of the type I and II alveolar pneumocytes (Sykes, Purchase, & Smith, 1977), and of the endothelium of the pulmonary capillaries (Dearden, Fairshter, Morrison, Wilson, & Brundage, 1982). Changes in vascular endothelial cells of the lung have also been reported in rodents following exposure to Pyrrolizidin alkaloids (Plestina & Stoner, 1972). And, the bronchiolar epithelium is particularly susceptible to chemicals requiring oxidative activation, such as 4-ipomeanol (Doster, Farrell, & Wilson, 1983), 2-methylfuran (Ravindranath, McMennamin, Dees, & Boyd, 1986), naphthalene (Mahvi, Bank, & Harley, 1977), and carbon tetrachloride (Boyd, Statham, & Longo, 1980).

Boyd (1980) introduced three possible mechanisms of activation which a systemically administered toxin could undergo to cause pulmonary toxicity (see Figure 1). Mechanism I depicts a reaction in which an "inert" parent chemical is metabolized *in situ* by the lung and undergoes activation to an ultimate toxin (see Figure 1-I). The ultimate toxin may cause damage to the cell in which it was formed or to other cells in the lung. In mechanism II, a compound which is primarily metabolized in the liver is transported to the lung and causes direct cell toxicity (see Figure 1-II). Mechanism III represents a pathway followed by a compound which undergoes cyclic reduction/oxidation metabolism, and thereby causes toxicity in the lung (see Figure 1-III). The reduction/oxidation cycling within pulmonary cells results in the production of reactive oxygen species and a compromised xenobiotic defense mechanism due to decreased NADPH pools. The relatively high oxygen tension of

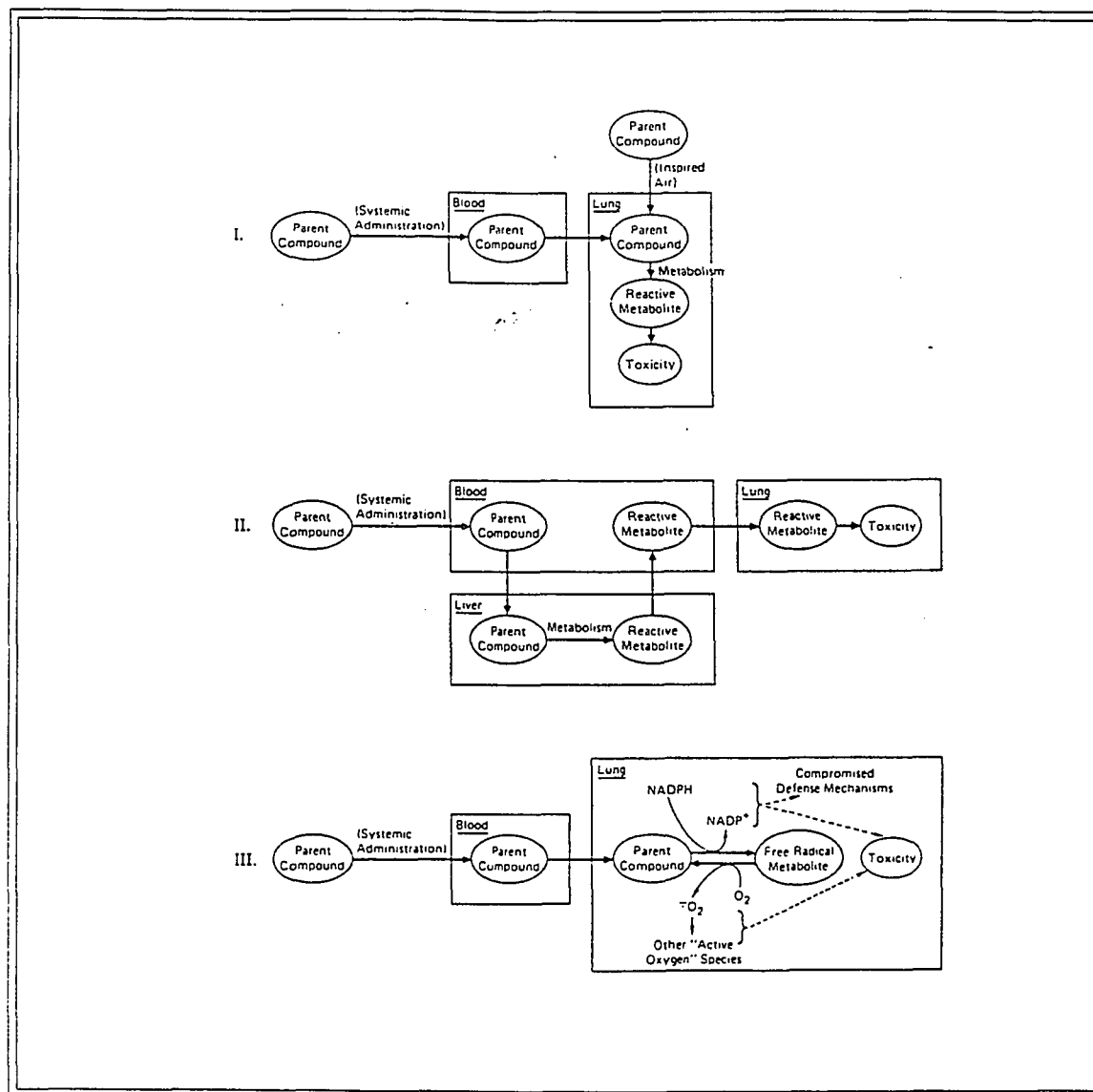


Figure 1. Schematic Representations of Three Potential Mechanisms of Chemical-Induced Lung Toxicity Involving Metabolic Activation.

Source: Boyd, M.R. (1980). Biochemical mechanisms in chemical-induced lung injury: Roles of metabolic activation. In H. P. Witschi (ref), CRC critical reviews in toxicology, Vol. 7 (pp. 103-176). New York: Academic Press

the lung could contribute to its preferential damage by agents acting through mechanism III.

It is apparent that mechanisms I, II, and III are highly simplified representations of early events which could occur in the complex step leading to lung damage by chemical toxins. These mechanisms do not take into account factors such as detoxification pathways and other cellular defense mechanisms, or the target cell's ability to repair itself after toxic assault. Yet interestingly, almost all known chemical toxicants which affect the lung can be grouped into one of these three mechanisms.

The Clara Cell

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 (Breeze & Wheeldon, 1977). The lung's airways are subdivided into various regions. In rat, the regions are named by their size and location in respect to the trachea. Bronchioles can be defined as the distal most branches of the airway, while bronchi are more proximal than bronchioles and are distal to the trachea (see Figure 2). Many of the epithelial cell types are restricted to specific regions of the airway and have specialized functions due to location. The bronchi contain a complex epithelial lining comprised of basal cells, ciliated cells, mucous goblet cells, and a variety of intermediate forms (Jeffery & Reid, 1975). The luminal surface of the bronchioles is lined with only two types of simple cuboidal epithelium consisting of a mixture of ciliated cells and non-ciliated secretory cells (Clara cells), which are not mucous goblet cells (Jeffery & Reid, 1975).

