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I am submitting herewith a dissertation written by Mary Elizabeth Pecquet Goad entitled "Acute Respiratory Failure Induced by Bleomycin and Hyperoxia." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Comparative and Experimental Medicine.

Hanspeter R. Witschi, Major Professor

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Vice Provost
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# ACUTE RESPIRATORY FAILURE INDUCED BY BLEOMYCIN AND HYPEROXIA

A Dissertation

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## ABSTRACT

Bleomycin, a chemotherapeutic agent, and oxygen at concentrations greater than 20%, induce acute pulmonary damage separately and when administered together. The interaction of 5 U/kg intratracheal bleomycin and 24 hours of exposure to 80% oxygen in hamsters produces delayed onset acute respiratory distress syndrome three days after treatment. As little as 12 hours of 80% $O_2$ exposure, after intratracheal bleomycin, induces severe pulmonary damage. Lung lesions are characterized as diffuse alveolar damage. Significant pulmonary edema, measured by iodine-125-bovine serum albumin and technetium-99m-diethylenetriaminepentaacetate, occurs 72 hours after treatment. Lesions progress from focal mild alveolar interstitial and air-space macrophage and granulocyte infiltrates at 24 hours to marked infiltrates and severe interstitial and air space edema with hemorrhages and hyaline membranes at 96 hours. Significant changes measured by electron microscopy morphometry are increases in volume fractions of neutrophils, alveolar tissue and mononuclear leukocytes. Morphological changes in alveolar capillary endothelial cells detected by electron microscopy are focal attenuations, overlapping cell junctions, gaps between cells or in cell cytoplasm, and focal segmental cell necrosis. Surfactant assay of bronchoalveolar lavage fluid shows a marked decrease in the lecithin/sphingomyelin ratio at 72 hours. Proposed mechanisms of bleomycin and hyperoxia synergism include enhanced production of superoxide radicals either directly or indirectly by increasing neutrophil activity or numbers, or by alteration of cell mediators. The pulmonary edema, without evidence of severe morphological changes, may be secondary to alterations of transalveolar transport mechanisms.

## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ABPE | atypical or acute bovine pulmonary emphysema and edema |
| ARDS | acute or adult respiratory distress syndrome |
| BA | bleomycin and air |
| BNF | 10% buffered neutral formalin |
| BO | bleomycin and 24 H 80% $O_2$ |
| DAD | diffuse alveolar damage |
| DPPC | dipalmitoyl phosphatidyl choline; lecithin |
| DTPA | diethylenetriaminepentaacetate |
| H | hours |
| HCl | hydrochloric acid |
| HMD | hyaline membrane disease |
| HPTLC | high-performance thin-layer chromatography |
| $^{3}$H-TdR | tritiated thymidine |
| $^{125}$I | iodine-125 |
| $^{125}$I-BSA | iodine-125-labelled bovine serum albumin |
| $LD_{50}$ | lethal dose [of substance] that will kill 50% |
| LI | labelling index |
| L/S | lecithin-sphingomyelin ratio, DPPC/SM |
| NaCl | sodium chloride, saline (0.9% solution in $H_20$) |
| $O_2$ | oxygen |
| PEAR | pulmonary extravascular albumin ratio |
| PED | pulmonary extravascular $^{99m}$Tc-DTPA ratio |
| RDS | respiratory distress syndrome |

| | |
|---|---|
| SA | saline and air |
| SM | sphingomyelin |
| SO | saline and 24 H 80% $O_2$ |
| $^{99m}Tc$ | technetium-99m |
| $^{99m}Tc$-DTPA | technetium-99m-labelled-diethylenetriaminepentaacetate |
| TLC | thin-layer chromatography |
| U | units |

# TABLE OF CONTENTS


| CHAPTER | | PAGE |
|---|---|---|
| I. | INTRODUCTION | 1 |
| | Purpose | 6 |
| II. | LITERATURE REVIEW | 7 |
| | Adult Respiratory Distress Syndrome | 7 |
| | Animal Models of Acute Respiratory Failure | 15 |
| | Conclusion | 27 |
| III. | METHODS TO STUDY ACUTE PULMONARY DAMAGE | 28 |
| | Introduction | 28 |
| | Pulmonary Edema | 29 |
| | Morphology and Morphometry | 31 |
| | Pulmonary Surfactant | 35 |
| IV. | PULMONARY EDEMA AND CELL KINETICS | 41 |
| | Introduction | 41 |
| | Materials and Methods | 44 |
| | Results | 57 |
| | Discussion | 79 |
| V. | PULMONARY MORPHOMETRY | 87 |
| | Introduction | 87 |

CHAPTER PAGE

Materials and Methods ..... 88
Results ..... 94
Discussion ..... 118

VI. PULMONARY SURFACTANT ..... 123
Introduction ..... 123
Materials and Methods ..... 124
Results ..... 129
Discussion ..... 134

VII. CONCLUSIONS ..... 137
Bleomycin and Hyperoxia in Hamsters ..... 137

LIST OF REFERENCES ..... 146

VITA ..... 164

## LIST OF TABLES


| TABLE | | PAGE |
|---|---|---|
| 2.1 | Models of ARDS | 17 |
| 4.1 | Hamsters Treatments for Initial Observations of Respiratory Failure and Death | 47 |
| 4.2 | Hamster Treatment and Exposure Groups | 48 |
| 4.3 | Initial Observations of Mortality After Bleomycin Treatment | 58 |
| 4.4 | Pulmonary Extravascular Albumin Ratio (PEAR) of Hamsters | 59 |
| 4.5 | Pulmonary Extravascular DTPA Ratio (PED) of Hamsters | 62 |
| 4.6 | Labelling Index (Percent Labelled Cells per 2000 Cells) of Pulmonary Alveolar Tissue | 63 |
| 4.7 | Labelled Alveolar Macrophages per 10,000 Cells | 63 |
| 4.8 | Labelled Type II Epithelial Cells per 10,000 Cells | 65 |
| 4.9 | Labelled Endothelial Cells per 10,000 Cells | 65 |
| 4.10 | Labelled Alveolar Interstitial Cells per 10,000 Cells | 66 |
| 5.1 | Pooled Volume Fractions from Point Counts Alveolar Tissues | 95 |
| 5.2 | Pooled Volume Fractions from Point Counts Total Alveolar Tissue | 96 |
| 5.3 | Pooled Volume Fractions from Point Counts Noncellular Spaces | 97 |
| 5.4 | Pooled Volume Fractions from Point Counts Alveolar Leukocytes-Vascular | 98 |
| 5.5 | Pooled Volume Fractions from Point Counts Alveolar Granulocytes-Vascular | 99 |
| 5.6 | Pooled Volume Fractions from Point Counts Alveolar Leukocytes-Nonvascular | 100 |
| 5.7 | Pooled Volume Fractions from Point Counts Alveolar Granulocytes-Air Space | 101 |
| 5.8 | Alveolar Ultrastructure-Cytoplasmic Surface and Edema | 104 |
| 5.9 | Alveolar Ultrastructure-Leukocytes and Exudates | 109 |
| 6.1 | Surfactant Values of Bronchoalveolar Lavage Samples | 130 |

## LIST OF FIGURES


| FIGURE | | PAGE |
|---|---|---|
| 4.1 | Radiolabelled alveolar macrophage | 52 |
| 4.2 | Radiolabelled type II alveolar epithelial cell | 53 |
| 4.3 | Radiolabelled alveolar capillary endothelial cell | 54 |
| 4.4 | Radiolabelled alveolar interstitial cell | 55 |
| 4.5 | Cumulative mortality of hamsters after Bleomycin treatment and 80% $O_2$ exposure | 60 |
| 4.6 | Pulmonary edema measured by PEAR | 61 |
| 4.7 | Autoradiography-derived labelling indices | 64 |
| 4.8 | Labelled alveolar macrophages per 10, cells | 67 |
| 4.9 | Labelled type II alveolar epithelial cells per 10,000 cells | 68 |
| 4.10 | Labelled alveolar capillary endothelial cells per 10,000 cells | 69 |
| 4.11 | Labelled alveolar interstitial cells per 10,000 cells | 70 |
| 4.12 | Photomicrograph of Saline and Air treated lungs at 96 H | 72 |
| 4.13 | Photomicrograph of Saline and 24 H 80% $O_2$ treated lungs at 96 H | 73 |
| 4.14 | Photomicrograph of Bleomycin and Air treated lungs at 96 H | 75 |
| 4.15 | Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 24 H | 76 |
| 4.16 | Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 48 H | 77 |
| 4.17 | Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H | 78 |
| 4.18 | Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H | 80 |
| 4.19 | Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 96 H | 81 |
| 5.1 | Electron micrograph of Saline and Air treated lungs at 24 H | 105 |

FIGURE PAGE

5.2 Electron micrograph of Saline and 24 H 80% $O_2$ treated lungs at 24 H ..... 106

5.3 Electron micrograph of Saline and 24 H 80% $O_2$ treated lungs at 24 H ..... 107

5.4 Electron micrograph of surfactant in Bleomycin and Air treated lungs ..... 108

5.5 Electron micrograph of Bleomycin and Air treated lungs at 24 H ..... 110

5.6 Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 24 H ..... 112

5.7 Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 48 H ..... 113

5.8 Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H ..... 114

5.9 Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H ..... 115

5.10 Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H ..... 116

5.11 Electron micrograph of Bleomycin and 72 H 80% $O_2$ treated lungs at 72 H ..... 117

5.12 Electron micrograph of Bleomycin and 72 H 80% $O_2$ treated lungs at 72 H ..... 119

6.1 DPPC values expressed as percent of control ..... 131

6.2 SM values expressed as percent of control, denoted group SA ..... 132

6.3 DPPC/SM ratios (lecithin/sphingomyelin ratios) indicate changes in amounts and quality of phospholipids ..... 133

# CHAPTER I

## INTRODUCTION

Acute respiratory failure without cardiovascular disease is called acute or adult respiratory distress syndrome (ARDS). A significant clinical entity of humans and animals, ARDS affects approximately 150,000 humans per year with approximately a 50% mortality rate (Petty 1982; Bernard and Brigham 1985). The incidence in animals is not known, however it is economically important in cattle as acute bovine pulmonary emphysema and edema (ABPE) (Dungworth 1985).

Clinical cases of ARDS present as severe, acute-onset pulmonary edema unrelated to cardiac abnormalities, with dyspnea, tachypnea, hypoxemia, decreased lung compliance, increased pulmonary capillary wedge presssures, and other signs of respiratory distress including radiographic evidence of alveolar and interstitial infiltrates (Petty 1982). Regardless of cause, clinical manifestations of ARDS are similar.

Circumstances leading to ARDS are numerous and many specific causes are known. Often the specific cause of an individual case of ARDS may never be known (Katzenstein and Askin 1982). Acute respiratory failure occurs as a response of the lung to a variety of injuries. The lung is responsible for gas exchange and is exposed to noxious components of inspired air and circulating blood. With an increase in inspired contaminants such as air pollutants as well as blood-borne substances such as therapeutic agents, the lung is exposed to an increasing array of potentially harmful substances. Toxins causing acute respiratory failure include paraquat, alpha-

napthylthiourea, phosgene, phorbol myristate acetate, and oleic acid. Therapeutic agents resulting in ARDS include methotrexate, cyclophosphamide, bleomycin, and high oxygen ($O_2$) concentrations. Other diseases associated with ARDS include shock, pancreatitis, head trauma, septicemia, viral pneumonia, and severe trauma (Rinaldo and Rogers 1982).

Response mechanisms leading to ARDS are poorly understood and poorly characterized, partly because of the difficulties of obtaining pulmonary tissue in man during the acute insult. Understanding the pathogenesis of ARDS is important to understand the lesions, to determine causes, to predict clinical cases, to arrive at prognoses, and to select therapy. Mechanisms studied to date have been based on known causes of ARDS and on histologic observations of lung from ARDS patients. The histological features of diffuse alveolar damage (DAD) are well defined; histologic characteristics include pulmonary epithelial and endothelial damage, interstitial and intraalveolar pulmonary edema, hyaline membranes, and type II cell hyperplasia (Katzenstein and Askin 1982).

To determine the events in the sequence of DAD and pulmonary edema, the lung should be studied microscopically. Alveolar septa are composed of several layers. From the capillary lumen to the alveolar lumen, the layers are capillary endothelium, capillary basement membrane, interstitium, epithelial basement membrane, and epithelium consisting of type I and type II pneumocytes. The alveolar surface is lined by a layer of surfactant. Previous studies of DAD and ARDS have emphasized the role of the endothelial layer; the remaining layers of alveolar septum should also be examined as sites of

edema fluid leakage, to enhance the understanding of alveolar flooding seen in ARDS (Hurley 1976, DeFouw 1983).

Use of an animal model of ARDS permits study and characterization of the initiating insult, the development, and the terminal stages of the pulmonary lesions, most notably the severe pulmonary edema. The initial event causing alveolar interstitial edema and the cause of alveolar air space edema is a topic of debate among researchers working with non-cardiogenic high-protein content pulmonary edema (Brigham 1982). The importance of the epithelial surface versus the endothelial surface as the main regulator of pulmonary edema, especially alveolar air space edema, is controversial (Crandall et al., 1983).

Specific unresolved problems in the pathogenesis of ARDS are the initiation and development of pulmonary edema and hyaline membranes, alterations of type II pneumocyte metabolism and hyperplasia, and surfactant alterations. The mechanisms of these changes have only been partially characterized. Current theories of the primary mechanism of ARDS include various neutrophil, macrophage, or lymphocyte responses, prostaglandin or leukotriene alterations, $O_2$ radical damage, and direct toxic or therapeutic agent damage (Rinaldo and Rogers 1982).

Experimental studies have concentrated on the mechanisms of acute pulmonary edema. A major model used for these studies is intravenous endotoxin in sheep. The main lesion produced in these sheep is alveolar interstitial edema, which is reversible when the endotoxin effect wears off (Coalson et al., 1970; Rinaldo et al., 1984). Other models include intravenous

phorbol myristate acetate in dogs or rabbits, intravenous oleic acid in dogs or rabbits, intravenous or oral alpha-naphtylthiourea in rats or dogs, removal of surfactant in mini-pigs, and intratracheal instillation of gastric contents or HCl in dogs or rabbits. Not all of these models have all the clinical signs or lesions of ARDS, but all have at least one major sign or lesion of acute respiratory failure (Rinaldo and Rogers 1982).

In man, even with large numbers of cases and high mortality rates, study of mechanisms is limited because of moral and ethical restraints. In human cases, lung sampling times, amounts, and frequencies are limited by patient care and survival. Animal experiments can be controlled and reproduced. Most current models are immediate-onset ARDS. These models use intratracheal HCl and, oleic acid, phorbol myristate actetate or bacterial endotoxins intravenously. Smoke inhalation and prolonged high $O_2$ concentrations have been used to produce delayed-onset ARDS. Delayed as well as immediate-onset ARDS occurs in man. Because the cause can not be definitively established in human cases, the time of initial pulmonary injury is frequently unknown and therefore the incidence of delayed-onset versus immediate-onset acute pulmonary edema with ARDS cannot be accurately determined (Rinaldo and Rogers 1982). However, many patients seem to have earlier, minor complaints prior to the onset of severe signs (Katzenstein and Askin 1982)..

Bleomycin is a chemotherapeutic agent that is known to cause acute pneumonitis and progressive pulmonary interstitial fibrosis by an unknown mechanism. Early pulmonary disease following therapeutic doses of bleomycin

can include ARDS (Porvik 1983). In pulmonary studies with intratracheal bleomycin and hyperoxia in mice and hamsters, lesions of acute DAD were noted to occur in animals dying 3 to 4 days after treatment. In several experiments with mice and rats, to investigate the interaction of $O_2$ with cyclophosphamide and $O_2$ with bleomycin, a few of the animals died with signs of respiratory failure and lesions of DAD. The most prominent pulmonary changes noted were edema and hyaline membranes. These were seen only in dead or moribund animals, not in healthy or depressed animals. To further characterize the lesion, rats were treated with intratracheal bleomycin and 70 or 80% $O_2$ until death. Dead or moribund animals had interstitial and alveolar pulmonary edema and hyaline membranes (Hakkinen et al., 1983). In additional studies with bleomycin and $O_2$, rats with respiratory failure were also noted to develop hemorrhages, neutrophil aggregates, microthrombi, and increased alveolar macrophages (Hakkinen et al., 1983).

Thus, it was decided to develop a model of acute respiratory failure induced by bleomycin and hyperoxia. Hamsters were selected as the experimental animal because the respiratory tract in this species and its reactions to injury are comparable to those of man (Hakkinen and Witschi 1985). Intratracheal bleomycin followed by $O_2$ exposure was chosen as the method to induce ARDS since it had produced the lesions in rats and since both agents are commonly used in man. Also, a model of slowly progressive interstitial fibrosis secondary to intratracheal bleomycin and subsequent oxygen exposure is well characterized in hamsters (Tryka et al., 1982).

## Purpose

The purpose of this work is to develop a model of ARDS in hamsters and to characterize the lesions produced by bleomycin and $O_2$ by measurement of pulmonary edema, histologic evaluation by light and electron microscopy, and determination of changes in surfactant of lung lavage samples.

# CHAPTER II

# LITERATURE REVIEW

## Adult Respiratory Distress Syndrome

The acute or adult respiratory distress syndrome (ARDS) is a clinical entity defined as acute-onset of dyspnea, pulmonary edema, hypoxemia, and decreased pulmonary compliance (Petty 1975; Lind et al., 1981; Rinaldo and Rogers 1982). The causes of ARDS and DAD are numerous and include widely disparate entities ranging from head trauma to hyperoxia (Petty 1982, Hudson 1982). Diffuse alveolar damage is the nonspecific response of the lung to a variety of insults. In the first few days after insult, the lesions are characterized by an acute or exudative phase of damage with intraalveolar fibrin and platelet microthrombi, intravascular neutrophil aggregates, and focal hemorrhages followed by degeneration and necrosis of type I epithelial cells and endothelial cells, interstitial and intraalveolar edema, and hyaline membranes. If the patient survives the acute phase, these lesions undergo a repair process, the subacute proliferative or organizing phase with type II epithelial cell hyperplasia and interstitial inflammation, and, occasionally fibrosis (Katzenstein et al., 1976; Lamy et al., 1976; Pratt et al., 1979; Katzenstein and Askin 1982).

The term ARDS was first coined when similarities were noticed between cases of adult acute respiratory failure and cases of respiratory distress syndrome (RDS) or hyaline membrane disease (HMD) in newborns (Petty 1975). The entity of ARDS was first observed and noted during World

War I in association with trauma, shock, and toxic gases especially mustard gas and phosgene (Cameron and Courtice 1946; Lind et al., 1981). Observations of ARDS increased following World War II and the Vietnam War (Pietra 1974). The first complete descriptions of ARDS came from Vietnam War physicians in the 1960's (Pietra 1974). Subsequently, as causes of ARDS or associations with other factors such as trauma or toxins were noted, more and more cases resulting from a variety of insults were recorded (Hudson, 1982).

Currently, any pulmonary insult or injury inducing noncardiogenic pulmonary edema is considered a cause of ARDS (Petty 1982; Robin et al., 1983). Noncardiogenic pulmonary edema is considered vascular permeability pulmonary edema, resulting from increased vascular leakage or direct vascular damage (Sibbald et al., 1979). Specific causes of ARDS in man and animals are shock; sepsis; bacterial or viral pneumonia; inhaled, ingested, or injected drugs or toxins; disseminated intravascular coagulopathy; immunologic reactions; thoracic irradiation; near-drowning; smoke inhalation and burns; prolonged surgery; and aspiration (Hudson 1982; Dungworth 1985). In cattle, causes of ARDS or ABPE include lush grass forages, especially new growth of rye grass containing high levels of tryptophan, moldy sweet potatoes containing 4-ipomeanol, and *Perilla* ketones (Dungworth 1985).

Diagnosis of ARDS in man or animals is based on history, clinical signs and symptoms, and lesions in biopsy or necropsy specimens. Onset of ARDS may follow the presumed initial insult by hours to days, and, although acute in presentation, the lesions are usually delayed relative to initial insult (Katzenstein and Askin 1982).

Studies performed on biopsy and autopsy specimens of acute respiratory failure cases have shown that the lesions and signs were more related to therapy than exposure to insult or duration of related illness (Pratt et al., 1979). Oxygen was implicated as the major therapeutic agent presumed to potentiate the lesions, since a good correlation between length of $O_2$ therapy and severity of lesions was determined (Pratt et al, 1979).

The mechanism of ARDS caused by hyperoxia exposure with or without simultaneous lung injury is considered to be the direct production of superoxide radicals and other reactive oxygen species (Rinaldo and Rogers 1982). Besides direct oxidant damage, other proposed mechanisms of permeability pulmonary edema are oxidants from neutrophils (Zimmerman et al., 1983) and related mediators of inflammation including complement and complement activators, coagulation and platelet factors, and prostaglandins (Rinaldo and Rogers 1982). Neutrophils and intravascular neutrophil aggregates are observed in DAD. Neutrophil-depleted animals given substances that usually produce ARDS have no edema or less severe, nonfatal lesions (Rinaldo and Rogers 1982).

Humoral factors causing ARDS include any intrinsic noncellular substances elicited by some stimulus or injury and produced by the body. Some of the humoral substances associated with ARDS are lipase (Hayes et al., 1974), histamine (Brigham 1977), elastase (McGuire et al., 1982), ketones (Brun-Buisson et al., 1985), and collagenase (Christner et al., 1985).

Intrinsic enzymes released during disease processes are good candidates to cause direct alveolar damage. Lipase is released during acute pancreatitis. Patients with acute pancreatitis are at risk for ARDS either because of

released enzymes such as lipase or other unexplained factors associated with the disease. However, lipase levels have not been shown to be associated with onset of ARDS in pancreatitis patients (Hayes et al., 1974).

In one group of ARDS patients, increased proteolytic activity was measured in bronchoalveolar lavage fluid. This proteolytic activity was identified as increased neutrophil elastase, an enzyme which degrades elastin. Elastase can directly damage endothelial cells. Neutrophil elastase is a normal product of neutrophils and can be elevated secondary to increased neutrophil activity or increased neutrophil numbers (McGuire et al., 1982).

Several diabetes mellitus patients with ketoacidosis have also developed ARDS. Since increased or decreased oncotic and hydrostatic pressures were eliminated as causes of pulmonary edema, ketoacids were suspected as a cause of increased pulmonary vascular permeability. Ketoacids may alter endothelial cell function or may alter surfactant function (Brun-Buisson et al., 1985).

Collagenase is another enzyme found in bronchoalveolar lavages from ARDS patients. Increased collagenase and serine protease levels were correlated with increased neutrophil and decreased macrophage counts in lavage samples of these patients. Collagenase and protease can damage the endothelial cells and the supporting connective tissue, leading to weakened vessels and permeability changes (Christner et al., 1985).

Other intrinsic compounds can lead to acute pulmonary edema and vascular changes. Histamine, and not serotonin, given intravenously in sheep causes increased pulmonary vascular permeability probably by changing endothelial cell function or active transport. Therefore toxins or stimuli causing release of histamine can cause pulmonary edema (Brigham 1977).

Prostaglandin and related compounds have also been associated with pulmonary edema and ARDS. Arachidonate metabolites, prostaglandins, may be a cause of lesions or a response to pulmonary damage. The lung releases protaglandins in several disease conditions, most notably endotoxemia. Prostaglandin modulators, such as non-steroidal anti-inflammatory compounds, can alter the degree of pulmonary edema caused by endotoxins (Brigham 1977; Brigham et al., 1983). Infusion of lipoxygenase an arachidonate metabolite infusion can produce increased pulmonary microvascular pressures similar to those caused by endotoxemia (Smith et al., 1981). Prostaglandin $E_2$, $F_2$, cyclic endoperoxides, thromboxane, and leukotrienes also can increase pulmonary microvascular pressure (Malik et al., 1985). Prostaglandins are implicated as local hormones and mediators of numerous inflammatory processes, but no cause-effect relationship has been determined (Rinaldo and Rogers 1982).

Relationships of ARDS, disseminated intravascular coagulopathy, and platelets are circumstantial in both natural cases and animal models. Animals treated with heparin before and after induction of respiratory failure show no differences in survivability or lesions from untreated animals.

Altered cells or cell mediators associated with ARDS are unfiltered whole blood transfusion or intratracheal instillation of (Van Zankwijk et al., 1979), formation or administration of antigen-antibody complexes (Pearson et al., 1980), platelets (Heffner et al., 1983), and neutrophil aggregation (Skasby et al., 1983; Zimmerman et al., 1983; Rinaldo et al., 1984; Fowler et al., 1984). Infusion of whole blood but not leukocyte-poor or platelet-poor blood has been shown to induce pulmonary damage. Neutrophils were sequestered in

the vessels and were the presumed source of pulmonary damage (Van Zandwijk et al., 1979).

Complement and mediators of the complement cascade may also cause pulmonary damage. Complement-induced pulmonary damage is usually associated with cellular activity such as neutrophil chemotaxis, or other active processes, such as antigen-antibody reactions (Larson et al., 1983). Sheep infused with activated complement develop ARDS-like pulmonary changes (Rinaldo and Rogers, 1980).

Immune complexes given intratracheally produce pulmonary lesions of DAD. The cause of the damage was presumed to be complement activation, since complement-depleted lungs had only minor changes after antigen-antibody complexes were given (Pearson et al., 1980). Platelets infused into lungs caused pulmonary edema, most likely by release of arachidonate metabolites (Heffner et al., 1983). Neutrophils from ARDS patients have increased chemotactic activity and increased release of superoxide radicals (Zimmerman et al., 1983). Injected endotoxin in rats caused sequestration of neutrophils in pulmonary vasculature; these neutrophils had increased chemotactic activity (Rinaldo et al., 1984). In another group of ARDS patients, neutrophils had decreased chemotaxis and increased release of lysosomal enzymes. Neutrophils from normal patients exposed to serum from ARDS patients also had inhibited chemotaxis and increased enzyme release. Therefore neutrophils in the lungs may also determine the status of humoral factors in cases of ARDS (Fowler et al., 1984).

**Pulmonary Edema and Acute Respiratory Failure**

The major lesion of ARDS, considered the essential lesion to reproduce in any animal model, is pulmonary edema. The mechanism of ARDS edema formation is considered to be increased vascular permeability (Demling 1980; Ayres 1982). A change in pulmonary vascular permeability can be due to functional or structural changes in pulmonary capillaries or endothelial cells (Brigham 1982).

Pulmonary edema is the accumulation of fluid in the interstitium, cells, and alveolar air spaces of the lungs. Edema fluid may result from heart failure, in which the fluid is of low protein content and is secondary to increased hydrostatic pressure (Lunde and Waaler 1969; Robin et al., 1982), or from increased pulmonary vascular permeability, in which the fluid is of high protein content (Butler et al.,1977; Sprung et al., 1981; Staub 1981) or from to hypoalbuminemia or decreased oncotic pressure (Robin et al., 1982). Pulmonary edema associated with ARDS is high in protein and is secondary to increased microvascular permeability (Staub 1981).

To understand the mechanisms and factors of vascular permeability pulmonary edema, the ultrastructure of the lung, especially the alveolar wall, must be examined. From the capillary lumen to the alveolar lumen, alveolar wall structures are endothelial cells, endothelial basement membrane, interstitial cells, noncellular interstitium, epithelial basement membrane, and epithelial cells. A layer of surfactant lines the epithelial surfaces (Hurley 1978, DeFouw 1983). Pulmonary capillary endothelial cells normally have gaps in the cell junctions with large interendothelial spaces and numerous cytoplasmic

pinocytotic vesicles. The interstitial cells are usually sparsely scattered fibroblasts among collagen fibers. Pulmonary epithelial cells are of two types. Type I epithelial cells are thin lining cells covering 95% of the alveolar surfaces; this 0.1 to 0.3 um thick surface is the site of oxygen-carbon dioxide gas exchange. Type II epithelial cells, covering 5% of the alveolar surface, are cuboidal epithelial cells with microvilli; these cells are type I precursors and synthesize surfactant. Epithelial cell junctions are tight and occluding; the zonula occludentes form a relatively impermeable seal between the interstitium and the alveolar air space. In the normal state, the epithelial surface is much less permeable than the endothelial surface. Thus, it is believed that the alveolar epithelium and not the endothelium is the main barrier to alveolar air space edema fluid (Harris and Heath 1977; Hurley 1978; DeFouw 1983).

In any form of pulmonary microvascular damage, pulmonary edema fluid begins to accumulate perivascularly and peribronchially in the interstitial tissues. Fluid then fills the alveolar interstitium. The flooding of alveolar air spaces then occurs on an individual alveolus basis (Staub et al., 1967). This is the sequence of pulmonary edema development, regardless of the cause, whether it is cardiac failure, hypoalbuminemia, or increased pulmonary vascular permeability (Staub et al., 1967; Funstig et al., 1982).

In ARDS, the cause of the alveolar interstitial pulmonary edema is considered to be increased pulmonary endothelial permeability (Brigham 1982). Endothelial permeability can be increased because of functional alterations or structural endothelial damage. Functional alterations of endothelial cells can include enhanced passage or enlargement of pinocytotic vesicles and widening of intercellular junctions. Electrolyte transport

mechanisms can also be affected. Structural damage can be mild to severe ranging from cell surface blebs to intracellular edema to necrosis with complete dissolution of cells (Hurley, 1978; Staub 1981; Brigham 1982).

The major component of pulmonary edema that seems to lead to clinical signs is alveolar air space flooding (Brigham 1982). The major barrier to alveolar air space edema is the epithelial surface, predominantly type I cells (Taylor et al., 1965; Gorin and Stewart 1979; DeFouw 1983). Leakage of fluid across alveolar epithelium into the air space can be due to functional or structural changes of epithelial cells (Egan 1983; Jones et al., 1983; Staub 1983). Functional alterations of epithelium include changes in active transport of electrolytes and proteins across the membranes; structural changes range from mild reversible damage to necrosis (Hurley 1978; Crandall 1983). Therefore, although most histologically documented cases of ARDS have endothelial damage and most good animal models of ARDS have functional or structural endothelial lesions, epithelial changes are also seen in ARDS and are considered the cause of the most severe clinical signs (Bachofen and Weibel 1982; Katzenstein and Askin 1982; Crandall 1983).

## Animal Models of Acute Respiratory Failure

Numerous animal models of acute respiratory failure have been developed and characterized to study the causes, mechanisms, lesions, and treatments of ARDS. Most of these animal models produce acute and immediate-onset pulmonary edema. Many models have very severe lesions, whereas others produce mild single or non-fatal lesions such as pulmonary

edema alone. Animals used include cattle, sheep, goats, pigs, primates, dogs, rabbits, rats, hamsters, and mice (Table 2.1). Agents used to induce acute respiratory failure or a single lesion, such as pulmonary edema, are numerous (Table 2.1).

Aconitine, injected into the brain of rats, produces vascular permeability pulmonary edema one hour after injection (Minnear and Connell 1981). Alloxan given intravenously to dogs causes endothelial and epithelial necrosis within an hour, and therefore structural damage resulting in vascular permeability pulmonary edema with fibrin thrombi, and necrosis (Cottrell et al., 1967, Meyer and Ottaviano 1973; Nelson et al., 1978). Whole blood, leukocyte-poor blood, and platelet-poor blood perfused through excised rabbit lungs produces perivascular and alveolar edema and hemorrhages within 60 minutes (van Zandwijk et al., 1979). By 24 hours, intraperitoneal butylated hydroxytoluene causes type I cell and endothelial cell damage in mice (Smith 1984). Intravenous cobra venom factor in mice produces endothelial injury of immediate-onset as well as intravascular aggregates of leukocytes, platelets and fibrin (Tvedten, et al., 1985). Endotoxin, a substance commonly used to induce immediate-onset pulmonary edema, produces lesions of endothelial damage and leukocyte aggregates in primates, sheep, dogs, and rats. The most commonly used endotoxin is from *Escherichia coli* (Coalson et al., 1970; Rinaldo et al., 1984). Hydrochloric acid, aspirated as gastric contents or given intratracheally, was one of the first agents noted to produce acute respiratory failure in all species as a result of direct damage to epithelium and endothelium (Glauser et al., 1979; Jones et al., 1979; Stothert et al., 1981). Hypovolemic shock is used to induce permeability pulmonary edema in dogs (Barrios et al., 1977).