The nonciliated secretory cells, known as Clara cells, are found in the distal bronchioles of both mammalian and avian lungs. These cells are arranged within

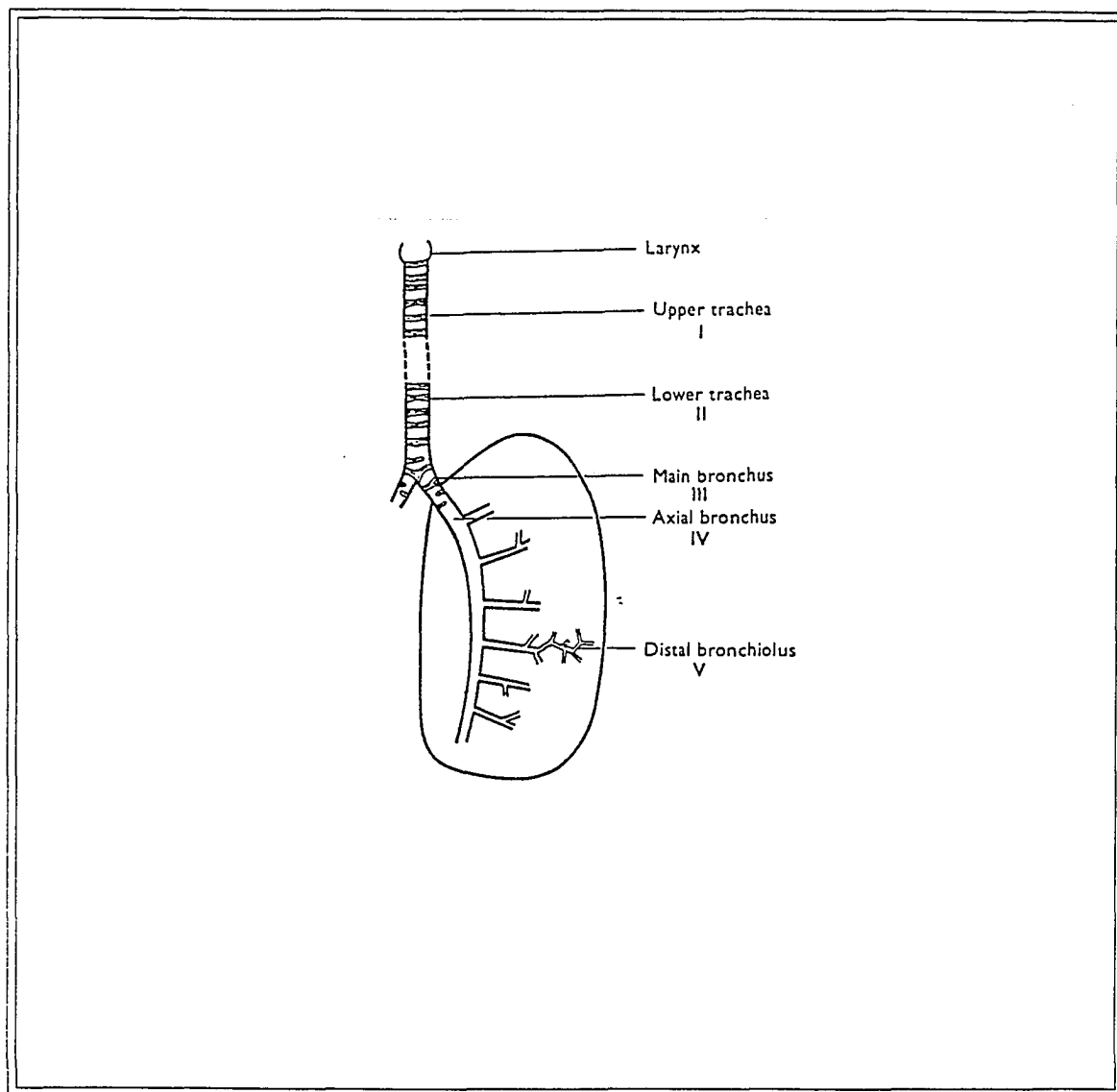


Figure 2. Diagrammatic Representation of Extra- and Intrapulmonary Airways of Rat Left Lung.

Source: Jeffery, P.K. & Reid, L. (1975). New observations of rat airway epithelium: A quantitative and electron microscopic study. Journal Anatomy, 120, 295-320.

bronchioles in linear arrays between the ciliated cells and have unique club-shaped surface projections, known as apicals (Plopper, Mariassy, & Hill, 1980). From these projections are secreted membrane-bound granules of choline-based phospholipid which form a secondary pulmonary layer below, but which are unrelated to alveolar surfactant (Stinson & 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 nonciliated bronchiolar epithelium (Smith, Heath & Moosavi, 1974).

The internal ultrastructure of the rat Clara cell was described by Plopper et al. (1980) as follows. An irregular shaped nuclei is localized in the basal one-third of the cell. Cytoplasm in the apical and lateral perinuclear region is filled mostly with smooth endoplasmic reticulum. Cylindrical mitochondria with abundant cristae and rough endoplasmic reticulum are also scattered throughout the cytoplasm. Round osmophilic granules, ranging in diameter from 0.3 to 0.6 microns, are localized in the apical cytoplasm and are enclosed by a single limiting membrane. Cytoplasmic glycogen is usually not observed.

Aside from being responsible for the production of phospholipid granules, Clara cells contain large reserves of cytochrome P-450-dependent monooxygenase activity in the lung (Boyd, 1977). These enzymes, in some cases, are able to mimic and even exceed the xenobiotic activation abilities associated with hepatocytes (Boyd, 1980). The high xenobiotic-metabolizing capacity and the high susceptibility of Clara cells to environmental chemicals conceivably could be an important factor in the pathogenesis of small airway disease, a problem of major public health significance.

1-Nitronaphthalene Toxicity

1-NN is a nitro-aromatic component of diesel exhaust particulates that has been shown to produce acute toxicity in the liver and lungs of rats. A single intraperitoneal injection of 100 mg/kg of 1-NN induces liver toxicity characterized by severe mitochondrial swelling and loss of matrix density in centrilobular hepatocytes (Lehmann et al., 1989). Using rat liver supernatant, El-Bayoumy and Hecht (1982) have shown that 1-NN is able to undergo ring oxidation. Based on their mass spectral and chemical properties, dihydrodiols and nitronaphthol isomers were found to be the major end metabolites from the liver supernatant. Concomitant lung toxicity involves the necrosis of bronchiolar epithelial cells and severe respiratory distress (Johnson et al., 1984). The metabolism and toxicity of 1-NN has also been shown to be modulated by pre-treatment with phenobarbital and SKF-525a in the rat (Johnson et al., 1984). *In vitro* studies (Rasmussen, 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.

DESIGN AND METHODOLOGY

Experimental Design

Fifteen male Sprague-Dawley rats (obtained from the Charles Rivers Co., Portage, MI 49008) ranging from 150-200 grams in weight were used as the test system for this study. Each animal received a single 100 mg/kg intraperitoneal injection of 1-NN (purchased from Sigma Chemicals Co., St. Louis Mo, 63178). The animals were observed for clinical signs of toxicity and then sacrificed at 0.16 (10 minutes), 1, 6, and 24 hours post 1-NN exposure. The animals' lung tissues were collected, processed and evaluated using light microscopy, scanning electron microscopy, and transmission electron microscopy. Photomicrographs were taken of 1-NN induced ultrastructural alterations, with specific emphasis on the epithelial cells lining the distal most bronchioles.

Procedures

Animal Husbandry and Dosing

The male Sprague-Dawley rats used in this experiment were housed in groups of three in wire mesh cages in a climate-controlled room with a 12:12 hour light/dark cycle. Food (Ralston-Purina Rodent Chow #5001; St. Louis Mo, 63148) and tap water were provided *ad libitum*. Animal care conditions conformed to present standards and were approved by the Institutional Animal Care and Use Committee at Western Michigan University (see Appendix A).

The treatment and necropsy schedule is shown in Table 1. Animals were randomly assigned to experimental groups and were fasted 24 hours prior to 1-NN administration. Each treatment animal received a single 100 mg/kg intraperitoneal injection of 1-NN in a vehicle of peanut oil, while the control animals received only vehicle. The animals were anesthetized with 35 mg/kg of sodium pentobarbitol and tissues were collected.