**Table 2.1. Models of ARDS**

| Substance Used | Reference |
|---|---|
| aconitine | Minnear and Connell 1981 |
| alloxan | Cottrell et al., 1967; Meyer and Ottaviano 1973; Neufeld et al., 1975; Nelson et al., 1978 |
| blood | van Zandwijk et al., 1979 |
| butylated hydroxytoluene | Smith 1984 |
| cobra venom factor | Tvedten et al., 1985 |
| endotoxin | Coalson et al., 1970; Rinaldo et al., 1984 |
| hydrochloric acid | Glauser et al., 1979; Jones et al., 1979; Stothert,et al., 1981 |
| hypovolemia | Barrios, et al., 1972 |
| immune complexes | Johnson and Ward 1974; Pearson et al., 1980 |
| 4-ipomeanol | Durham et al., 1985 |
| lipoxygenases | Smith et al., 1981; Malik et al., 1985 |
| lung lavage | Albert et al., 1980; Kuckeldt et al., 1981 |
| lung re-inflation | Pavlin et al., 1981 |
| 3-methylindole | Dickinson et al., 1976 Hammond et al., 1979; Atwal and Persofsky 1984 |
| alpha-naphthylthiourea | Gardiner 1978; Vivet et al.,1983 |
| N-nitroso-N-methylurethane | Ryan 1972; Ryan et al., 1976 |
| oleic acid | Dickey et al., 1981 |
| oxygen | Deneke and Fanburg 1980 |
| paraquat | Gin et al.,1980 |
| petrol vapour | Lykke et al., 1979 |
| phorbol myristate acetate | Canham et al., 1983; McCall et al., 1983; Taylor et al., 1985 |
| phosgene | Cameron and Courtice 1946 |
| x-irradiation | Slauson et al., 1976; Gross 1980; Adamson and Bowden 1983 |

Immune complexes given intravenously or intratracheally produce hemorrhagic pulmonary edema within several hours in rats (Johnson and Ward 1974; Pearson et al., 1980). Endothelial cell damage and pulmonary edema can be induced immediately in mice with intravenous 4-ipomeanol (Durham et al., 1985). Lipoxygenases or arachidonic acid metabolites; including prostaglandins, thromboxanes and leukotrienes; induce immediate-onset pulmonary edema in sheep (Smith et al., 1981; Malik et al., 1985). Acute immediate-onset pulmonary edema can be produced in pigs by repeated pulmonary lavage, thereby removing the surfactant layer (Kuckeldt et al., 1981). Isolated perfused dog and rabbit lungs which are deflated and reinflated develop pulmonary edema (Albert et al., 1980; Pavlin et al., 1981). Oral 3-methylindole produces delayed-onset ARDS (ABPE) in goats and cattle (Dickinson et al., 1976; Hammond et al., 1979; Atwal and Persofsky 1984). Oral, intravenous, or intraperitoneal alpha-naphthylthiourea causes severe endothelial damage, immediate-onset pulmonary edema, and hemorrhages in rats (Gardiner 1978; Vivet et al., 1983). Subcutaneous N-nitroso-N-methylurethane in hamsters and dogs produces ARDS in 1 to 3 days (Ryan 1972; Ryan et al., 1976). Immediate-onset ARDS develops in rats given intravenous oleic acid; however many of the lesions appear to be pulmonary infarcts (Dickey et al., 1981). Intraperitoneal paraquat produces pulmonary edema and subsequent pulmonary damage in mice (Giri et al., 1980). Petrol vapour produces mild acute pulmonary necrosis and progressive fibrosis in rats (Lykke et al., 1979). Immediate-onset hemorrhagic pneumonitis is produced by intravenous phorbol myristate acetate in rabbits (Canham et al., 1983; McCall et al., 1983; Taylor et al., 1985). In rabbits, dogs, and goats, phosgene

inhalation results in pulmonary edema 6 to 12 hours after exposure (Cameron and Courtice 1946). Thoracic x-irradiation causes delayed-onset ARDS in mice, hamsters, and dogs (Slauson et al., 1976; Gross 1980). One of the most important models of ARDS, producing delayed-onset signs and lesions of DAD in all species studied is exposure to high $O_2$ concentrations (Rinaldo and Rogers 1982).

The earliest reported histologic lesions, regardless of cause or mechanism, in ARDS cases are epithelial and endothelial cell damage, hyaline membranes, and interstitial edema (Katzenstein et al., 1976; Bachofen and Weibel 1983). A good model of ARDS should have a delayed-onset of acute edema, clinical dyspnea, and lesions similar to those seen in man. One of the best models is long-term exposure to 100% $O_2$. Animals exposed to high $O_2$ concentrations are considered good models of ARDS because of the delayed onset of lesions, similarity of lesions to ARDS cases, and because $O_2$ therapy is used in most cases of ARDS (Rinaldo and Rogers 1982).

**Oxygen and Acute Respiratory Failure**

Oxygen, at concentrations higher than 20 to 40%, produces physiological and structural lesions in lungs; the severity of the lesions and the ability of the lungs to recover depends on the length of $O_2$ exposure. Effects of hyperoxia exposure are important to study since 100% $O_2$ was used to supply air for pilots, divers, astronauts, and others in chambers separate from the normal atmosphere. Currently $O_2$ mixtures with other gases such as neon, krypton and argon, are used to avoid $O_2$ toxicities. Hyperoxia is also used

therapeutically, often in patients with pre-existing pulmonary lesions. Therefore the study of pulmonary $O_2$ toxicity in normal and damaged lungs is of utmost importance (Adamson et al., 1970; Bowden and Adamson 1971; Deneke and Fanburg 1980).

Experimental studies of the effects of hyperoxia first focused on morphologic changes. Lesions in rats, observed 2 to 3 days after 98.5% $O_2$ exposure, included pulmonary edema secondary to endothelial cell damage and necrosis followed by epithelial damage, inflammatory cell infiltrates, and fibrin thrombi and deposits (Kistler et al., 1967). Systematic examination of human lung tissue obtained at autopsy showed the same lesions after exposure to $O_2$ concentrations ranging from 40 to 100%. Earliest changes are in endothelial and type I epithelial cells followed by pulmonary interstitial and alveolar edema, neutrophil infiltrates, hyaline membranes, and fibrin thrombi in vessels (Gould et al., 1972). The earliest functional change of $O_2$ toxicity was seen in rats exposed to 90 to 95% $O_2$. Pulmonary edema was detected in rats by radiolabelled tracer molecules as early as one day after $O_2$ exposure (Valimaki et al., 1974). Labelling of cells synthesising DNA secondary to $O_2$ toxicity revealed increased endothelial cell activity, reflected by increased label, as early as 2 days after $O_2$ exposure (Bowden and Adamson 1974). Type II epithelial cells and interstitial fibroblasts prolifrate in $O_2$-exposed lungs 5 to 7 days after the start of exposure (Crapo et al., 1978b). Exposure to 85% $O_2$ for 14 days causes necrosis, regeneration, and hyperplasia of endothelial cells and proliferation of type II epithelial cells in mice (Crapo et al., 1980). Longer exposures to 100% $O_2$ produces increasing pulmonary vascular permeability to albumin (Matalon and Egan 1981). Increased numbers of neutrophils are

found as microaggregates and infiltrates in rats exposed to 95% $O_2$ (Fox et al., 1981). Hyperoxia in normal human lungs for 24 hours or less results in reversible changes of increased pulmonary permeability (Davis et al., 1983).

Therefore in humans and animals exposed to $O_2$ concentrations of 40% or greater, the lesions include interstitial and alveolar pulmonary edema; endothelial damage, necrosis and type I epithelial cell necrosis, and type II cell alteration and regeneration; increased interstitial fibroblasts and leukocytes; deposits of fibrin and hyaline membranes; microthrombi; and neutrophil aggregates. The major proposed mechanism of $O_2$ reactions and damage is oxidant damage. Oxygen itself is an oxidant, however $O_2$ radicals such as superoxide radical, hydroxyl radical, hydrogen peroxides, and other free radicals are believed to be the most damaging reactants. Free radicals can be produced by $O_2$ reactions which damage cells, by enzyme systems, by neutrophils, and by macrophages. Free radicals cause damage to cells and membranes by lipid peroxidation, interferences with enzyme systems, nucleic acid damage, and protein damage (Mustafa and Tierney 1978; Deneke and Fanburg 1980; Kappus and Sies 1981; Jenkinson 1982; Taylor et al., 1983).

Hyperoxia alone produces ARDS; $O_2$ therapy or $O_2$ at low concentrations along with other compounds also can produce pulmonary lesions. Generally the lesions produced by $O_2$ interactions with other substances require less $O_2$ exposure time and lower $O_2$ concentrations than hyperoxia alone to produce similar lesions (Deneke and Fanburg 1980; Warnock 1982).

The interaction or synergism of $O_2$ with other pulmonary insults to produce pulmonary lesions in man was first noted in burn patients treated with $O_2$ concentrations of up to 75%. Patients not treated with $O_2$ or who had

minimal length of $O_2$ exposure did not have lesions as severe as those exposed to high or prolonged $O_2$. Lesions were those of DAD, including hyaline membranes and alveolar edema (Nash et al., 1974). Many ARDS patients have a history of prolonged $O_2$ therapy as well as an initial pulmonary insult, such as trauma, septicema, hypovolemia, shock, prolonged surgery, head trauma, drugs, or a pre-existing underlying lung disorder such as emphysema, pneumonia or heart failure edema (Katzenstein et al., 1976). Oxygen used as therapy for ARDS or acute respiratory failure may enhance or promote the initial disease process (Pratt et al., 1979).

Several experiments have been designed to examine the interactions of $O_2$ and other agents causing pulmonary lesions. Butylated hydroxytoluene in mice causes acute alveolitis and subsequent fibrosis. Butylated hydroxytoluene and 70% $O_2$ causes significantly more severe lesions, indicating synergism of butylated hydroxytoluene and hyperoxia (Witschi et al 1980). Mice exposed to ozone develop acute endothelial damage. If these mice are then exposed to 61% or higher concentrations of $O_2$, regeneration and repair are significantly inhibited (Hackney et al., 1981). Prolonged exposure to 50% or higher $O_2$ concentrations also causes potentiation of pulmonary lesions caused by other agents, thus demonstrating that $O_2$ potentiates lung damage initiated by other agents (Witschi et al., 1981; Haschek et al., 1983). Regardless of the original cause of pulmonary damage, exposure to a second insult, especially hyperoxia can potentiate the damage such that it may develop into irreversible injury (Warnock 1982).

**Bleomycin and Pulmonary Damage**

One of the drugs producing pulmonary lesions that are potentiated by $O_2$ is the chemotherapeutic agent bleomycin (Tryka et al., 1982; Hakkinen et al., 1983). Bleomycin, a complex glycopeptide, is a fermentation product of the bacteria *Streptomyces verticillus*. The chemotherapeutic action of bleomycin is ascribed to its ability to cause changes in DNA. Bleomycin binds to DNA and causes DNA strand scission, decreased DNA replication, and decreased RNA and protein synthesis (Crooke and Bradner 1976; Porvik 1983).

To be active, bleomycin must be an oxygenated iron-drug complex. This iron-drug complex activated by $O_2$, has the ability to cleave DNA (Burger et al., 1981). The mechanism of action has been associated with superoxide radicals, since superoxide dismutase prevents the cytotoxicity of bleomycin (Cunningham et al., 1983). *In vitro* DNA cleavage by bleomycin requires DNA and iron; cleavage of DNA occurs equally with or without $O_2$ therefore $O_2$ is not an absolute requirement as is iron for *in vitro* activity (Burger et al., 1982).

As a chemotherapeutic agent, bleomycin can be used alone or in combination with other drugs or treatments such as irradiation. Bleomycin is used to treat squamous cell carcinoma, lymphoproliferative disorders, and testicular tumors. Bleomycin is not myelosuppressive, as are many other cancer chemotherapeutic agents, such as cyclophosphamide. Because of its lack of myelosuppression, bleomycin is used in combination with myelosuppressive chemotherapeutic drugs (Crooke and Bradner 1976; Povik 1983).

The major toxicities of bleomycin are interstitial pneumonitis and additive, dose-related pulmonary fibrosis (Crooke et al.,et al., 1976; Porvik 1983). After bleomycin use was initiated in cancer therapy, many patients treated with bleomycin developed acute interstitial pneumonitis. Lesions observed included pulmonary edema, hyaline membranes, type II cell hyperplasia, and interstitial fibrosis; these changes are compatible with DAD (Luna et al., 1972). Ultrastructural examination revealed lesions similar to those seen in ARDS patients and in animal models of ARDS, including interstitial edema, type I epithelial cell damage, and fibrosis (Bedrossian et al., 1973).

Some forms of combination therapy, such as radiation or cyclophosphamide treatment, enhance the toxic effects of lower doses of bleomycin (Sostman et al., 1977). Lesions progress from endothelial damage to DAD to pulmonary interstitial fibrosis. Therapy for bleomycin toxicity is withdrawal of the drug; corticosteroids are clinically ineffective (White and Stover 1984). Although low doses of bleomycin alone do not cause pulmonary toxicities, low doses along with radiation, supportive $O_2$ therapy or hyperoxia during anesthesia may produce severe disease (Goldiner et al., 1978; Gockermann 1982; Bauer et al., 1983; Luursema et al., 1983; van Barneveld et al., 1984).

Experiments were performed to investigate the lesions and mechanisms of bleomycin pulmonary toxicity. Mice, given 120 mg/kg bleomycin intravenously or intraperitoneally, had pulmonary endothelial damage, type I cell necrosis, and fibrosis (Adamson 1976). Further studies in mice showed that type II cells become hyperplastic and metaplastic in response to the injury

(Adamson and Bowden 1977; Adamson and Bowden 1979). A hamster model of pulmonary fibrosis was developed by intratracheal instillation of bleomycin. The major change produced was chronic interstitial pulmonary fibrosis (Snider et al., 1978). Bleomycin was also used to develop a mouse model of chronic interstitial pulmonary fibrosis (Sikic et al., 1978; Raisfeld and Brodowsky 1982).

In hamsters, intratracheal bleomycin resulted in acute hemorrhagic interstitial pneumonia followed by interstitial fibrosis (Giri et al., 1980). Bleomycin-induced lung lesions in hamsters progresses from pneumonia over 5 to 7 days, to fibrosis by 14 days (Chandler et al., 1983; Giri et al 1983). In rats, intratracheal bleomycin produces functional pulmonary edema 5 days after treatment, without structural changes (Wangensteen et al., 1983).

Rat lung explants, exposed to bleomycin, induced granulocytes to produce superoxide anions; increased free radical production is thought to be the mechanism of bleomycin pulmonary toxicity (Moseley et al., 1984). More direct proof that superoxide or free radical production is the method of bleomycin damage is that pretreatment of rats with N-acetyl cysteine, an antioxidant, significantly decreases lung damage induced by intratracheal bleomycin (Berend 1985). The mechanism of bleomycin-induced DAD and fibrosis is considered to be enhanced production of superoxide radicals.

Factors appearing to enhance or promote bleomycin toxicity have been examined. One of the first agents associated with acute respiratory failure and death with bleomycin was hyperoxia; excess fluid therapy was also considered a possible cause of the pulmonary edema (Goldiner et al., 1978). Experiments in animals were then performed to determine if there was a synergism or

interaction of bleomycin and $O_2$. Mice treated with both bleomycin and prolonged exposure to 40% $O_2$ had significantly decreased lifespans compared to mice treated with either substance alone (Toledo et al., 1982). Mice treated with bleomycin and exposed to 80% $O_2$ had decreased survival times as well as increased pulmonary collagen (Hakkinen et al., 1982). Hamsters treated with intratracheal bleomycin and exposed to 60 to 100% $O_2$ also had decreased survival times as well as decreased lung function (Rinaldo et al., 1982). In bleomycin treated hamsters, mortality increased in hamsters exposed to 70% $O_2$; the amount of bleomycin required to cause an $LD_{50}$ decreased from 0.73 U/100 g body weight for bleomycin alone to 0.23 U/100 g body weight for bleomycin and 70% $O_2$ exposure.. Bleomycin-treated $O_2$-exposed hamsters also had more severe pulmonary lesions at earlier time points than hamsters treated with either bleomycin or 70% $O_2$ alone (Tryka et al., 1982). The length and timing of $O_2$ exposure subsequent to bleomycin treatment was also found to be significant. Synergistic effects of $O_2$ and bleomycin only occurred if they were given simultaneously and not if $O_2$ exposure was delayed relative to bleomycin treatment (Tryka et al., 1984). Most studies done on bleomycin-$O_2$ interactions have concentrated on subacute and chronic changes, such as fibrosis; one study showed more severe acute pulmonary lesions including edema and DAD in rats given intratracheal bleomycin and exposed to 100% $O_2$ for 24 or 48 hours (Berend 1984). Therefore, simultaneous bleomycin and hyperoxia result in increased pulmonary disease (Hakkinen et al., 1982; Rinaldo et al., 1982; Toledo et al., 1982; Tryka et al., 1982; Tryka et al., 1984).

## Conclusion

Acute respiratory failure is a life-threatening disease of man and animals which is caused by numerous etiologies. Many experimental models have been developed to study the lesions and mechanisms, however they are still not completely understood. To develop an animal model, several parameters should be studied including agents causing pulmonary insult, such as bleomycin and $O_2$, morphology of the lesions, and accessible biochemical parameters such as surfactant production. The characterization of a model of ARDS can only help in the goal of understanding mechanisms of respiratory disease.

# CHAPTER III

# METHODS TO STUDY ACUTE PULMONARY DAMAGE

## Introduction

To understand the mechanisms of acute respiratory failure, including ARDS and bleomycin-$O_2$ damage several parameters should be examined. Initial studies have included observations of human cases and of experimental animals. Morphologic studies, including gross observations, light and electron miroscopy, and light and electron morphometry are used to define and characterize the lesions. Functional studies emphasize development of pulmonary edema; various techniques of edema measurement are available. Edema studies also attempt to locate the source and cause of pulmonary fluid. Cell kinetic experiments using autoradiography indirectly examine the regeneration and repair responses of lungs after insult. Biochemical assays of surfactant phospholipids from bronchoalveolar lavage reflects changes induced in the surfactant layer as well as possible changes in type II epithelial cell metabolism. Therefore the techniques used to study acute pulmonary damage can include clinical observations, measurements of pulmonary edema, gross and microscopic examination of pulmonary tissue, cell kinetic studies, and pulmonary surfactant assays.

Clinical signs of humans or animals with respiratory disease include dyspnea or abnormal breathing, increased respiratory rate, increased sounds of breathing or stridor and rales, and other nonspecific subjective observations such as lack of comfort. Functional and morphologic studies, which are more

objective, can be better used to diagnose and characterize disease than subjective observations alone.

## Pulmonary Edema

Pulmonary edema and its effects are important, measurable phenomena in ARDS. Original observations of edema were made by thoracic auscultation; edema was determined by presence and degree of respiratory stridor and auscultation of rales, wheezes, and other noises. Thoracic radiographs allow detection of changes compatible with edema. Radiographic changes typical of pulmonary edema are increased or radioopaque densities of interstitium, alveoli, and perivascular tissue. Progressive changes can be monitored easily with radiographs; however, pulmonary edema detected by radiography is usually at an advanced stage. Earlier changes of pulmonary edema are better determined by functional studies, usually done with tracer substances (Hudson 1982, Petty 1982).

There are two major methods to measure pulmonary edema development with tracers. The tracer substance can be injected into the blood or other non-pulmonary body compartment and then the appearance of the substance in lung tissue or fluids, or disappearance from blood can be measured. The second method is intratracheal instillation of tracer or exposure of lungs to a tracer substance with subsequent measurement of tracer in blood or disappearance from lungs. Generally, appearance of a substance in lungs after intravenous injection is considered a measure of pulmonary vascular permeability; whereas pulmonary exposure to a tracer followed by its

appearance in blood is considered a measure of epithelial permeability. In practice, the separation of vascular and epithelial permeabilities may be less distinct (Pearce et al., 1965; Taylor et al., 1965; Gorin and Stewart 1979; Crandall et al., 1983; Effros and Mason 1983; Jones et al., 1983).

Substances, injected intravenously or intraperitoneally to measure edema, include albumin, urea, mannitol, inulin, catalase, ferritin, hemoglobin, transferrin, ethylenediaminetetraacetate, and dextrans of varying molecular weights. The most commonly injected substances are iodine-125-labelled albumin and iodine-131-labelled albumin. The injection technique of edema measurement involves sampling of blood or plasma and simultaneous sampling of pulmonary fluids or tissues to detect the injected substance. Tracer substance amounts are measured in blood and lung samples; lung tracer substance amounts divided by blood tracer amounts is the value used to compare degrees of pulmonary edema (Pearce et al., 1965; Pietra, Szidon et al., 1969; Alpert et al., 1971; Johnson and Ward 1974; Nelson et al., 1978; and Crandall et al., 1983).

Measurement to detect the permeability in pulmonary lining layer and epithelium associated with edema, especially alveolar air space flooding, is performed by intratracheal instillation of, or aerosol exposure to, tracers. These tracers include diethylenetriaminepentaacetate, albumin, sodium, potassium, and ethylenediaminepentaacetate. The most commonly used inhaled aerosol tracer is radiolabelled technetium-99m-diethylenetriaminepentaacetate, which has a radioactive half-life of 6 hours. Tracer substances are aerosolized or instilled intratracheally; blood or plasma samples are assayed for appearance

and amount of tracer. With nuclear imaging, disappearance of aerosolized radiolabelled tracer can be followed dynamically without drawing blood samples. Aerosolized tracers are more acceptable for use in human studies, however they are very difficult to adapt for use in laboratory animals (Gorin and Stewart 1979; Rinderknecht et al., 1980; Crandall et al., 1983; Effros and Mason 1983; Jones et al., 1983).

## Morphology and Morphometry

Morphologic changes in lungs with acute respiratory failure or edema need to be studied objectively and thoroughly. Thorough examination includes gross examination, and systematic light and electron microscopy of multiple tissues. To do complete studies of DAD and ARDS, alveolar structures which should be examined include alveoli, type I and II epithelial cells, interstitium, perivascular tissues, capillaries, and endothelial cells.

### Autoradiography

One method to study morphology of pulmonary injury and repair is by labelling of cells going through DNA synthesis. The most common technique used to label cell synthesis is autoradiography. Autoradiography utilizes radio-labelled thymidine which is incorporated into DNA at time of synthesis. Tritiated thymidine ($^3$H-TdR) is the radiolabelled substance used. The $^3$H-TdR is injected intraperitoneally or intravenously in experimental animals prior to sacrifice, but following pulmonary insult. Lung samples are then fixed in formalin, fixed and processed, and embedded in glycol methacrylate, a

plastic. Plastic-embedded lungs can be sectioned at 1 um for light microscopic examination; 1 um sections allow better definition of cells for identification. Methacrylate or plastic sections labelled with $^{3}$H-TdR are dipped in photographic emulsion and treated as film. Resulting slides have developed black granules of silver halides over nuclei with incorporated radiolabelled DNA. Cells thus labelled can be identified and quantified for objective analysis. Labelled cells are considered an indirect measure of pulmonary injury and repair, since dividing cells must go through synthesis before division, and increased division indicates regeneration (Evans et al., 1973; Aherne et al., 1977; Adamson and Bowden 1983; Adamson 1985).

**Morphometry**

Objective microscopic evaluation of lesions in any tissue can be accomplished by morphometry or stereology. Morphometry and stereology are quantitative evalutions of three-dimensional tissues from two-dimensional sections yielding sizes and numbers of tissues for comparison. Morphometry is especially useful in the lung for comparing alveolar wall structures in diseased and non-diseased tissue. Pulmonary morphometry can quantitate morphologic changes such as alveolar intracellular and interstitial edema and increased cell size and numbers. The results of lung morphometry studies are quantitative and qualitative numbers (Weibel 1970; Elias and Hyde 1980; Cruz-Orive and Weibel 1981; Muller et al., 1981; Weibel 1984; Barry and Crapo 1985).

Pulmonary alveolar morphometry studies requires electron microscopic examination of tissues. For electron microscopy to be successful, especially in

the lung, the tissues must be adequately and properly fixed. Fixation for transmission electron microscopy requires rapid, thorough fixation with appropriate chemicals under defined conditions. The fixative most successfully and commonly used is glutaraldehyde in a phosphate buffer solution. Lungs can be preserved by immersion fixation, by intratracheal fixation, or by vascular perfusion fixation. Immersion fixation penetrates the tissue poorly and creates many artifacts. Intratracheal fixation penetrates and fixes the tissue well, however the surfactant lining layer is disrupted and alveolar structure shapes can be altered. Fixation by vascular perfusion, with simultaneous 20 to 30 cm of intratracheal air pressure, fixes the tissue well and maintains normal alveolar architecture. Vascular perfusion fixation, to avoid induced artifacts, must be rapid, and the solutions must be near the osmolality of plasma. Therefore, for pulmonary morphometry the best method of fixation is well-controlled vascular perfusion (Gil and Weibel 1969; Gil 1971; Mathieu et al., 1978; Warheit et al., 1981; Brain et al., 1984).

Lung tissue sample selection for morphometry is very important. Samples are not random, but are the same standard sections from lung to lung so that comparisons can be properly made. For light microscopy, an adequate number of representative samples should be taken from the cranial, middle, and caudal portions of both right and left lungs. Sampling for electron microscopy must take into account what tissues are of interest. Vessels, large airways, and pleura are to be avoided in favor of mostly alveolar tissue in studies concentrating on alveoli. The number of samples for electron microscopy should be enough for good statistical methods. Samples at the electron microscope level must be random to a certain degree, but also selective

to avoid tissues not under study, therefore the numbers of samples must be large enough to yield results statistically significant (Cruz-Orive and Weibel 1981; Muller, Cruz-Orive et al., 1981; Weibel 1984; Barry and Crapo 1985).

The resulting values for light or electron microscopy morphometry are point counts of alveolar structures on glass slides, photographic prints of glass slides, or electron micrograph prints. Light microscopic evaluations are usually performed on standard hematoxylin and eosin 5 um thick sections on glass slides; electron micrographs result in black and white prints (Muller et al., 1981). Light microscope slides can also be developed as black and white prints for ease of point counting (Barry and Crapo 1985). For point counting, specific grids with lines or points are designed as overlays or eyepiece reticules with certain numbers of points such that the points or lines are standard, but fall randomly on the samples or prints. The points counted are the points or ends of lines falling on structures of interest; these numbers are used in the quantitative analyses (Weibel 1984; Barry and Crapo 1985).

Specific pulmonary structures that should be identified and examined morphometrically are type I epithelial cells; type II epithelial cells; interstitium or noncellular tissue including basement membrane; interstitial cells; endothelial cells; leukocytes in alveolar lumens, interstitial spaces, and capillary lumens; alveolar air spaces; and capillary lumens. Aside from morphometric parameters, electron microscopy samples should be observed for qualitative changes not adequately described by numbers alone. Specific as well as nonspecific morphologic changes have been described in human cases of ARDS as well as in experimental animal studies.

Because of the significance of pulmonary edema and increased vascular permeability, the attention of microscopists in ARDS cases has focused on the alveolar vascular structures. Early changes that have been seen are microthrombi, hemorrhages, leukocyte infiltrates, alveolar interstitial and air space edema, epithelial and endothelial necrosis, and hyaline membranes. Subacute changes are hyperplasia and metaplasia of type II epithelial cells, and early fibrosis (Lamy et al., 1976; Bachofen et al., 1982; Katzenstein et al., 1982; Tomashefski et al., 1983).

In experimental animal studies, ultrastructural changes can be well-characterized because of the ability to properly fix lung samples and because samples can be taken at various timepoints (Gil 1971). Endotoxin models of respiratory failure have a progression of changes from type I epithelial cell edema, interstitial edema, increased endothelial cell pinocytotic vesicles, intravascular neutrophil aggregates, and epithelial cell necrosis to endothelial cell degeneration (Coalson et al., 1970). In hemorrhagic shock, endothelial cell junctions do not change, endothelial cell pinocytotic vesicles increase, and epithelial cell junctions become disrupted (Barrios et al., 1977).

## Pulmonary Surfactant

Measurement of surfactant quality and quantity can also provide information about pulmonary function in general and type II epithelial cells in particular. Surfactant alterations of either amount or composition have been suggested as potential causes of pulmonary failure and ARDS (Morgan 1971; Petty 1982).

Pulmonary surface-active material or surfactant forms a thin lining layer on alveolar epithelial surfaces. The surfactant layer functions to maintain functional alveolar surface tension by decreasing it, thus preventing collapse of the alveoli. Other important functions of surfactant include prevention of fluid transudation into alveolar air spaces, transport and medium for alveolar macrophages and inhaled particles, and site of secretory immunoglobulin A (Morgan 1971; Clements 1977).

The major components of surfactant are composed of phospholipids; proteins, plasma filtrates, and water are some of the minor components. Quantities of specific phospholipids varies from animal to animal, but the overall composition of surfactant is similar. Dipalmitoyl phosphatidyl choline comprises 74 to 88% of the total phospholipid content, phosphatidyl glycerol 7.8 to 10%, phosphatidylethanolamine 3.1 to 7.2% phosphatidyl inositol and phosphatidyl serine 2.5 to 5%, and sphingomyelin 0.3 to 5%. Dipalmitoyl phosphatidyl choline or lecithin, the major component of surfactant in all animals, is unique to surfactant and has the greatest *in vitro* effect on reducing surface tension (Kikkawa and Smith 1983).

The alveolar type II epithelial cell of the alveolus is the source of surfactant. Type II cells have numerous large cytoplasmic lamellar bodies, which are the site of phospholipid metabolism. When processed by the cell, the phospholipids are released into the alveolar lumen. Ultrastructurally, lamellar bodies unwind in the alveolar air space to form tubular myelin figures. Disease states that alter type II cell function and replication may also affect the production, appearance, and composition of surfactant (Mason and Williams

1977; Smith and Kikkawa 1978; Smith et al., 1980; Baritussio et al., 1981; Ward and Nicholas 1984).

Altered surfactant quality and quantity in association with disease states is best known in the disease process of newborns known as hyaline membrane disease (HMD) or respiratory distress syndrome (RDS) of the newborn. Many premature babies or those with altered metabolic states, such as infants of diabetic mothers, are born with decreased amounts of surfactant or surfactant which does not have the normal ratios of phospholipid components. Because of abnormal surfactant, infants with HMD have increased alveolar surface tension resulting in atelectasis. Affected infants develop hyaline membrane formation from the high-protein content alveolar edema. The cause of this process is related to immature type II epithelial cells or interference with type II cell metabolism (Morgan 1971; Farrell and Avery 1975; Brown and Duck-Chong 1982; Kikkawa and Smith 1983).

Because of similarities between newborn respiratory distress and ARDS, pulmonary surfactant in adult cases of ARDS has been examined. Surfactant from trauma and shock patients has increased compressibility, or increased surface tension, and contains several classes of protein-phospholipid aggregates which are abnormal (Petty et al., 1977; Petty et al., 1979). The composition of fatty acids in the phospholipids of surfactant from ARDS patients is significantly but variably altered from that of normal (Baughman et al., 1984). Protein content of surfactant from bronchoalveolar lavage fluid from ARDS cases is increased possibly due to interaction of the phospholipids with the protein-laden edema fluid. A proposed mechanism of altered surfactant in ARDS is direct or indirect damage to type II cells (Kikkawa and Smith 1983).

Surfactant has also been assayed in experimental lung disease. In mice exposed to 90% $O_2$, surfactant samples have increased surface tension; ultrastructure examination of alveoli showed areas of alveolar epithelium devoid of lining layer including lack of type II cells (Adamson et al., 1970). Subacute changes following 90% $O_2$ exposure revealed increased surfactant associated with hyperplasia and metaplasia of type II cells (Bowden and Adamson 1971). Rats exposed to 99% $O_2$ have increased amounts of protein and decreased phospholipids in bronchoalveolar lavage samples (Brashear and Christian 1973). Isolated *in vitro* rat and rabbit type II cells treated with bleomycin have increased diphosphatidyl choline synthesis (Kikkawa et al., 1976). Rats exposed to trichloroethylene, carbon tetrachloride, gasoline vapor or cigarette smoke have significantly decreased surfactant levels (LeMesurier et al., 1980). Surfactant was increased in vitamin C-deficient guinea pigs exposed to nitrogen dioxide (Selgrade et al., 1981). Rats exposed to 85% $O_2$ for 7 days developed twice the number of type II cells and lamellar bodies, and had twice the amount of surfactant (Young et al., 1982). In mice treated with butylated hydroxytoluene, proliferating type II cells decreased and the number and size of lamellar bodies decreased (Smith 1983). Mice pre-treated with steroids and then exposed to 98% $O_2$ had significantly decreased dipalmitoyl phosphatidyl choline (Gross and Smith 1984). *In vitro* type II pneumocytes exposed to x-irradiation had an increased release of surfactant (Shapiro et al., 1984).

Combining ultrastructural examination of type II cells and surfactant studies in ARDS cases and experimental studies, the type II cells tend to increase in number with increased lamellar bodies, however the quality and

quantity of phospholipids varies. An important consideration in the interpretation of these studies is the technique used to measure phospholipid content and composition in each experiment.

Functional tests of surface tension do not adequately measure surfactant. The amount and composition of phospholipids in surfactant are the determinants necessary to characterize it. Current techniques used to assay surfactant include morphologic studies of lamellar bodies fractionated lung samples, type II epithelial cell cultures, and bronchoalveolar lavage samples by electron microscopy. Morphology can denote amount, size, and shape of lamellar bodies and type II cells, but not phospholipid content. Minced or fractionated samples of lung include tissue proteins and phospholipids which may interfere with assays of phospholipids found in the lining layer alone (Kaytal et al., 1977). Bronchoalveolar lavage samples or lung tissue samples are often centrifuged at high speeds; this may alter or disrupt some surfactant components. Isolated type II cell cultures are used to study surfactant production, however they do not interact with other cells and processes such as type I cell necrosis or alveolar edema. Bronchoalveolar lavage with gentle centrifugation of the fluid obtained is one of the best methods for collecting good samples and is the technique used in man and in many experimental animal studies (Kikkawa and Smith 1983).

The most common method of determining phospholipid quantity and composition is separation of components and measurement of amounts. The best technique for accomplishing this is thin-layer chromatography (TLC) of surfactant-containing samples. Total phosphorus assays are also used, but may be inaccurate due to the small amounts of samples and because other sources

of phosphorus, besides the phospholipids, may be present. These methods of TLC were first developed and employed in the diagnosis of hyaline membrane disease in infants (Gluck et al., 1971; Brown and Duck-Chong 1982). Surfactant quantity and quality can be simply yet adequately characterized by thin-layer chromatography of bronchoalveolar lavage samples (Touchstone et al., 1983).