Table 1
Necropsy Schedule

Groups	Time of tissue collection post injection			
	10min	1hr	6hr	24hr
Control	-	1 [*]	1	1
1-NN treated	3	3	3	3

* - number of animals in group

Tissue Collection and Preparation

The lung tissues were fixed *in situ* by cardiac perfusion with 3% glutaraldehyde in 0.1M cacodylate buffer, using a modified procedure from Kuhn, Callaway and Askin (1974). The periphery of the inferior lobe of the right lung was cut into eight 1 x 0.3 cm longitudinal blocks, and immersed in the 3% glutaraldehyde buffer for twenty five additional minutes. The tissues were rinsed in 0.1M cacodylate buffer, followed by postfixation with 1% osmium tetroxide in 0.1M cacodylate buffer. The tissues were again rinsed in 0.1M cacodylate buffer and then dehydrated with ascending concentrations of ethyl alcohol into a final concentration of 100%. Four blocks of tissue from each animal

were labeled as transmission samples and cleared with propylene oxide. The transmission samples were then embedded in Polybed 812 (Polyscience, Inc., Warrington, PA 18976) and polymerized in flat embedment molds. The remaining four blocks were labeled scanning samples and dehydrated in hexamethyldisilazane (Polyscience, Inc., Warrington, PA 18976).

Section Preparation and Electron Microscopy

After polymerization, the transmission samples were trimmed and 1 micron sections were cut using a glass knife. The sections were stained with toluidine blue and examined with a light microscope. Special consideration was given to the sample orientation so that transverse bronchiolar section could be obtained from the lung samples. The blocks were retrimmed and 80-100 nanometer ultra-thin sections were prepared using a diamond knife. The sections were collected on 200 mesh, 3.05mm copper grids. Methanolic uranyl acetate (Dawes, 1988) and Reynold's lead citrate (Reynold, 1963) were used to stain the ultra-thin sections prior to observation. The samples were examined with a Siemens 101 Elmiskop electron microscope at an accelerating voltage of 80 kV.

The scanning samples, following dehydration with hexamethyldisilazane, were mounted to aluminum stubs with conductive carbon glue. The samples were then coated with a 100 angstrom thick layer of gold using an E5200C Polaron SEM Autocoater (Polaron Equipment Limited, Watford, CT 37144). The freshly coated tissues were examined with an ISI-DS 130 scanning electron microscope at an accelerating voltage of 19 kV.

Transmission electron microscopy photomicrographs were taken of selected areas which represented the most common ultrastructural alterations observed in all lung tissues for that time period. The same selectivity was used in deciding the areas which

would be micrographed with the scanning electron microscope.

RESULTS

Clinical and Gross Observations

Clinical observations were taken at 0.16 , 1, 6 and 24 hours following 1-NN exposure. Within 6 hours of 1-NN treatment, all animals exhibited severe respiratory distress characterized by labored breathing and severe gasping, as well as mucous deposits around the mouth and external nares. All 1-NN treated animals also displayed morbidity and urogenital staining at 6 and 24 hours. Upon lung dissection of the 6 and 24 hour 1-NN treated animals, focal red-brown consolidated areas of minimal severity were evident in all lobes of the lungs, possibly indicating an acute suppurative inflammation. No clinical or gross changes were apparent in the vehicle control, 0.16 or 1 hour animals.

Light Microscopic Observations

Examination of the 1 micron sections of experimental lung showed characteristic 1-NN induced toxicity in the smaller bronchioles as described by Johnson et al. (1984). Microscopic examination revealed that the majority of lesions occurred in the terminal bronchioles in all 1-NN treated animals, but at later time intervals, lesions were found in alveolar sacs and larger bronchioles. No changes were evident in the 0.16 hour exposure animals as compared to the controls. A general constriction of the smaller bronchioles, as indicated by the bronchiolar folding, was observed at 1 hour and continued to 24 hours post 1-NN exposure.

At 6 hours, in the distal most bronchioles, swelling and cytoplasmic vacuolization was seen in both the Clara and ciliated cells. A number of exfoliated epithelial cells were observed as debris in the lumen of the terminal bronchioles. Severe pneumonitis was also observed with a significant number of inflammatory cells infiltrating the interstitium around the terminal bronchioles. Adhesion of inflammatory cells to endothelium located in pulmonary arteries which run parallel to affected bronchioles was also evident at 6 hours. Interstitial edema was also observed along with the organization of blood in alveolar spaces and bronchioles.

At 24 hours post 1-NN exposure, the terminal bronchioles were almost completely denuded of epithelium and clogged with cellular debris. Intra-alveolar hemorrhages, organization of blood in alveolar spaces and bronchioles, as well as alveolar proteinosis were also evident at 24 hours. Severe interstitial edema was accompanied by a mixed inflammatory response, including activated mast cells, small lymphocytes and neutrophils in both the interstitium around affected bronchioles and alveolar spaces. Endothelium of the pulmonary arteries was severely paved with inflammatory cells, even to a greater degree than the 6 hour animals.

Electron Microscopic Observations

Upon ultrastructural examination of vehicle control samples (see Figures 3 and 4), bronchiolar epithelium appeared similar in structure to that described by Plopper et al. (1980). No ultrastructural alterations were detected in any of the animals in the 0.16 hour treatment group.

Dilation of smooth endoplasmic reticulum and mitochondrial distension of Clara cells were apparent as early as 1 hour after exposure in the terminal bronchioles, and only minor changes were observed in the adjacent ciliated cells