# CHAPTER IV

# PULMONARY EDEMA AND CELL KINETICS

## Introduction

The two major routes of lung exposure to toxic insults or inciting agents that can induce respiratory failure are via inhalation or the bloodstream. Food contaminants and air pollutants are toxins one can avoid or try to avoid. Therapeutic agents are purposely ingested or inhaled substances which can also be toxic (Rosenow 1972; Warnock 1982; Witschi and Lindenschmidt 1985). The category of cancer chemotherapeutic agents includes several substances that cause acute pulmonary failure. Chemotherapeutic agents that can induce acute lung damage include bleomycin, methotrexate, busulphan, cyclophosphamide, 6-mercaptopurine, azathioprine, melphalan, chlorambucil, procarbazine, and nitrosoureas (Luna et al., 1972; Rosenow 1972; Sostman et al., 1977; Gockerman 1982). Bleomycin is used as therapy for squamous cell carcinoma, lymphoproliferative disorders, and testicular tumors (Crooke and Bradner 1976; Porvik 1983). Unfortunately it also causes dose-dependent acute pneumonitis and pulmonary fibrosis (Crooke and Bradner 1976; Porvik 1983).

Oxygen therapy alone or in combination with other pulmonary insults can also cause ARDS. Oxygen toxicity usually occurs at inspired concentrations of 40% or higher (Robin et al., 1980). Controversy exists over whether patients treated with bleomycin and exposed to 40% or higher $O_2$ concentrations are at greater risk to develop ARDS than patients treated with bleomycin alone (Goldiner et al., 1978). Patients with pre-existing lung

damage or ARDS may also be at greater risk to develop more severe lung damage if exposed to hyperoxia (Pratt et al., 1979; Warnock 1982).

Interactions of $O_2$ and various substances have been studied in several experimental models. Agents have been given simultaneously with $O_2$ exposure, prior to $O_2$ exposure, or following $O_2$ exposure (Witschi and Hakkinen 1982). Exposure to 50% or higher $O_2$ concentrations potentiates lung damage caused by agents such as oleic acid (Cheney et al., 1980), bleomycin (Tryka et al., 1982; Haschek et al., 1983), ozone (Hackney 1981), and cyclophosphamide (Hakkinen et al., 1983).

Studies on subacute and chronic effects of exposure to both bleomycin and $O_2$ have been undertaken in various animal models. Hyperoxia, at a concentration of 70% $O_2$, and intraperitoneal bleomycin, 100 mg/kg for up to 3 weeks in mice, potentiated acute lung damage from thoracic irradiation and caused increased pulmonary type II cell hyperplasia in mice (Hakkinen et al., 1982). Exposure of hamsters to 60 to 100% $O_2$ for up to 21 days following 0.5 U/100 g intratracheal bleomycin produced more severe interstitial fibrosis than either agent alone (Rinaldo et al., 1982). Mice treated with 40 mg/kg bleomycin twice per week had decreased survival times when exposed to 40% $O_2$ (Toledo et al., 1982).

Hamsters treated with simultaneous sub-lethal doses of intratracheal bleomycin and hyperoxia had increased interstitial fibrosis and decreased survival times. Mortality and $LD_{50}$ studies were done with bleomycin alone and with bleomycin and 70% $O_2$. The $LD_{50}$ of bleomycin alone was 0.73 U/100 g body weight; the $LD_{50}$ of bleomycin and exposure to 70% $O_2$ was 0.23 U/100

g of body weight. Lesions, in animals dying up to day 6 after bleomycin treatment, were hemorrhages, DAD, hyaline membranes, and scattered prominent interstitial granulocytes. Animals treated with bleomycin and $O_2$ had more severe lesions than those treated with bleomycin alone, suggesting that the effect of combined bleomycin and $O_2$ treatment was synergistic (Tryka, et al., 1982).

The synergism of bleomycin and hyperoxia was also studied in mice and rats. All mice treated with 4 U/kg intratracheal bleomycin and kept in 80% $O_2$ for 6 days died during the $O_2$ exposure. Rats, treated with 3 U/kg bleomycin intratracheally, died either after 4 to 5 days of 70% $O_2$ or on removal to room air after 6 days of $O_2$ exposure. Lesions in both mice and rats were diffuse necrotizing bronchiolitis, severe interstitial pneumonitis, and extensive hyaline membranes. To further study these acute changes, additional mice were treated with 4 U/kg intratracheal bleomycin and exposed to 80% $O_2$. Histologic lesions similar to those seen in the first study were seen only in mice with respiratory distress and in dead mice, and not in sacrificed mice which had no signs of respiratory distress. Thus the hyaline membrane formation and other pulmonary lesions were associated with respiratory failure and death secondary to bleomycin and hyperoxia (Hakkinen et al., 1983).

Hamsters, mice and rats have been shown to develop acute lung damage secondary to interaction of intratracheal bleomycin amd 70% to 80% $O_2$ exposure (Tryka et al., 1982; Hakkinen et al., 1983). Since human patients also have had severe acute lung damage associated with bleomycin and hyperoxia (Goldiner et al., 1978), these animals were considered good models to characterize interactions of bleomycin and hyperoxia.

It was decided to develop and characterize acute changes of bleomycin and hyperoxia since these agents are used in man and have been associated with acute respiatory failure. Hamsters were chosen as the expermental animal since well-defined $LD_{50}$ studies and bleomycin-$O_2$ subacute and chronic studies have already been done (Snider et al., 1978; Tryka et al., 1982; Tryka et al., 1983).

## Materials and Methods

### General Methods

Animals were LVG/LAK male Syrian golden hamsters, *Mesocricetus auratus*, 100 to 120 g body weight, approximately 3 months old, from Charles River Breeding Laboratories, Inc., Wilmington, MA or Lakeview NJ. Hamsters were housed five per cage in standard (17 × 30 × 36 cm) shoe box plastic cages with metal bar tops. Bedding was pine wood shavings, approximately 1½ cm deep, changed once per week or as needed. Food was ad libitum Purina$^R$ rodent chow. Water was from the laboratory's general source, 16 ppm chlorine, neutralized with HCl, available ad libitum in two 150 ml containers with metal spouts. Serum samples from hamsters and mice, kept on hamster-soiled bedding in the same room, were negative when screened for antibodies to Kilham rat virus (rodent parvovirus), Sendai virus, and pneumonia virus of mice. New hamster arrivals were quarantined only if they were from a different source than hamsters already in the room.

Blenoxane$^R$, sterile bleomycin sulfate, 15 units per vial, was donated by Bristol Laboratories Division of Bristol-Meyers Co., Syracuse, NY. The

lyophilized bleomycin was reconstituted with sterile 0.9% NaCl (saline). Saline was also used as the control substance. Bleomycin, at a dose of 5 U/kg in 0.5 to 0.6 ml saline was given intratracheally to anesthetized hamsters. The bleomycin was drawn up in 1 ml plastic tuberculin or insulin syringes and administered with blunted 18 or 20 gauge 2″ metal cannulas. Saline as a control was administered in a similar volume for body weight.

Hamsters were anesthetized with methoxyflurane (Metofane$^{R}$ Pitman-Moore, Inc., Washington Crossing, NJ) poured onto cotton and placed in large glass jar exposure chambers with the hamsters. Anesthesia was deep enough if a hamster did not move when placed on hamster holders.

Hamster holders were slanted metal sheets with two pegs 3 to 4 cm apart on which a rubber band was placed. A hamster was placed in on its back against the metal sheet such that the teeth were over the rubber band and the mouth held open. The trachea was visualized by intense transillumination with a light source 1 to 2 cm cranial to the thoracic inlet. The cannula was then inserted into the trachea and bleomycin or saline was slowly injected. After treatment, hamsters were allowed to recover in cages, six per cage. Recovery was defined as the time when a hamster could walk without difficulty.

Hamsters were then exposed to 80% $O_2$ or room air (20% $O_2$). Oxygen exposures were in 60 × 60 × 110 cm Stasafe SD-4205 840A plastic gas exposure chambers from G-F Supply Division of Standard Safety, Palatine, IL. The gas flow rate was set at 16 to 20 liters/minute as measured by a Lab Crest Century gas flowmeter. Oxygen was from the general laboratory supply, and was adjusted to 80% by addition of compressed air. Oxygen concentration was measured by a Taylor Servomex Oxygen Analyzer type OA 250 made in

England under B. P. Chemical Science. Room air was air in the space adjacent to the chamber and in the room in the Laboratory Animal Facilities. Room and chamber temperature was 20 to 22°C; humidity was 50 to 60%, measured by an Airguide relative humidity gauge, Airguide Instrument Company, Chicago.

**Initial Observations**

The purpose of the initial observations was to watch for time of onset of respiratory failure. For the first observations, 40 hamsters were treated with 5 U/kg intratracheal bleomycin. Hamsters were divided into 5 cages with 8 animals in each cage. Each cage of hamsters was exposed to 80% $O_2$ for a specified length of time to determine the length of $O_2$ exposure necessary to induce respiratory failure. Cage 1 was exposed to 80% $O_2$ for 12 hours (H). Cage 2 was exposed to 80% $O_2$ for 24 H, cage 3 for 48 H, cage 4 for 72 H, and cage 5 for 96 H (Table 4.1). After the specified $O_2$ exposure, hamsters were removed from the $O_2$ chamber and placed in room air. Hamsters were observed for signs of respiratory failure such as dyspnea and tachypnea. Animals were also monitored for deaths; times of death were recorded to determine the length of $O_2$ exposure required to induce respiratory failure and cause death.

The lungs of all hamsters were examined for gross lesions. Selected representative lungs from hamsters in each $O_2$ exposure group were fixed in 10% buffered neutral formalin (BNF). For histologic examination, samples of

**Table 4.1. Hamster Treatments for Initial Observations of Respiratory Failure and Death**

| Treatment Group | Length of Exposure to 80% $O_2$ |
|---|---|
| Cage 1[a] | 12 Hours[b] |
| Cage 2 | 24 Hours |
| Cage 3 | 48 Hours |
| Cage 4 | 72 Hours |
| Cage 5 | 96 Hours |

[a]All groups had 8 hamsters. All hamsters were treated with 5 U/kg bleomycin intratracheally prior to 80% $O_2$ exposure.

[b]Animals were placed in room air, 20% $O_2$, after the period of 80% $O_2$ exposure.

lungs were embedded in paraffin, sectioned at 5 to 6 um, mounted on glass slides, and stained with hematoxylin and eosin.

**Edema Studies**

**Iodine-125-Labelled Bovine Serum Albumin.** Since pulmonary edema occurred in the initial observations, it was important to follow the development of edema and determine its onset. The development of pulmonary edema was followed with the radioactive tracer, iodine-125-labelled bovine serum albumin ($^{125}$I-BSA). The radiolabelled albumin, 1.3 uCi/mg, 2.7 uCi/ml, molecular weight 60,000 daltons, was obtained from New England Nuclear, Boston, MA. The albumin was diluted with saline to 100 ml and kept frozen in sterile glass vials, 10 ml per vial, until use. One hour prior to use, $^{125}$dRBSA was removed from the freezer and allowed to thaw at room temperature. Hamsters were

injected intraperitoneally with 0.1 to 0.5 ml or 1 uCi of $^{125}$I-BSA two hours prior to measurement to allow for absorption and equilibration of the albumin throughout the tissues. This equilibration period was determined by test studies done with hamsters prior to the main experiment.

Treatment groups were intratracheal saline and air (20% $O_2$) (SA), intratracheal saline and 24 H 80% $O_2$ (SO), intratracheal bleomycin and air (BA), and intratracheal bleomycin and 24 H 80% $O_2$ (BO) (Table 4.2).

**Table 4.2. Hamster Treatment and Exposure Groups**

| Treatments | Exposures | |
|---|---|---|
| 5 U/kg Bleomycin | 20% $O_2$, room air (BA) | 24 H 80% $O_2$, hyperoxia (BO) |
| Saline | 20% $O_2$ (SA) | 24 H 80% $O_2$ (SO) |

Hamsters were divided six per cage into groups of 24 per treatment group and 24 per day for five days for a total of 120 animals. Six animals were killed in each treatment group at the end of 24 H time period for four days. Twenty-four hamsters were killed at the end of each 24 H, six from group SA, six from group SO, six from group BA, and six from group BO. Six animals from each treatment group were also killed at the end of 48, 72, and 96 H. Timepoints were designated as hours after intratracheal bleomycin or saline.

Animals were deeply anesthetized by intraperitoneal injection of 0.25 ml Na pentobartital, 1 mg/ml Nembutal$^R$ (Abbott Laboratories, North Chicago,

IL). From each hamster, one ml of blood was collected from the jugular area to avoid contamination with peritoneal $^{125}$I-BSA. Blood was collected in 3 ml plastic syringes. Lungs were perfused intravascularly with 5 to 8 ml of saline via the pulmonary artery. This was to remove all pulmonary intravascular labelled albumin. Lungs were then removed and rinsed in clear water to remove $^{125}$I-BSA in the blood on the pleural surfaces. Each sample of lungs or blood was placed in 10 × 75 mm borisilicate glass test tubes (Kimble, Owens-Illinois).

Samples were kept numbered in pairs, one blood and one lung, from each hamster. The radioactivity of each sample was measured and counted by an Intertechnique CG4000 gamma counter (IN/US, Fairfield, NJ). Each sample was counted for 4 minutes or approximately 5000 disintegrations on the channel set for $^{125}$I, 35 and 66 KeV.

The final number obtained, the pulmonary extravascular albumin ration (PEAR), was defined as counts per minute (cpm) $^{125}$I in lung divided by cpm $^{125}$I in one ml of blood (Johnson and Ward 1974).

**Technetium-99m Diethylenetriaminepentaccetic acid**

Pulmonary edema development was also followed with technetium-99m-diethylenetriaminepentaacetic acid ($^{99m}$Tc-DTPA). A small radiolabelled substance was selected to see if leakage of substances smaller than albumin occurred at earlier timepoints. Radiolabelled DTPA, molecular weight 493 daltons, 42.4 mCi/ml was prepared by Nuclear Pharmacy Incorporated (Knoxville, TN) from $^{99m}$Tc and stannous DTPA pentatate. The $^{99m}$Tc-DTPA

was diluted to 20 uCi per ml with saline. Because of the short radioactive half-life of 6 hours, $^{99m}$Tc-DTPA was prepared and diluted each day of use. One tenth ml of tracer, 2 uCi, was injected intraperitoneally in hamsters one hour prior to sacrifice to allow for absorption and tissue equilibration. This equilibrium time was determined by test studies done with hamsters prior to the main experiment.

Animals were divided into six hamsters per group. Treatment and exposure groups were SA and BO at 24, 48, and 72 hours for a total of 36 hamsters. Sacrifice and lung and blood sample collection were the same as for $^{125}$I-BSA. Each sample was counted for 1 minute or approximately 20,000 disintegrations. The Intertechnique gamma counter was used, set for 57 Cobalt, 122 KeV, the available pre-set channel closest to $^{99m}$Tc emissions. The resulting number, the pulmonary extravascular DTPA ratio (PED), was defined as cpm $^{99m}$Tc-DTPA per lung divided by cpm $^{99m}$Tc-DTPA per 1 ml blood (Effros and Mason 1983).

**Autoradiography**

Cell kinetic studies, by autoradiography, were done to measure pulmonary repair and regeneration responses secondary to damage. Tritiated thymidine ($^{3}$H-TdR), specific activity, 1.9 Ci/mmole, 1 mCi/ml, 99% labelled, in a 2% ethanol solution, was obtained from New England Nuclear, Boston, MA. To label cells synthesizing DNA, 200 uCi or 0.2 ml of $^{3}$H-TdR was injected intraperitoneally in each hamster one hour prior to sacrifice. Hamsters were treated and exposed as for $^{125}$I-BSA edema studies. Six hamsters from

each group, SA, SO, BA, and BO (Table 4.2) were killed at 24, 48, 72, and 96 H after for a total of 120 hamsters. Animals were killed by intraperitoneal injection of 0.25 ml of Na pentobarbital and subsequent laceration of the abdominal aorta.

Lung airways were filled *in situ* with 10% BNF until full at 25 to 30 cm of pressure; filled lungs remained under that pressure for 10 minutes. The trachea was ligated and the lungs were removed and placed in 10% BNF for a minimum of 48 H. Three samples were cut from each set of lungs; one was from the left lobe, one from the right cranial lobe, and one from the right caudal lobe. Samples were processed and embedded in the plastic medium, glycol methacrylate. One micron thick sections of tissue were cut and mounted on glass slides which were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, New York) and kept in the dark for 14 days, then developed in Kodak D-19 Developer and fixed with Kodak Fixer. Slides were then stained with Lee's methylene blue and basic fuchsin.

Radiolabelled cells were defined as cells with 5 or more silver grains over the nucleus. Only cells in alveolar tissue were counted. The number of labelled cells per 2000 total cells were counted; 1000 cells were counted in the left lung lobe section and 500 in each of the two smaller right lung sections. Airways, large blood vessels, pleura, and interlobular septa were excluded. The labelling index (LI) of the lung was defined as the percent of labelled cells per 2000 cells counted.

Labelled cells were differentiated into one of four cell types: macrophage (Figure 4.1), type II epithelial cell (Figure 4.2), endothelial cell (Figure 4.3) or interstitial cell (Figure 4.4). Type I epithelial cells do not

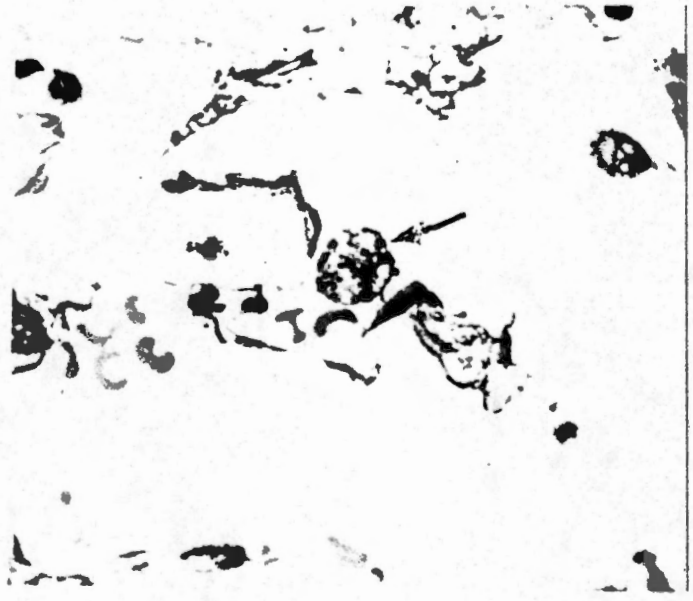

**Figure 4.1. Radiolabelled alveolar macrophage.** (arrow) From 48 H Bleomycin and Air group. Lee's methylene blue and basic fachsin. (Magnification 400X).



**Figure 4.2. Radiolabelled type II alveolar epithelial cell.** (arrow) From 48 H Bleomycin and Air group lung. Lee's methylene blue and basic fuchsin. (Magnification 400X).

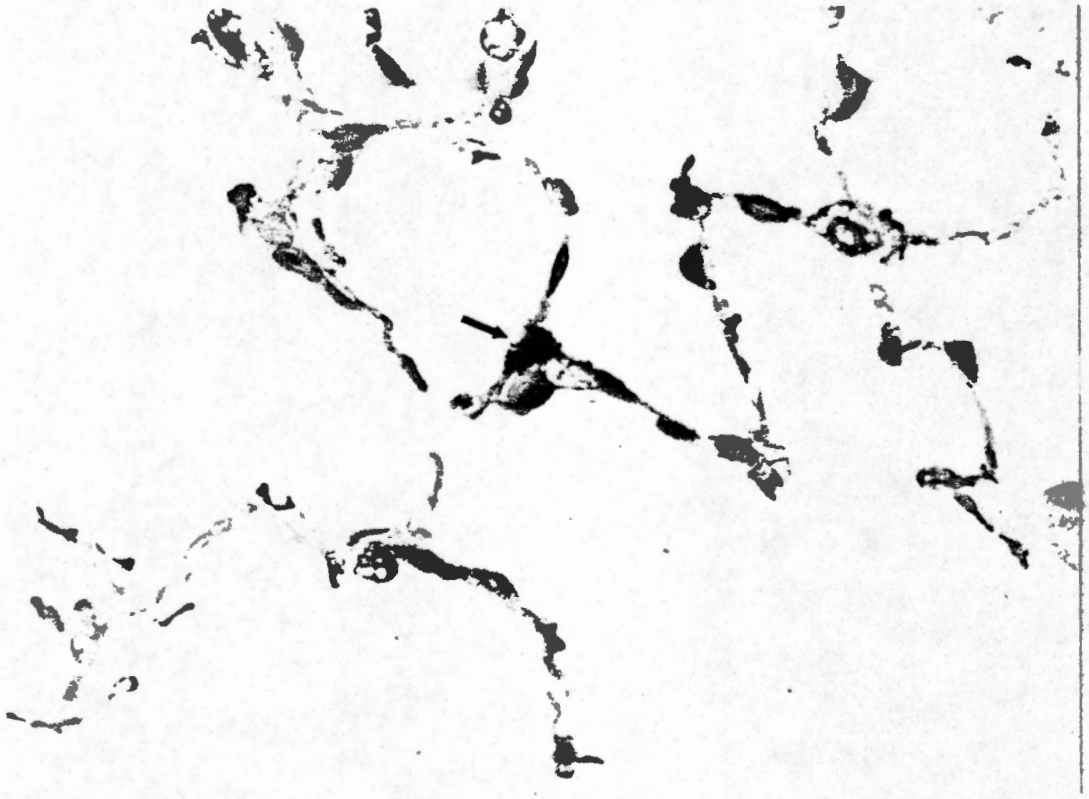

**Figure 4.3. Radiolabelled alveolar capillary endothelial cell.** (arrow) From lung of 48 H Bleomycin and Air group. Lee's methylene blue and basic fuchsin. (Magnification 400X).

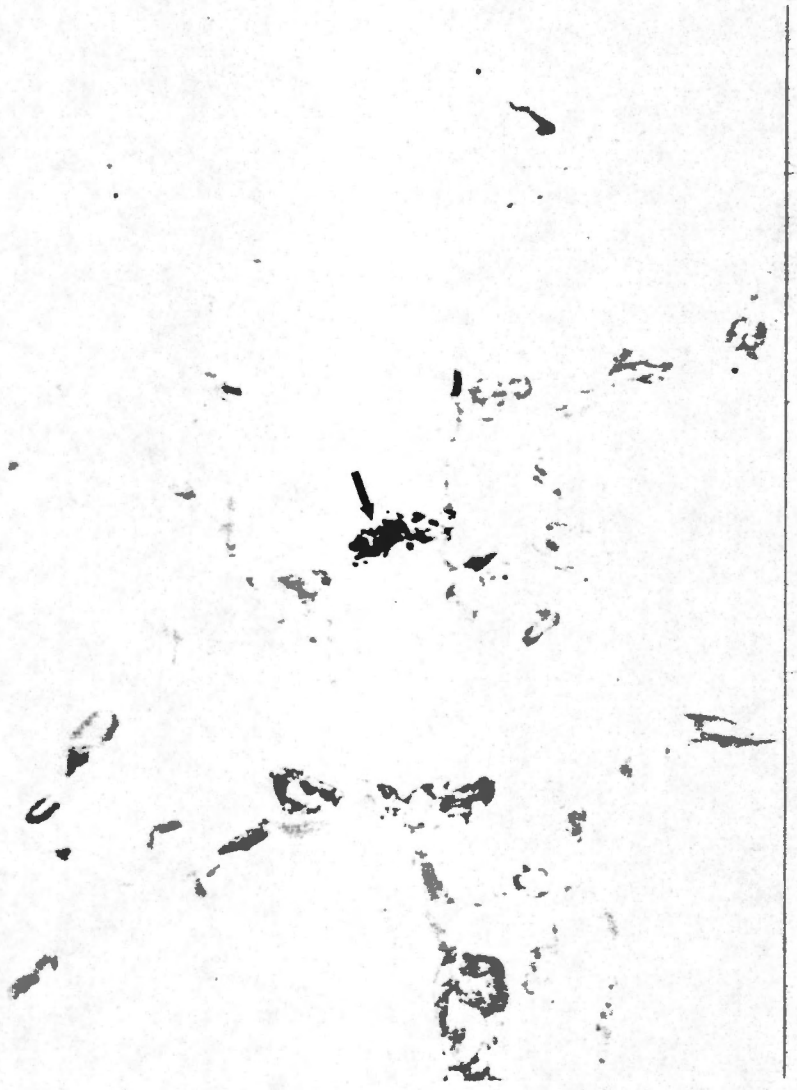

**Figure 4.4. Radiolabelled alveolar interstitial cell.** (arrow) From Bleomycin and Air 48 H group lung. Lee's methylene blue and basic fuchsin. (Magnification 400X).

synthesize DNA and can be labelled only as a result of type II cell division. Endothelial cells, were defined as cells forming capillaries, generally triangular or polyhedral to spindle-shaped cells located in alveolar corners; the nucleus may protrude into the vessel lumen. Macrophages were defined as large cells in alveolar air spaces or along epithelial surfaces with single irregular-shaped nuclei and abundant often basophilic cytoplasm usually containing multiple irregular vacuoles. Type II epithelial cells were defined as large, often polyhedral cells lining alveoli, usually found in corners and often with prominent fairly uniform cytoplasmic vacuoles and pale eosinophilic cytoplasm. Interstitial cells were defined as the fibroblasts in the alveolar interstitium. Other labelled cells, predominantly leukocytes other than macrophages, were not counted.

If 100 labelled cells were not counted for the labelling index a differential count was also done; 100 labelled cells, 50 from the left lung lobe and 25 from each of the right lobes, were counted and differentiated. If 100 total labelled cells could not be found, all labelled cells observed were differentiated. The differential cell counts were all recorded as percents, usually number of labelled specific cells per 100 labelled cells. Results of differential counts were reported as number of labelled specific cell type per 10,000 cells. Labelled specific cell types per 10,000 cells was determined as LI X 10,000 X Differential of specific cell type.

### Analysis of Data

Paired samples were analyzed by student's paired t-test. Other values were compared using one-way analysis of variance and Tukey's honestly significant difference tests (Daniel 1983). Statistical programs used were those on the IBM SAS program system (IBM 3033). Differences in values were considered significant if a p value of less than 0.05 was obtained. The saline and air group values were considered the control values.

## Results

### Initial Observations

Hamsters treated with bleomycin intratracheally and exposed to from 12 to 120 H of 80% $O_2$ began to die at 72 H (Table 4.3). Most of the animals, 75%, died between 72 and 120 H after bleomycin treatment regardless of length of $O_2$ exposure (Figure 4.5). All animals were dead by 120 hours. Moribund animals had marked dyspnea, rales, and often serosanguinous foam around the nose and mouth. Gross examination of lungs from animals dying at 72 H and later revealed marked diffuse pulmonary edema and congestion, multifocal hemorrhages, and atelectasis. On histologic examination, lungs had changes characteristic of DAD. Major lesions were diffuse focally intense interstitial and alveolar edema. Alveolar and interstitial infiltrates of macrophages and some neutrophils, variably sized foci of perivascular hemorrhages, and small foci of epithelial cells with karyolysis and karyorrhexis

**Table 4.3. Initial Observations of Mortality After Bleomycin Treatment**

| Duration of Exposure of 80% $O_2$[a] (Hours) | Number of Dead Hamsters Found in each Time Period | | | | |
|---|---|---|---|---|---|
| | Time of Observation in Hours | | | | |
| | 24 | 48 | 72 | 96 | 120 |
| 12 | 0 | 0 | 1 | 1 | 6 |
| 24 | 0 | 0 | 0 | 1 | 7 |
| 48 | 0 | 0 | 0 | 0 | 8 |
| 72 | 0 | 0 | 2 | 3 | 3 |
| 96 | 0 | 0 | 2 | 4 | 2 |

[a]Hamsters in groups of 8 were exposed to 80% $O_2$ in chambers for specified length of time after 5 U/kg intratracheal bleomycin.

were also in sections examined. Hyaline membranes were few, scattered, and small.

**Pulmonary Edema**

All results, PEAR and PED measurements, were compared to values of group SA, the control group at the same timepoint. Results of PEAR studies are summarized in Table 4.4. Edema, measured by the 60,000 dalton substance $^{125}$I-BSA, did not occur in groups SA, SO, or BA at any timepoint. Group BO hamsters had no measurable edema at 24 and 48 H. Significant edema, as determined by an increased PEAR, did occur in group BO at 72 and 96 H (Table 4.4, Figure 4.6).

**Table 4.4. Pulmonary Extravasular Albumin Ratio (PEAR)[g] of Hamsters**

| Treatment | Hours After Treatment | | | | |
|---|---|---|---|---|---|
| | 24 | 48 | 72 | 80 | 96 |
| SA[a] | 0.135[e] ±0.008 | 0.126 ±0.016 | 0.136 ±0.013 | 0.139 ±0.007 | 0.0140 ±0.017 |
| SO[b] | 0.138 ±0.022 | 0.182 ±0.052 | 0.112 ±0.011 | 0.153 ±0.052 | 0.162 ±0.023 |
| BA[c] | 0.216 ±0.020 | 0.168 ±0.007 | 0.211 ±0.023 | 0.159 ±0.040 | 0.194 ±0.047 |
| BO[d] | 0.152 ±0.016 | 0.151 ±0.018 | 0.344[f] ±0.036 | 0.333[f] ±0.049 | 0.317[f] ±0.033 |

[a]Saline and air.
[b]Saline and 80% $O_2$.
[c]Bleomycin and air.
[d]Bleomycin and 80% $O_2$.
[e]Mean ± Standard deviation.
[f]Significant at $p < 0.05$, compared to values for group SA at the same timepoints.

$$^{g}\text{PEAR} = \frac{^{125}\text{I}-\text{BSA cpm in lung}}{^{125}\text{I}-\text{BSA cpm in blood}}$$

Results of PED studies are summarized in Table 4.5. Edema, measured by the 493 dalton substance $^{99m}$Tc-DTPA, did not occur in group SA at any timepoint. Leakage of $^{99m}$Tc-DTPA was not measured in groups SO or BA. Edema, determined by increased PED, did not occur in group BO at 24 and 48 H, but was significant in group BO at 72 H.

Therefore, significant increases in PEAR and PED occurred only in Group BO animals and only at 72 H and later. Both a large molecular weight

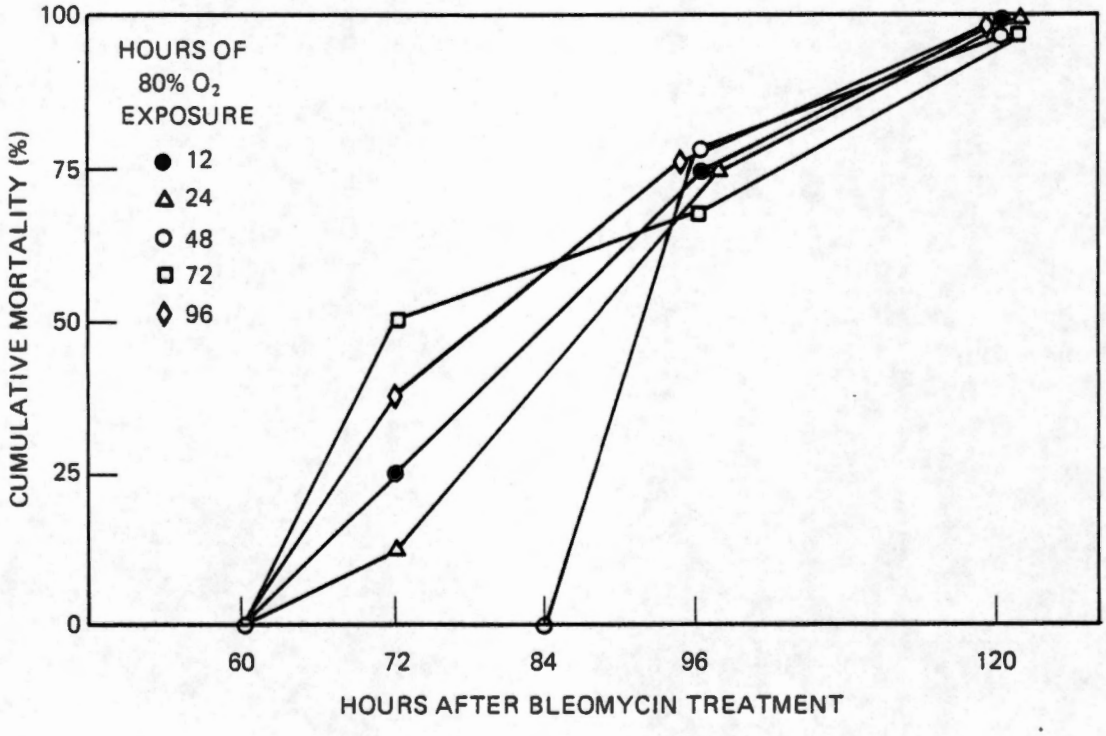


**Figure 4.5. Cumulative mortality of hamsters after Bleomycin treatment and 80% $O_2$ exposure.** Hamsters in all exposure groups, except the 48 H group, began to die 72 H after 80% $O_2$ exposure. By 96 H, hamsters in all exposure groups were dying. All hamsters were dead by 120 H after initial 80% $O_2$ exposure.