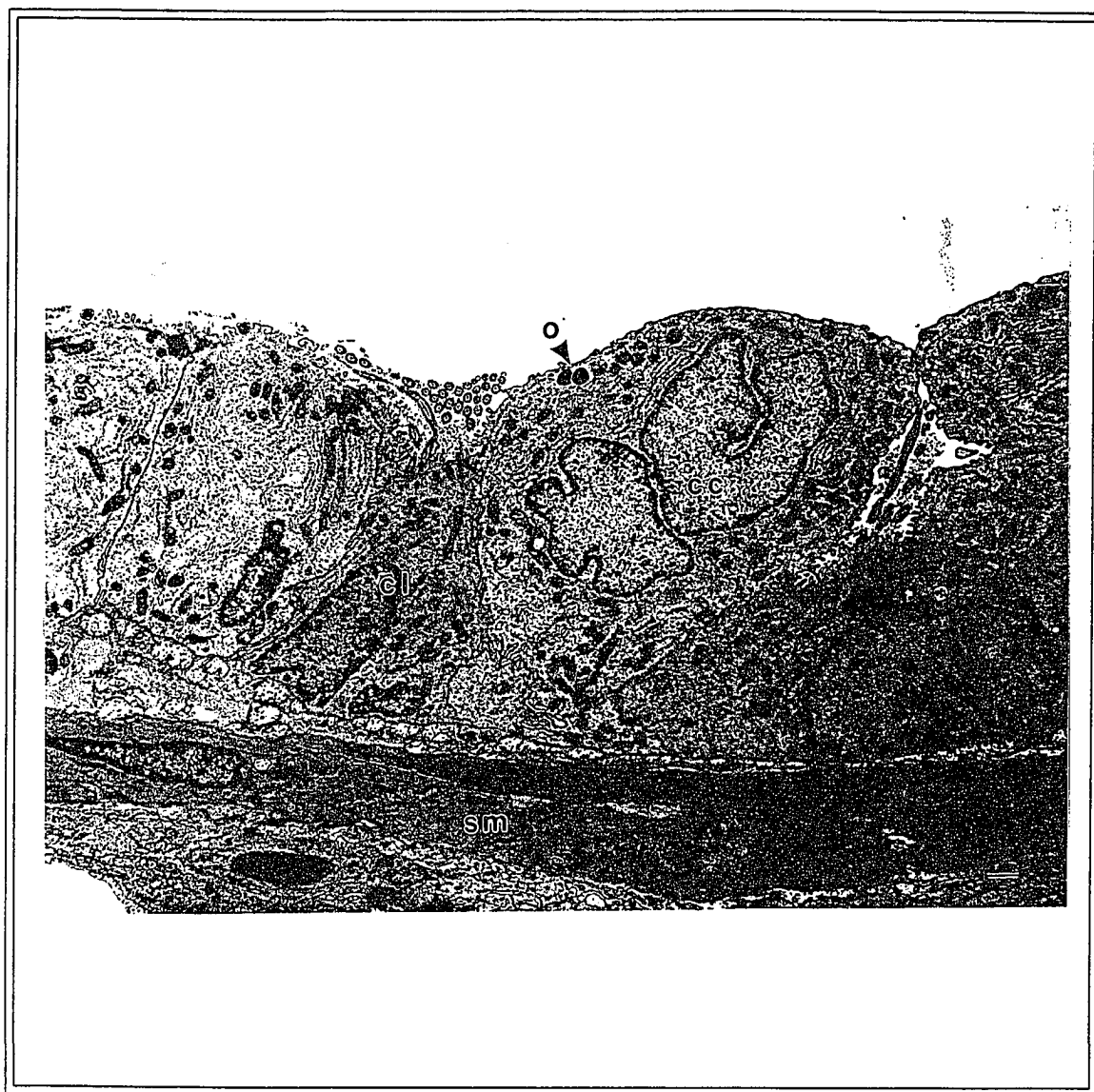


Figure 3. A Transmission Electron Micrograph of Bronchiolar Epithelium From a Terminal Bronchiole of an Animal Treated With Vehicle (Peanut Oil).

Commonplace feature of both Clara and ciliated cells have been labelled. cc; Clara cell, cl; ciliated cell, o; osmophilic granules, sm; smooth muscle. Bar: 1 micron.

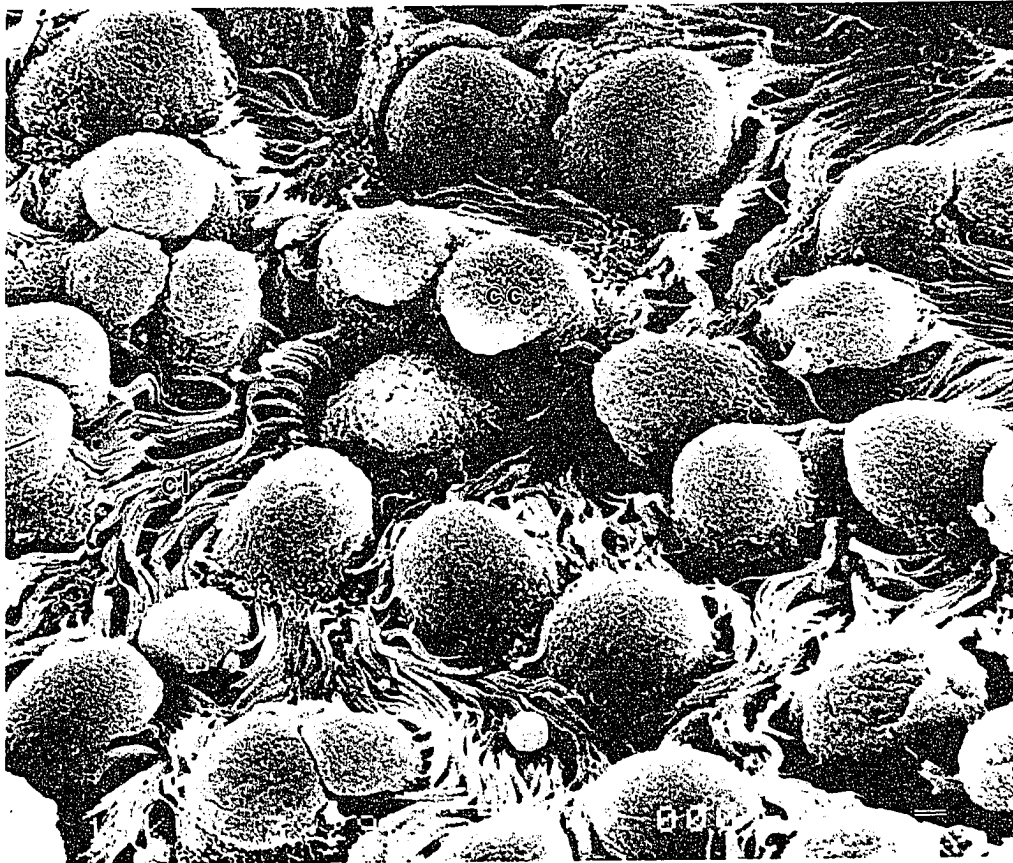


Figure 4. A Scanning Electron Micrograph of Bronchiolar Epithelium From an Animal Treated With Vehicle (Peanut Oil).

Clara cells and ciliated cells have been highlighted: cc; Clara cell, cl; ciliated cell.
Bar: 1 micron.

(see Figure 5). Clara cells of the terminal bronchioles appeared slightly swollen, and because of general bronchiolar constriction, the orientation of the bronchiolar epithelium was changed (see Figure 6).

At 6 hours, Clara cells exhibited chromatin clumping, loss of osmophilic granules, phagocytosis of mitochondria and severe cytoplasmic vacuolization (see Figure 7). Focal swelling, loss of microvilli and exfoliation of Clara cells were apparent in terminal bronchioles. In some cases, regression of Clara cell apicals was also observed (see Figure 8 and 9). Also at 6 hours, ciliated cells exhibited minor cytoplasmic vacuolization and mitochondrial degeneration. The cilia of many ciliated cells were matted with fibrin and red blood cells in several of the bronchioles examined but no internal or external ultrastructural changes of the cilia were noted. The interstitial area around the small bronchioles was infiltrated with inflammatory cells; ultrastructurally identified as small lymphocytes, mast cells and neutrophils.

By 24 hours, smaller bronchioles were almost completely denuded of epithelial cells (see Figure 10) and both Clara and ciliated cells in larger bronchioles exhibited dilation of the smooth endoplasmic reticulum, cytoplasmic vacuolization, nuclear changes, and mitochondrial distension and phagocytosis (see Figure 11). Fibrin deposition and basement membrane degradation were evident in denuded bronchioles. The debris in the bronchioles was ultrastructurally identified to be exfoliated bronchiolar epithelium and macrophages. The pneumonitis persisted and inflammatory cells were observed to be actively degranulating in the interstitial areas and alveolar sacs of treated animals. Type II alveolar cells showed cytoplasmic vacuolization and swelling of the mitochondria and smooth endoplasmic reticulum, similar to the early damage observed in ciliated cells. Endothelial cell damage, vacuolization and minor swelling was also evident at 24 hours.