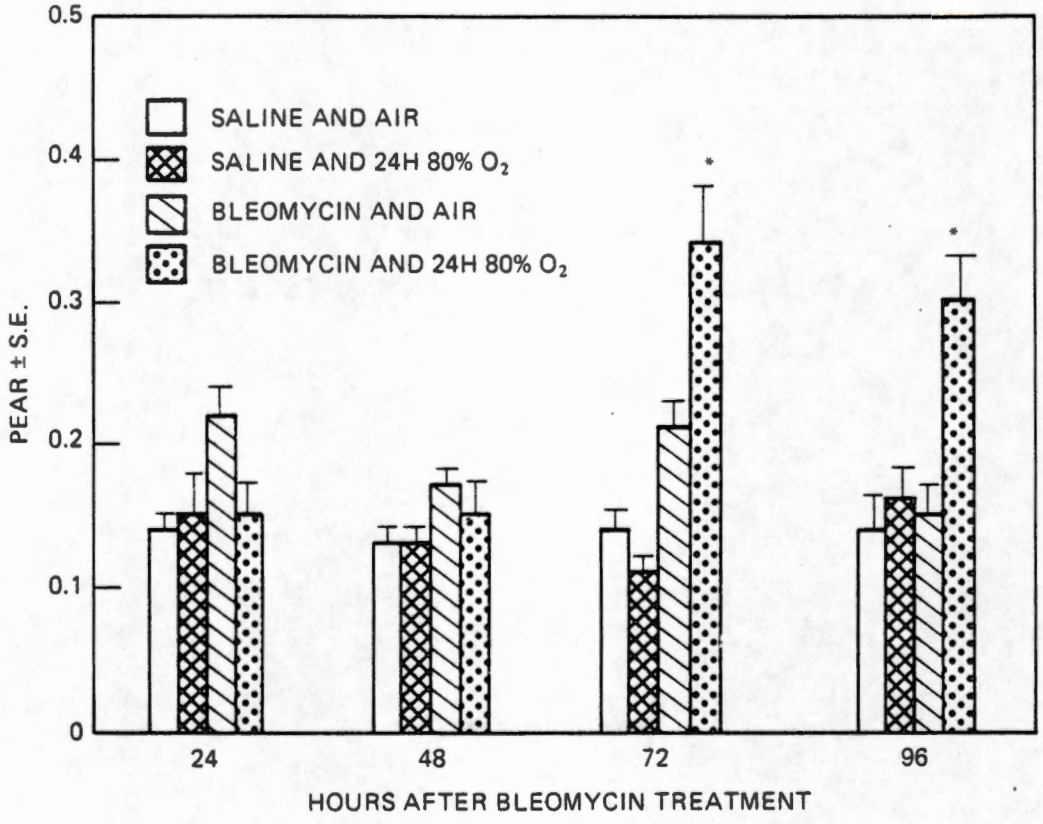


**Figure 4.6. Pulmonary edema measured by PEAR[a].** PEAR was not increased in any treatment group at 24 or 48 H after treatment. At 72 H and 96 H after treatment, PEAR was significantly increased only in the Bleomycin and 24 H 80% $O_2$ treament group.

$$^{a}\text{PEAR} = \frac{^{125}\text{I}-\text{BSA cpm in lung}}{^{125}\text{I}-\text{BSA cpm in blood}}$$

[b]Significant at $p < 0.05$ compared to values of Saline and Air, Saline and 24 H 80% $O_2$, and Bleomycin and Air groups.

**Table 4.5. Pulmonary Extravascular DTPA Ratio (PED) of Hamsters**

| Treatment[c] | Hours After Treatment | | |
|---|---|---|---|
| | 24 | 48 | 72 |
| Saline and Air | 0.283 ± 0.025[a] | 0.191 ± 0.018 | 0.206 ± 0.019 |
| Bleomycin and 24 H 80% $O_2$ | 0.343 ± 0.021 | 0.348 ± 0.037 | 0.821 ± 0.073[b] |

[a]Mean ± standard error.

[b]Significant at $p < 0.05$ compared to Saline and Air values at the same timepoints.

[c]Six hamsters were in each treatment group.

substance, $^{125}$I-BSA, and a small molecular weight substance, $^{99m}$Tc-DTPA, were significantly increased in nonvascular lung tissue at 72 H and not before.

**Autoradiography**

**Cell Kinetics.** Group SA was the control group; results of groups SO, BA, and BO were compared to group SA values at the same timepoint. Results are summarized in Tables 4.6 through 4.10. No significant changes were observed in the labelling indices of groups SA or SO at 24, 48, 72 or 96 H. At 96 H, groups BA and BO had significant increases in the labelling index (Figure 4.7).

Groups SA and SO had no significant changes in macrophage labelling at any timepoint. Groups BA and BO had no significant change at 24, 48, or 72 H. At 96 H, both groups BA and BO had significantly increased

**Table 4.6. Labelling Index (Percent Labelled Cells Per 2000 Cells) of Pulmonary Alveolar Tissue**

| Treatment[a] | Hours After Treatment | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| SA[b] | 0.6 ± 0.2[f] | 1.3 ± 0.3 | 1.1 ± 0.4 | 0.7 ± 0.2 |
| SO[c] | 1.1 ± 0.4 | 0.9 ± 0.3 | 0.5 ± 0.2 | 0.9 ± 0.2 |
| BA[d] | 0.7 ± 0.2 | 0.9 ± 0.1 | 1.2 ± 0.2 | 1.9 ± 0.5[g] |
| BO[e] | 0.5 ± 0.1 | 0.7 ± 0.2 | 1.0 ± 0.2 | 1.8 ± 0.3[g] |

[a]Six hamsters wre in each treatment group at each time point.

[b]Saline and 20% Air.

[c]Saline and 24 H 80% $O_2$.

[d]Bleomycin and 20% Air.

[e]Bleomycin and 24 H 80% $O_2$.

[f]Mean ± standard error.

[g]Significant at $p < 0.05$ compared to group SA values at the same timepoints.

**Table 4.7. Labelled Alveolar Macrophages per 10,000 Cells**

| Treatment[a] | Hours After Treatment | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| Saline and Air | 18 ± 6[b] | 29 ± 6 | 23 ± 11 | 19 ± 5 |
| Saline and 80% $O_2$ | 29 ± 8 | 31 ± 10 | 14 ± 7 | 23 ± 7 |
| Bleomycin and Air | 10 ± 5 | 19 ± 7 | 32 ± 7 | 74[c] ± 17 |
| Bleomycin and 80% $O_2$ | 6 ± 3 | 16 ± 3 | 42 ± 10 | 93[c] ± 18 |

[a]Six hamsters were in each treatment group at each timepoint.

[b]Mean ± standard error.

[c]Significant at $p < 0.05$ compared to group SA values at the same timepoints.

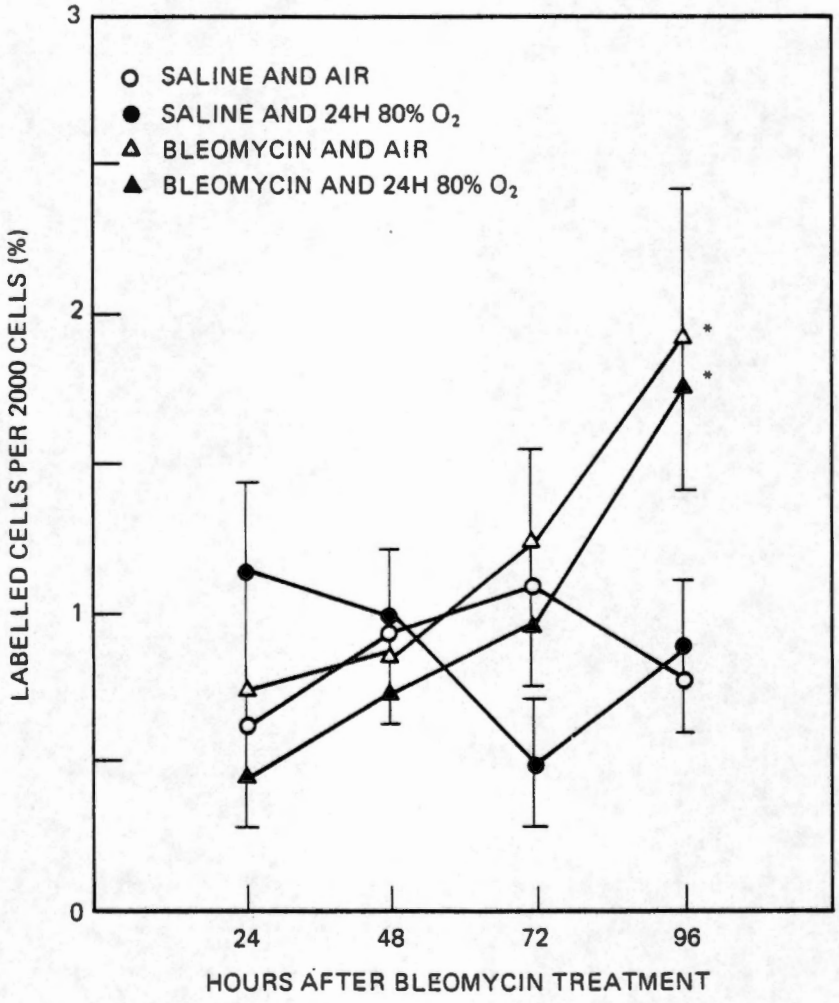


**Figure 4.7. Autoradiography-derived labelling indices.** At 96 H, the Bleomycin and Air and Bleomycin and 24 H 80% $O_2$ groups had significant increases in the labelling index.

*Significant at $p < 0.05$ compared to values of the Saline and Air group at the same timepoints.

**Table 4.8. Labelled Type II Epithelial Cells per 10,000 Cells**

| Treatment[a] | Hours After Treatment | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| Saline and Air | 4 ± 1[b] | 11 ± 2 | 15 ± 6 | 8 ± 3 |
| Saline and 80% $O_2$ | 8 ± 4 | 24 ± 11 | 6 ± 3 | 8 ± 3 |
| Bleomycin and Air | 1 ± 1 | 4 ± 0 | 6 ± 1 | 19[c] ± 7 |
| Bleomycin and 80% $O_2$ | 2 ± 1 | 3 ± 1 | 4 ± 2 | 4 ± 2 |

[a]Six hamsters were in each treatment group at each timepoint.

[b]Mean ± standard error.

[c]Significant at $p < 0.05$ compared to group SA values at the same timepoints.

**Table 4.9. Labelled Endothelial Cells per 10,000 Cells**

| Treatment[a] | Hours After Treatment | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| Saline and Air | 21 ± 8[b] | 33 ± 5 | 35 ± 14 | 24 ± 5 |
| Saline and 80% $O_2$ | 40 ± 12 | 20 ± 5 | 15 ± 5 | 30 ± 5 |
| Bleomycin and Air | 34 ± 12 | 31 ± 4 | 41 ± 8 | 52 ± 17 |
| Bleomycin and 80% $O_2$ | 18 ± 4 | 27 ± 6 | 21 ± 4 | 30 ± 7 |

[a]Six hamsters were in each treatment group at each timepoint.

[b]Mean ± standard error.

**Table 4.10. Labelled Alveolar Interstitial Cells per 10,000 Cells**

| Treatment[a] | Hours After Treatment | | | |
|---|---|---|---|---|
| | 24 | 48 | 72 | 96 |
| Saline and Air | 20 ± 8[b] | 26 ± 4 | 36 ± 15 | 21 ± 4 |
| Saline and 80% $O_2$ | 37 ± 17 | 16 ± 3 | 13 ± 4 | 27 ± 9 |
| Bleomycin and Air | 28 ± 7 | 33 ± 5 | 42 ± 9 | 48 ± 12 |
| Bleomycin and 80% $O_2$ | 19 ± 5 | 26 ± 5 | 31 ± 6 | 43 ± 8 |

[a]Six hamsters were in each treatment group at each timepoint.
[b]Mean ± standard error.

macrophage labelling; group BO macrophage labelling was significantly greater than the group BA increase in label (Figure 4.8).

Statistically significant changes in type II epithelial cell labelling when compared to group SA values were not seen in groups SO or BO at 24, 48, 72 or 96 H. Group BA had a significant increase in labelled type II cells at 96 H. Group BO type II cell label was at a constant low level; however these values were not significantly different from those of groups SA or SO at any timepoint (Figure 4.9).

Endothelial cell label values had no significant changes at any timepoint (Figure 4.10). Values for groups SA, SO, BA, and BO were not statistically different at any time point. Interstitial cell label was similar to endothelial cell values; no result for groups SA, SO, BA, and BO at 24, 48, 72, and 96 H was statistically significant (Figure 4.11).

**Histologic Observations.** Lungs prepared for autoradiography were examined for lesions by light microscopy. Group SA hamsters, the control

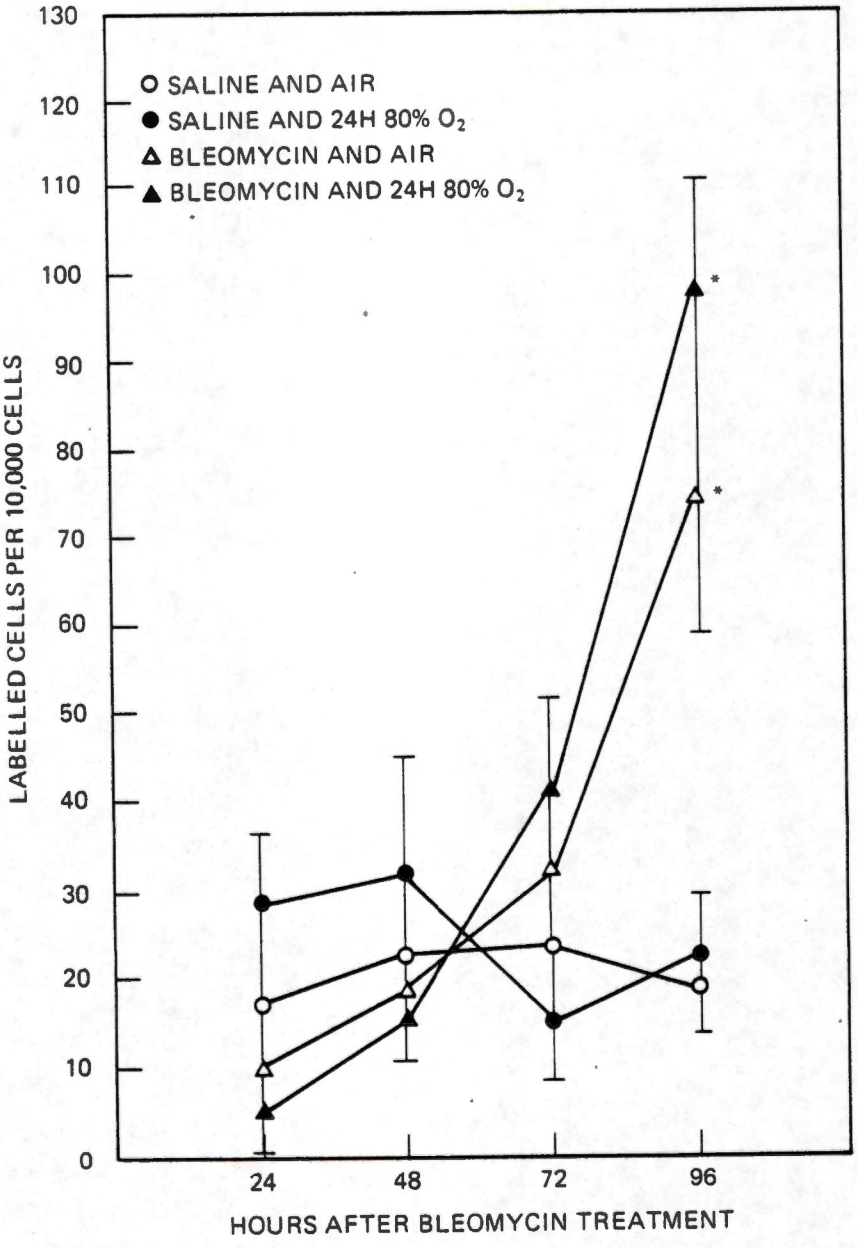


**Figure 4.8. Labelled alveolar macrophages per 10,000 cells.** Significant increases in labelled macrophages occurred at 96 H in the Bleomycin and Air and the Bleomycin and 24 H 80% $O_2$ treatment groups.

*Significant at $p < 0.05$ compared to values of the Saline and Air group at the same timepoints.