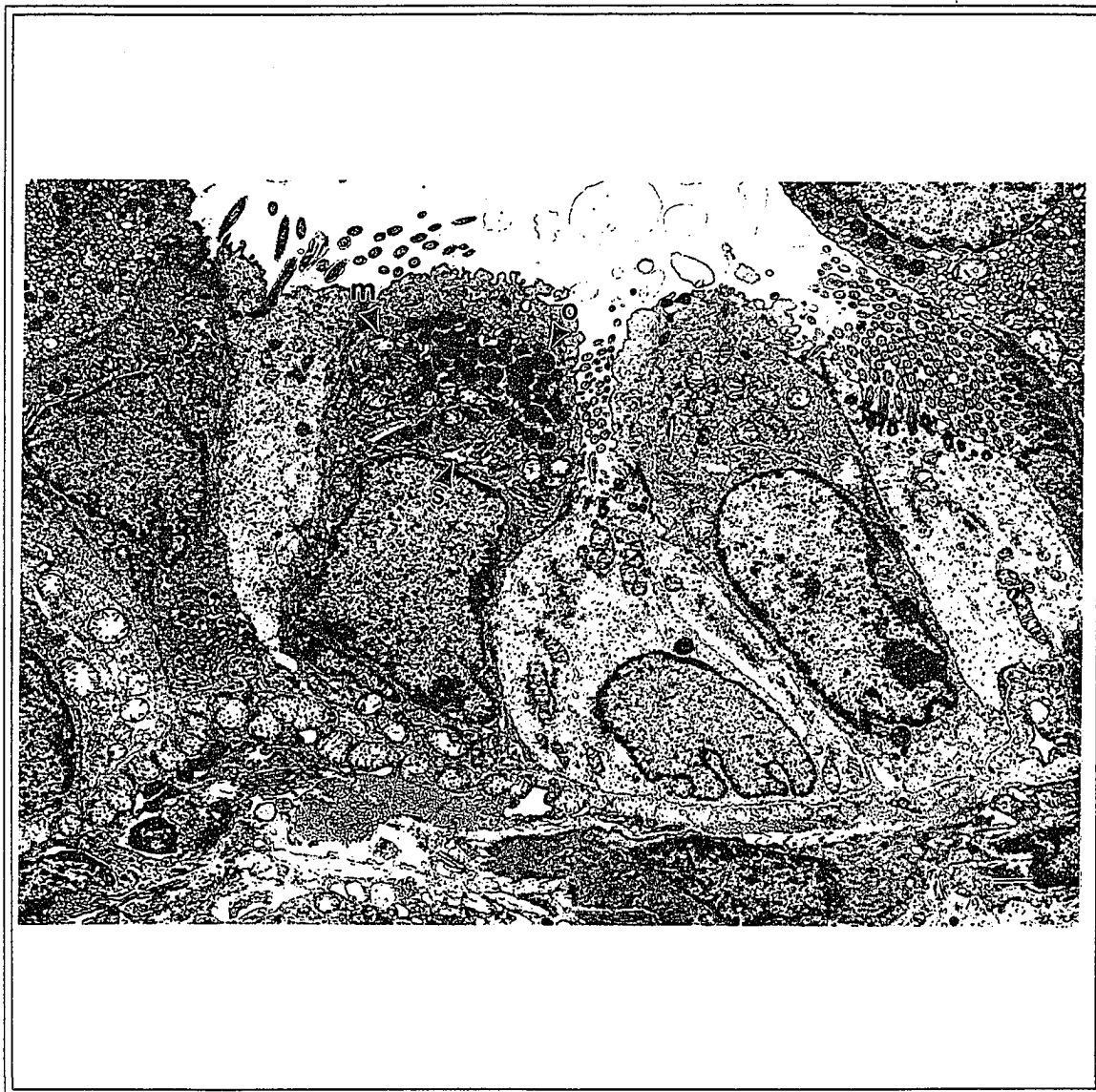


Figure 5. A Transmission Electron Micrograph of Epithelium in a Terminal Bronchiole of an Animal Treated for 1 Hour With 1-NN.

Note the dilation of the smooth endoplasmic reticulum and mitochondrial distension in the Clara cells, while ciliated cells show only minimal changes. s; smooth endoplasmic reticulum, m; mitochondria, o; osmophilic granules. Bar: 1 micron

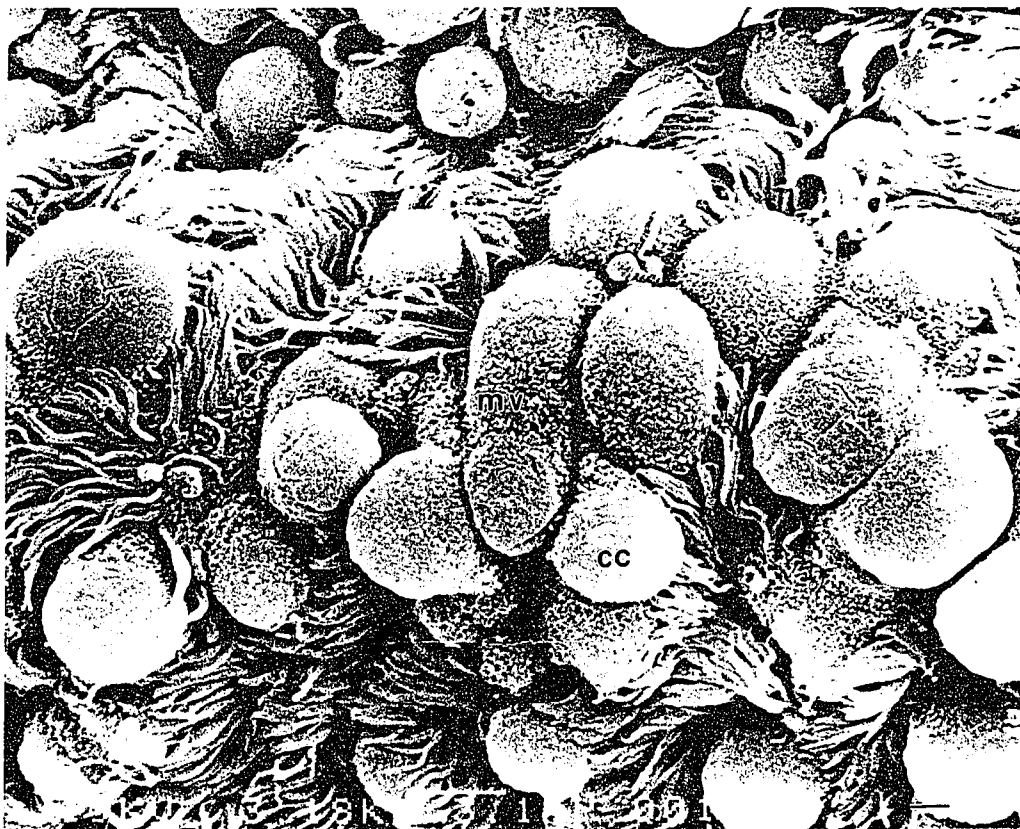


Figure 6. A Scanning Electron Micrograph of Epithelium Located in a Terminal Bronchiole of an Animal Treated With 1-NN for 1 Hour.

The epithelium shows minimal changes except for slight swelling of the Clara cells and a change in orientation due to bronchiolar constriction. cc; Clara cell, mv; microvilli. Bar: 1 micron.

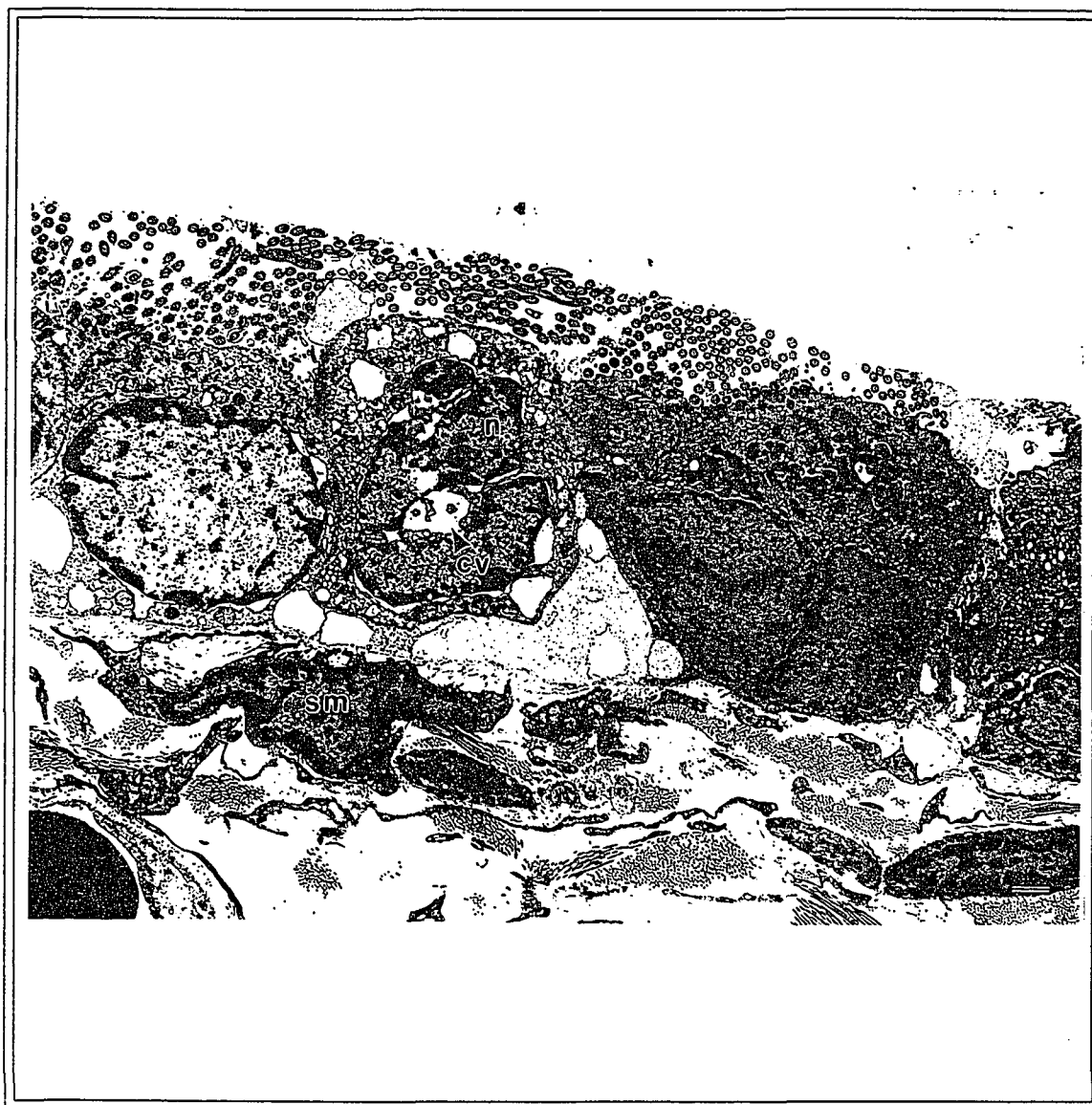


Figure 7. A Transmission Electron Micrograph of Epithelium Located in a Terminal Bronchiole of a Rat Treated With 1-NN for 6 Hours.

Note the severe cytoplasmic vacuolization, and overall degeneration of both Clara and ciliated cells. Disruption of underlying smooth muscle cells due to severe edema can also be observed. cv; cytoplasmic vacuole, n; nucleus, sm; smooth muscle. Bar: 1 micron

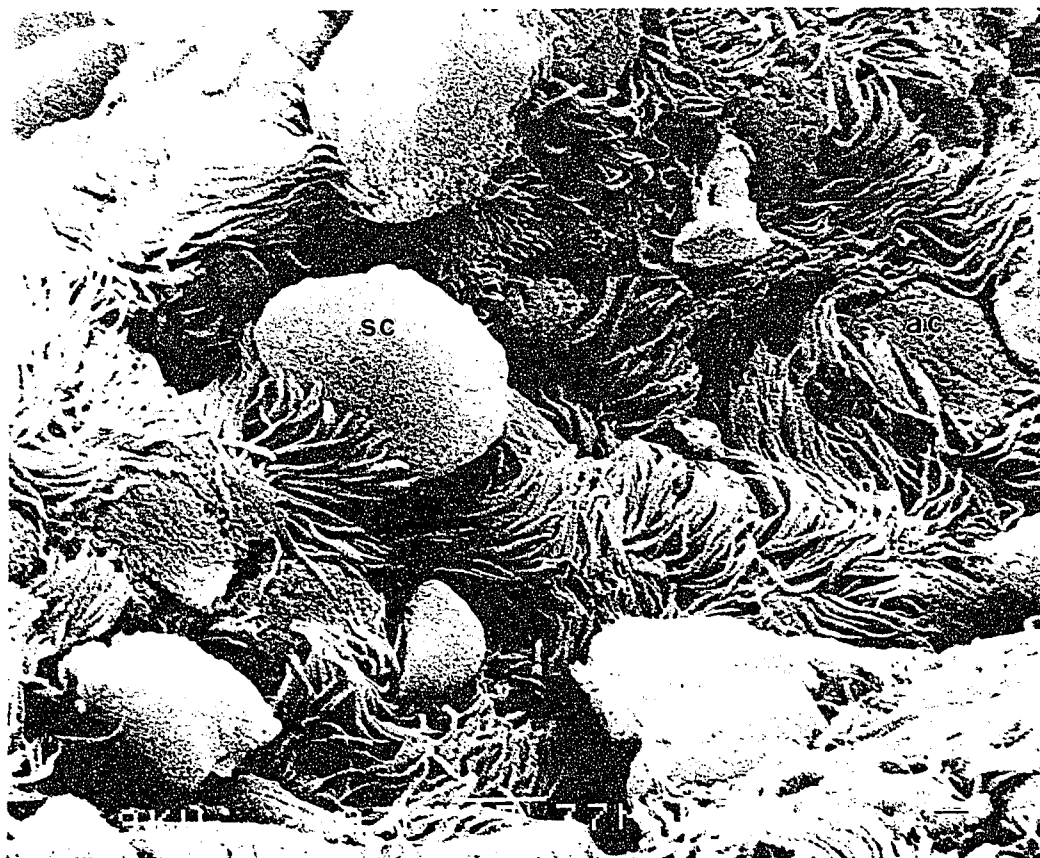


Figure 8. A Scanning Electron Micrograph of Terminal Bronchiolar Epithelium Taken From an Animal Treated With 1-NN for 6 Hours.

Note the contrast in morphology; some Clara cells demonstrate severe swelling while other have lost their apicals and are decreased in size. sc; swollen Clara cell, ac; Clara cell which lost its apicals. Bar: 1 micron.

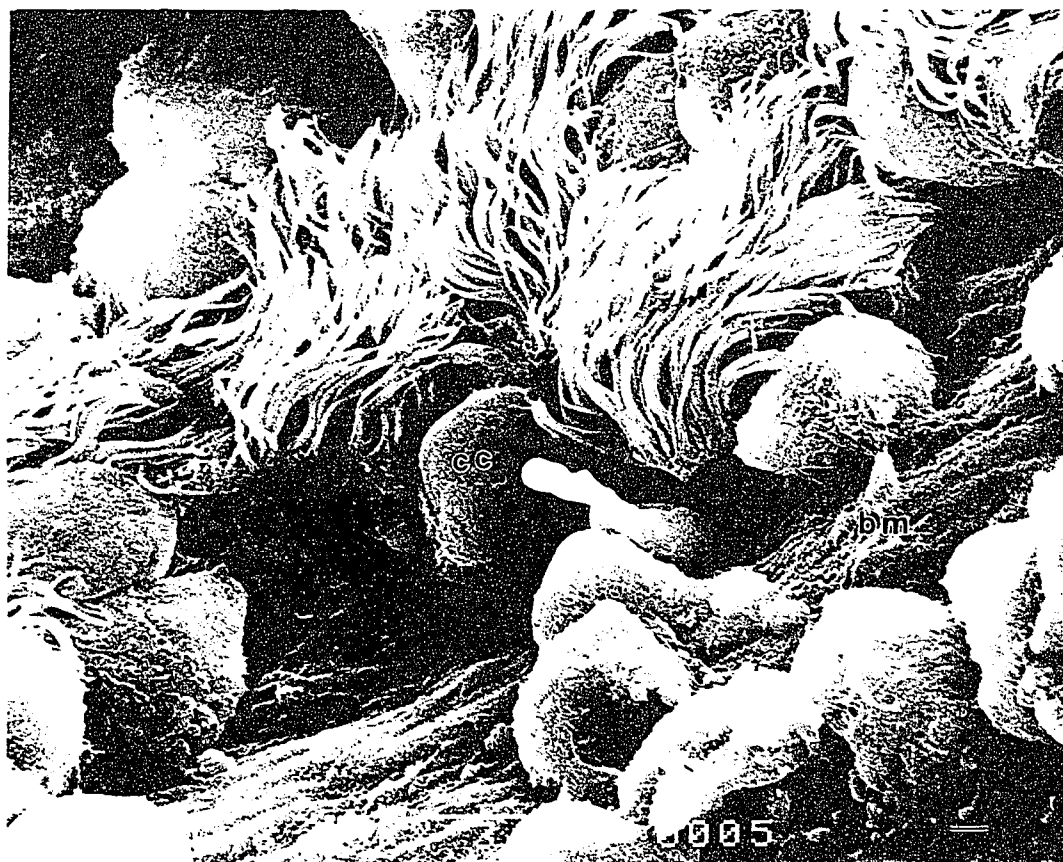


Figure 9. A Scanning Electron Micrograph of Epithelium Located in a Terminal Bronchiole of a Rat Treated With 1-NN for 6 Hours.

Focal areas where both Clara and ciliated cells exfoliated from the basement membrane were noted in all of the animals treated with 1-NN for 6 hours. bm; basement membrane, cc; Clara cell. Bar: 1 micron.

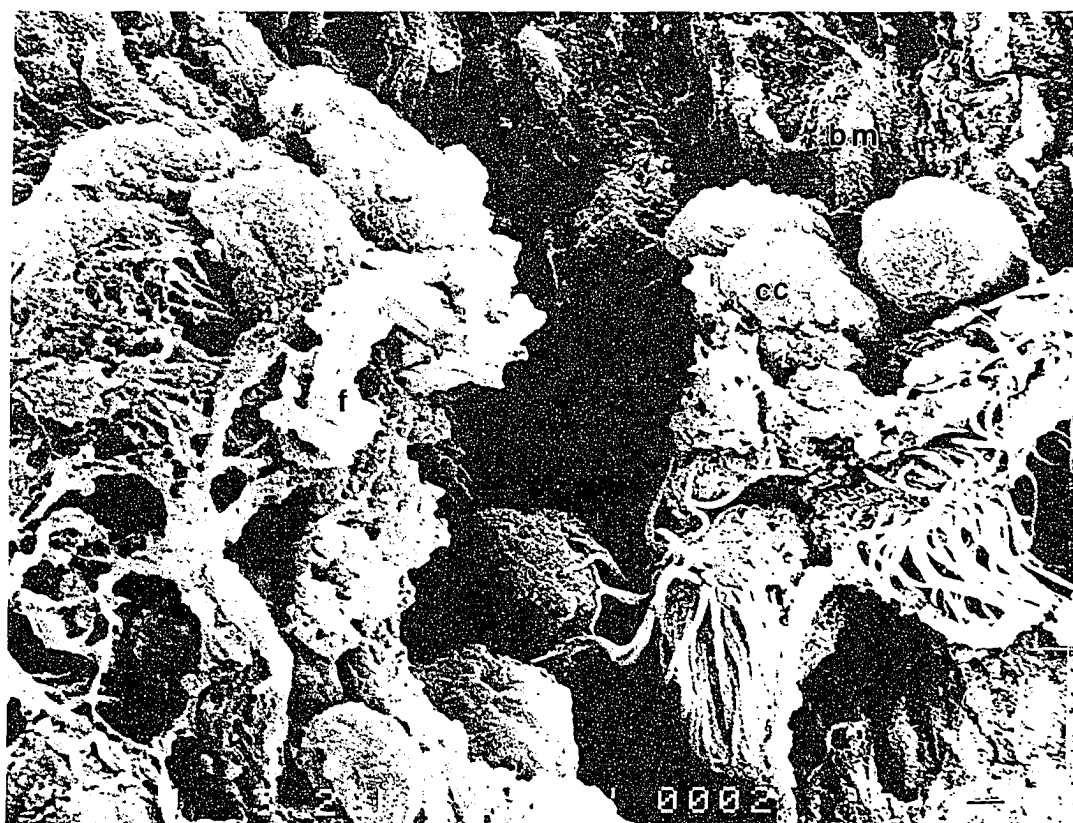


Figure 10. A Scanning Electron Micrograph of Epithelium Located in a Terminal Bronchiole of an Animal Treated With 1-NN for 24 Hours.

The epithelium has completely exfoliated from the basement membrane and remaining cells are covered with fibrin. f; fibrin, cc; Clara cell, bm; basement membrane. Bar: 1 micron.



Figure 11. A Transmission Electron Micrograph of Epithelium Located in a Large Bronchiole of a Rat Treated With 1-NN for 24 Hours.

Note the severe cytoplasmic vacuolization, and overall degeneration of both Clara and ciliated cells, as well as the disruption of underlying smooth muscle cells due to severe edema and influx of inflammatory cells. m; mitochondria, sm; smooth muscle, mg; mast cell granule. Bar: 1 micron

DISCUSSION

Compounds structurally similar to 1-NN, including naphthalene (Warren, Brown, & Buckpitt, 1982), 2-methylnaphthalene (Griffen, Johnson, Berger, & Franklin, 1981), and compounds of unrelated structure, including 4-ipomeanol (Doster et al., 1983) and bromobenzene (Reid, Ilett, Glick & Krishna, 1973), have been shown to exert similar types of pulmonary toxicity in rodents. The primary ultrastructural lesion caused by 1-NN in the lung occurs in the Clara cells of the terminal bronchioles. The metabolism of 1-NN has been previously shown, using specific inhibitors of cytochrome P-450 isoenzymes, to require oxidative activation by cytochrome P-450 IIB1 (Verschoyle & Dinsdale, 1990), an enzyme found in both Clara cells and hepatocytes. Ciliated cells, which contain only limited cytochrome P-450 mixed function oxygenase activity, are affected subsequent to the primary lesions in adjacent Clara cells. The *in situ* formation of reactive intermediates and/or superoxide in Clara cells likely causes the generalized damage associated with the bronchiolar epithelium, while non-bronchiole lesions apparently are caused by the influx and activation of inflammatory cells.

Lehmann et al. (1989), in a recent study, reported that the first sign of liver toxicity occurred at eight hours following a 100 mg/kg intraperitoneal injection of 1-NN and consisted of ultrastructural changes in the centrilobular hepatocytes. These hepatic changes occurred after the initial pulmonary injury described in this study. The vast difference in the time course of damage between the liver and the lung may be due to tissue specific differences in the distribution of cytochrome P-450 content or differences in related metabolic contributors available in these organs.

For example, the oxygen tension of the lung is much higher than that found in the liver and may influence the rates of 1-NN metabolism and activation. Moreover, 4-ipomeanol, a substituted furan compound which requires oxidative activation in order to cause lung and liver toxicity, has also been found to cause damage to the lung before affecting the liver (Boyd, 1980).

The two major pulmonary cytochrome P-450 isoenzymes (P-450I and P-450II) are known to be involved in xenobiotic metabolism, and are in some cases much more active than the hepatic P-450 in bioactivation functions (Boyd, 1984). The pulmonary P-450 system is also present in the bronchial and alveolar epithelium, but immunohistochemical, electrophoretic and metabolic evidence shows that its specific activity is much less than in the Clara cells for many xenobiotics (Voigt et al., 1989). In the liver, cytochrome P-450 appears to be somewhat evenly distributed throughout the parenchyma, thereby lessening the chance of an enriched hepatocyte producing a lethal concentration of toxic metabolites (Baron et al., 1984). On the other hand, conditions that favor the metabolism of compounds similar to 1-NN, naphthalene and 4-ipomeanol appear to be optimal in the Clara cells of the lung. 1-NN in this study was shown to primarily affect the Clara cells, and in other studies (Rasmussen, 1986; Verschoyle & Dinsdale, 1990) to be activated within the lung. Thus, 1-NN appears to exert its toxicity in a fashion similar to Boyd's mechanism I (see Figure 1-I), where the parental compound requires direct metabolism in the lung to cause pulmonary toxicity. Although 1-NN has been shown to be metabolized in both the liver and lungs *via* an oxidative pathway, other studies have shown that 1-NN can also undergo a reductive metabolism *in vivo* (Johnson & Cornish, 1978). The reduction of 1-NN and other nitroaromatics can produce toxic products by a cyclic reduction/oxidation pathway which could cause the activation of oxygen and the formation of radical metabolites (Hewick, 1982). Boyd (1980) described a mechanism of xenobiotic activation in the lung which allowed for such

a reductive pathway, mechanism III (see Figure 1-III).

Johnson et al. (1984) have shown that 2-nitronaphthalene (2-NN), at equivalent dosage levels to 1-NN, does not produce acute liver or lung toxicity in the rat. Since both 1-NN and 2-NN undergo nitroreduction (Johnson & Cornish, 1978) and the *para* position is open in 1-NN and blocked in 2-NN, the *para* position may be the site of reactivity leading to toxicity. In essence, dihydrodiols or other intermediates which are formed during the metabolism of 1-NN are unable to be made during the metabolism of 2-NN due to the location of its nitro moiety. Clara cells are known to contain large reserves of epoxide hydrolase which is responsible for the hydration of arene oxides to their corresponding *trans*-dihydrodiols. The acute target organ toxicity caused by 1-NN is therefore likely mediated by the formation of a specific arene oxide, similar to the mechanism believed to cause 3-methylnaphthalene toxicity (Griffen et al., 1981). McCoy, Rosenkranz, Petrullo, Rosenkranz, and Mermelstein (1981) have shown that nitroarenes may exert their mutagenic activity through mechanisms other than by nitroreduction, which is consistent with evidence that the nitro functional group exercises a direct effect on ring oxidation. If this is the case with 1-NN, it may account for the greater toxicity of 1-NN vs. 2-NN.

Previous studies investigating the activation and metabolism of carbon tetrachloride have shown that the cytochrome P-450 mixed function oxygenase system is involved in the formation of highly reactive toxic metabolites that result in macromolecular binding (Sipes, Krishna, & Gillette, 1977). The liver and lungs are target organs for carbon tetrachloride toxicity in rodents, and like 1-NN, the first indication of lung toxicity consists of damage to the bronchiolar epithelium (Boyd, 1980). The ultrastructural pulmonary lesions induced by carbon tetrachloride, as reported by Boyd et al. (1979), appeared to be restricted to the Clara cells of smaller bronchioles, even at later time points. Although at different time intervals, 1-NN causes damage to

both Clara and ciliated cells. This difference in toxicity may be related to the degree of reactivity possessed by the toxic metabolites from these compounds. Carbon tetrachloride is known to form trichloromethane radicals due to the homolytic cleavage of a carbon-chlorine bond (Recknagel, Glende & Hruszkewycz, 1977), whereas 1-NN likely forms arene epoxide intermediates. The epoxides formed by the oxidative metabolism of 1-NN may be stable enough to diffuse from Clara cells into surrounding ciliated cells, thus causing adjacent cell damage. The highly reactive trichloromethane radicals, on the other hand, may not escape the Clara cell to cause injury to neighboring cells.

Respiratory distress syndrome (RDS)--an acute respiratory failure due to noncardiogenic pulmonary edema (Rinaldo & Rogers, 1982)--was the major clinical observation seen in 1-NN toxicity. Nitrofurantoin, an antibacterial agent used in the treatment of urinary tract infection, also causes acute bronchiolar cell damage and RDS (Boyd, Catignani, Sasame, Mitchell, & Stiko, 1979). Several investigators have associated the RDS caused by nitrofurantoin with an immune-complex mediated reaction and post activation of inflammatory cells (Pearsall, Ewalt & Tsoi, 1974). It is possible that the RDS accompanying 1-NN toxicity is mediated by an immune event similar to that seen with nitrofurantoin. The interstitial pneumonitis caused by 1-NN toxicity was observed as early as 6 hours after exposure and was associated with the severe necrosis of the bronchiolar epithelium. Mast cells, neutrophils and small lymphocytes were all observed to be present in the area around the affected bronchioles. Diesel exhaust particulates, like 1-NN, have been found to act as powerful adjuvants for the production of IgE (Takafuji et al., 1987). This ability to act as an adjuvant may explain the recruitment and activation of inflammatory cells, as well as bronchiolar constriction, edema and RDS during 1-NN toxicity in the lung.

Other organs which possess cytochrome P-450 may also be targets for 1-NN toxicity. The kidney, which is exposed to relatively high levels of 1-NN or its metabolites

during an acute exposure, may be such a target. Although naphthalene has been shown to have very little interaction with kidney microsomes (Buckpitt & Warren, 1982), a mono-substituted naphthalene, 3-methylnaphthalene, has been shown to be readily metabolized by kidney microsomes resulting in macromolecular binding (Griffen et al., 1981). 1-NN has not been shown to cause renal toxicity, but because of the large potential for its occurrence, further investigation is required to ascertain the entire extent of 1-NN toxicity *in vivo*.

Conclusion

This study has shown that Clara cells are the primary targets of 1-NN in the lung, due likely to the high concentration of cytochrome P450 within these cells. The initial degenerative changes observed in Clara cells of the smaller bronchioles consisted of dilation of the smooth endoplasmic reticulum, as well as mitochondrial changes. These cellular changes are probably caused by the *in situ* formation of an ultimate toxin. Degeneration and necrosis of adjacent ciliated epithelial cells occurred subsequent to the changes initially noted in the Clara cells. In addition to bronchiolar epithelial changes, the infiltration of leukocytes, lymphocytes and mast cells was observed in the interstitial areas around the smaller bronchioles 6 hours after 1-NN exposure. Between 6 and 24 hours, endothelial and alveolar cell damage, as well as bronchiolar fibrin deposition and interstitial edema were seen in the 1-NN treated animals. These factors, in concert with inflammatory cell activation, contribute to the adult respiratory distress syndrome seen in rats treated with 1-NN.

Appendix A
IACUC Approval Form

INVESTIGATOR CERTIFICATION

Title of Project The Detection of Pulmonary Ultrastructural Changes
Induced by 1-Nitronaphthalene Toxicity in Rat

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.

Signature: John Miller Green Biological Sciences 4/17/90
Principal Investigator Department Date

REVIEW BY THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

 Disapproved

✓ Approved

_____ Approved with the provisions
listed below

Provisions:

or

Explanation

Alan Poling
IACUC Chairperson

4/16/92
Date

Researcher's Acceptance of Provisions:

Signature: Principal Investigator

Date

IACUC Chairperson Final Approval

Date

Approved IACUC Number 0056

Revised June, 1988

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