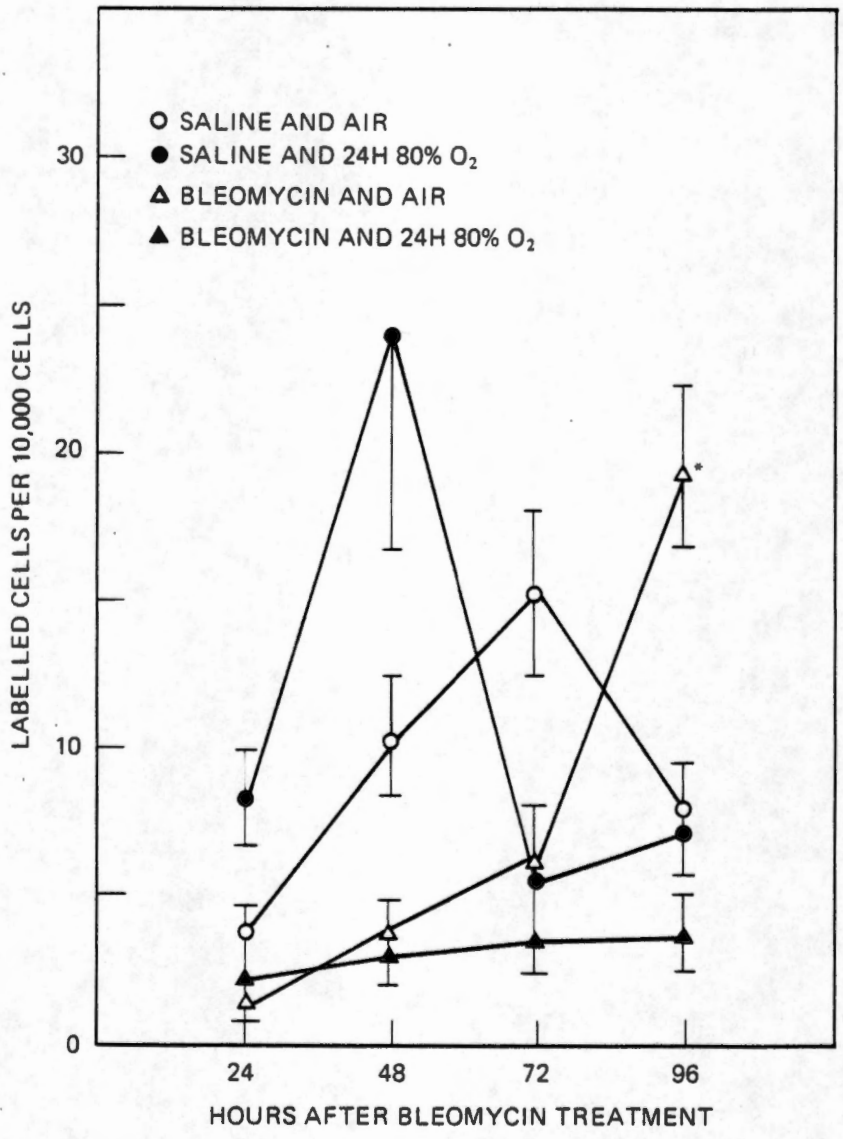


**Figure 4.9. Labelled type II alveolar epithelial cells per 10,000 cells.** No significant change in label was observed until 96 H. The Bleomycin and Air group had an increased type II cell label at 96 H.

*Significant at $p < 0.05$ compared to Saline and Air vaues at the same time point.

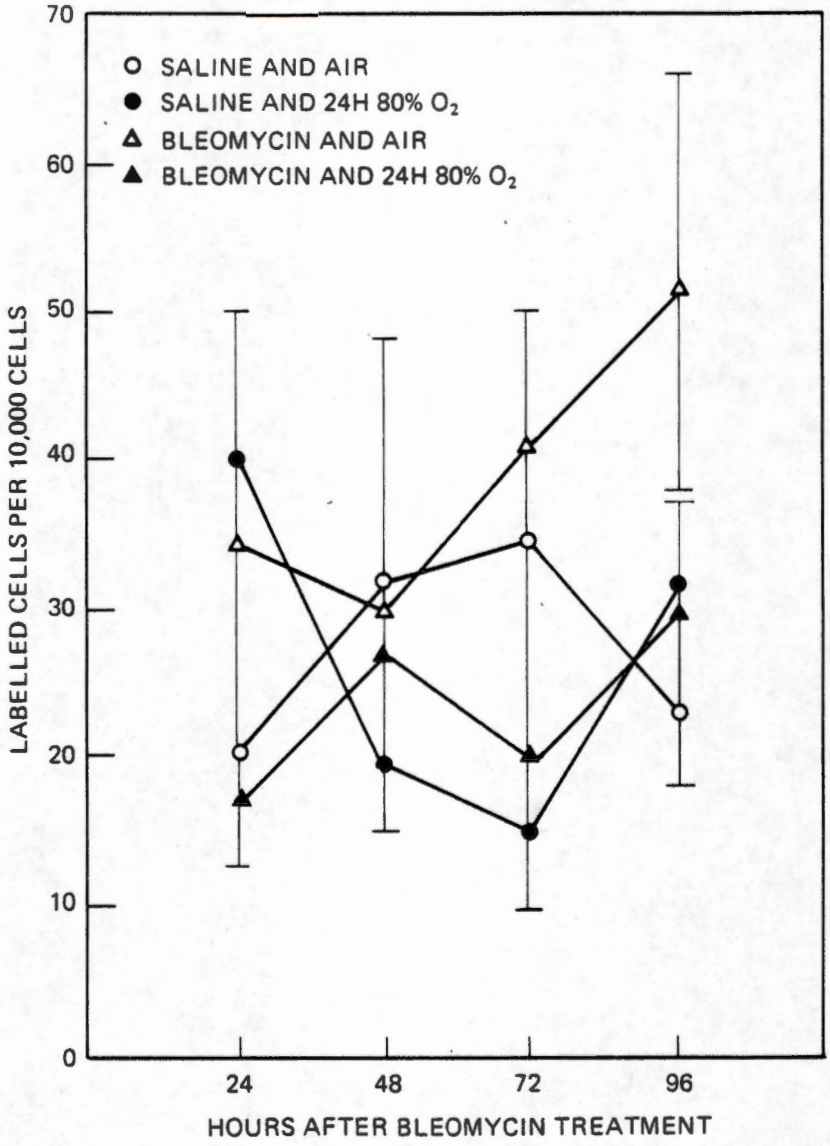


**Figure 4.10. Labelled alveolar capillary endothelial cells per 10,000 cells.** No significant increase in label was observed at any timepoint in any treatment group. Saline and Air values are the control values.

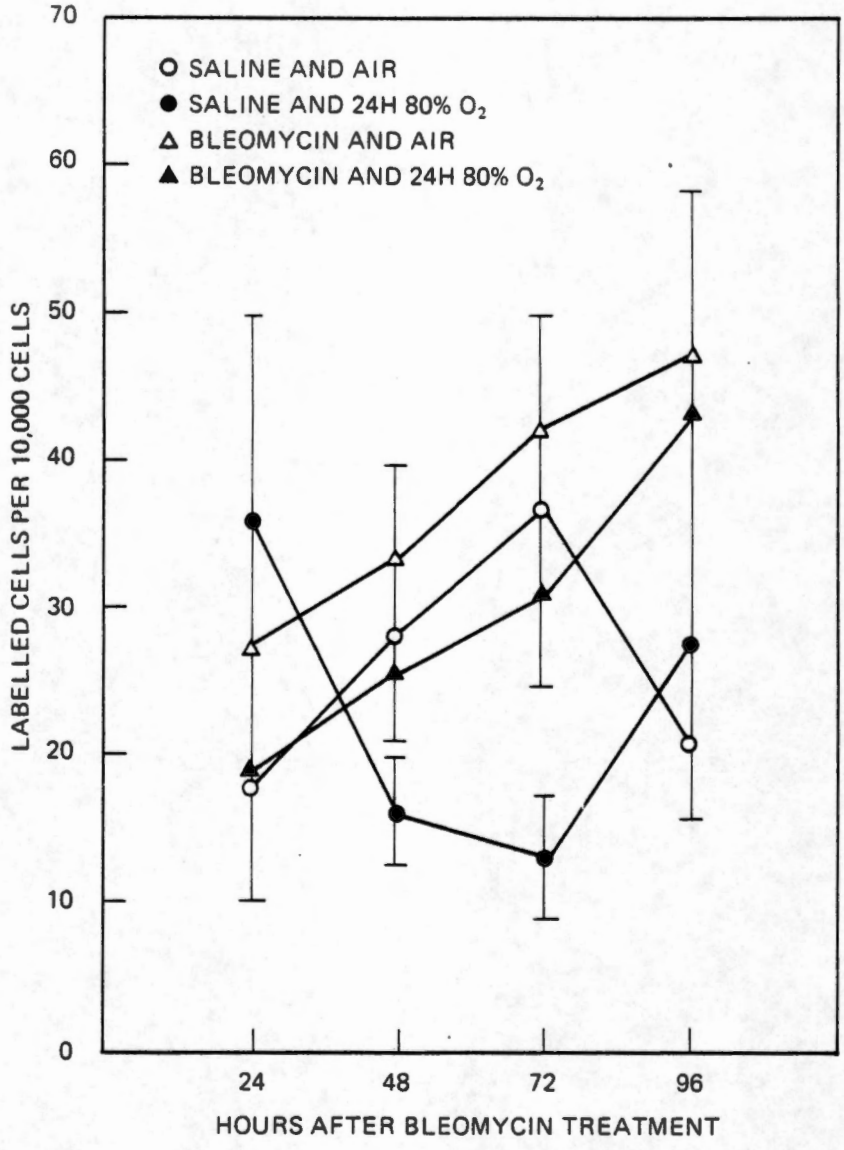


**Figure 4.11. Labelled alveolar interstitial cells per 10,000 cells.** No significant labelling increase was observed at any timepoint in any treatment group. The Saline and Air group values are the controls.

animals, had no significant lesions at any timepoint. The bronchi and bronchioles, vessels, and pleura were normal. In the alveolar air spaces and septa were a few very small foci of slightly increased numbers of macrophages at all timepoints (Figure 4.12).

Lungs from hamsters in group SO had mild changes. At 24 H small foci of interstitial and alveolar macrophages and a few associated granulocytes were scattered throughout the parenchyma. By 48 H mild perivascular and peribronchial edema was observed around the larger vessels and airways. A few macrophages and granulocytes were in the perivascular spaces. The foci of macrophages and granulocytes were slightly larger. The 72 H sections had diffuse often focally increased interstitial granulocytes. At 96 H the edema around vessels and airways was mild to moderate and extended to bronchioles, arterioles, and vessels. A mild infiltrate of macrophages and granulocytes was seen in the edema spaces. Alveolar septa had slight diffuse thickening. Occasional areas had large foci of macrophages and some granulocytes in alveolar air spaces and interstitium (Figure 4.13). Pleura and airways were normal.

Group BA animals at 24 H had changes similar to but more severe than those seen in group SO animals at 24 H. At 48 H, the perivascular and peribronchial edema was slightly increased in group BA hamsters. The alveolar air space and interstitial infiltrate of macrophages and granulocytes was mild and diffuse. Lungs at 72 H had mild to moderate infiltrates of macrophages and some granulocytes in alveolar air spaces, interstitium, and perivascular spaces. The 96 H BA hamster lungs had mild to moderate perivascular and



**Figure 4.12. Photomicrograph of Saline and Air treated lungs at 96 H.** Vessels, airways, and alveoli are normal. In some small foci are increased numbrs of alveolar macrophages. Lee's methylene blue and basic fuchsin. (Magnification 200X).



**Figure 4.13. Photomicrograph of Saline and 24 H 80% $O_2$ treated lungs at 96 H.** Small foci of macrophages and interstitial and air space granulocytes and erythrocytes are scattered throughout the parenchyma. Lee's methylene blue and basic fuchsin. (Magnification 200X).

peribronchial edema with macrophages and s,ome granulocytes and alveolar infiltrates similar to that seen at 72 H. Therefore lungs of group BA animals progressed from mild to moderate edema around vessels and airways and developed a mild to moderate infiltrate comprised primarily of foamy macrophages and some granulocytes (Figure 4.14). Alveolar septa were also moderately thickened.

Hamster lungs from group BO had variable mild to moderate perivascular and peribronchial edema at 24 H. Small foci of macrophages, as previously described in group SA, SO, and BA lungs, were larger and more numerous on day one in group BO than in those of the other groups. These foci of macrophages had associated small hemorrhages. The alveolar septa were mildly thickened in these foci. Macrophages were prominent with foamy, vacuolated cytoplasm. Granulocytes, prominent around vessels, were also mildly increased in numbers (Figure 4.15, page 76 ). At 48 H the edema was increased and extended along smaller vessels and airways. Alveolar septa in more foci were mildly thickened. The foci of macrophage and granulocyte infiltrates were larger and more moderate. Scattered single alveoli or small groups of alveoli contained edema fluid and cellular debris in the airspaces. Occasional alveoli were atelectatic (Figure 4.16, page 77). The 72 H lung sections had moderate edema along vessels and airways (Figure 4.17, page 78 ); pleural surfaces were normal. Endothelial cells in most vessels were edematous and bulged into vessel lumens; endothelial cell cytoplasm often had clear spaces indicating cell swelling. Alveolar septa had moderate diffuse thickening. A diffuse, marked infiltrate of macrophages also had associated scattered alveolar interstitial and air space granulocytes, hemorrhages, hyaline

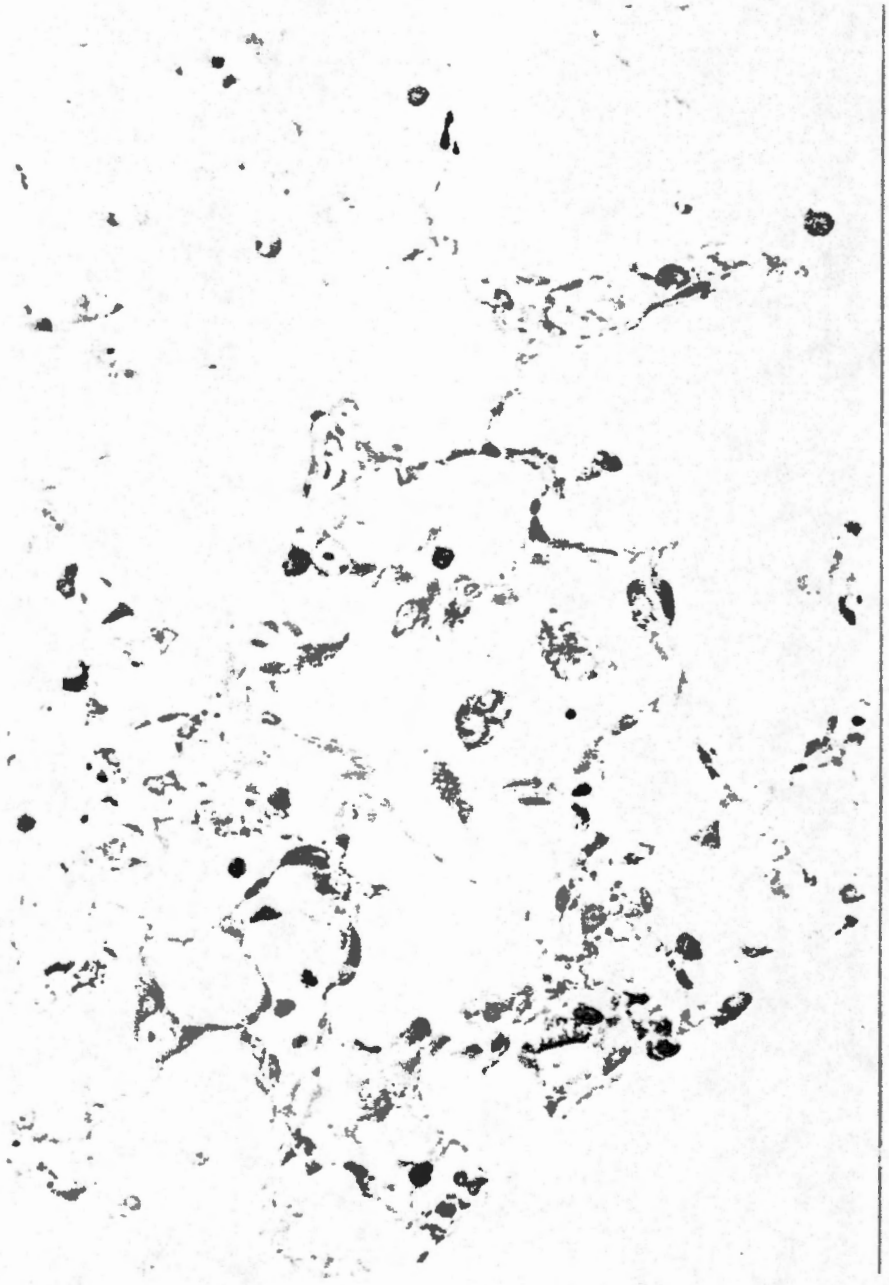

**Figure 4.14. Photomicrograph of Bleomycin and Air treated lungs at 96 H.** Mild to moderate focally intense infiltrates of foamy macrophages and interstitial and air space granulocytes are scattered through the alveoli. Lee's methylene blue and basic fuchsin. (Magnification 200X).

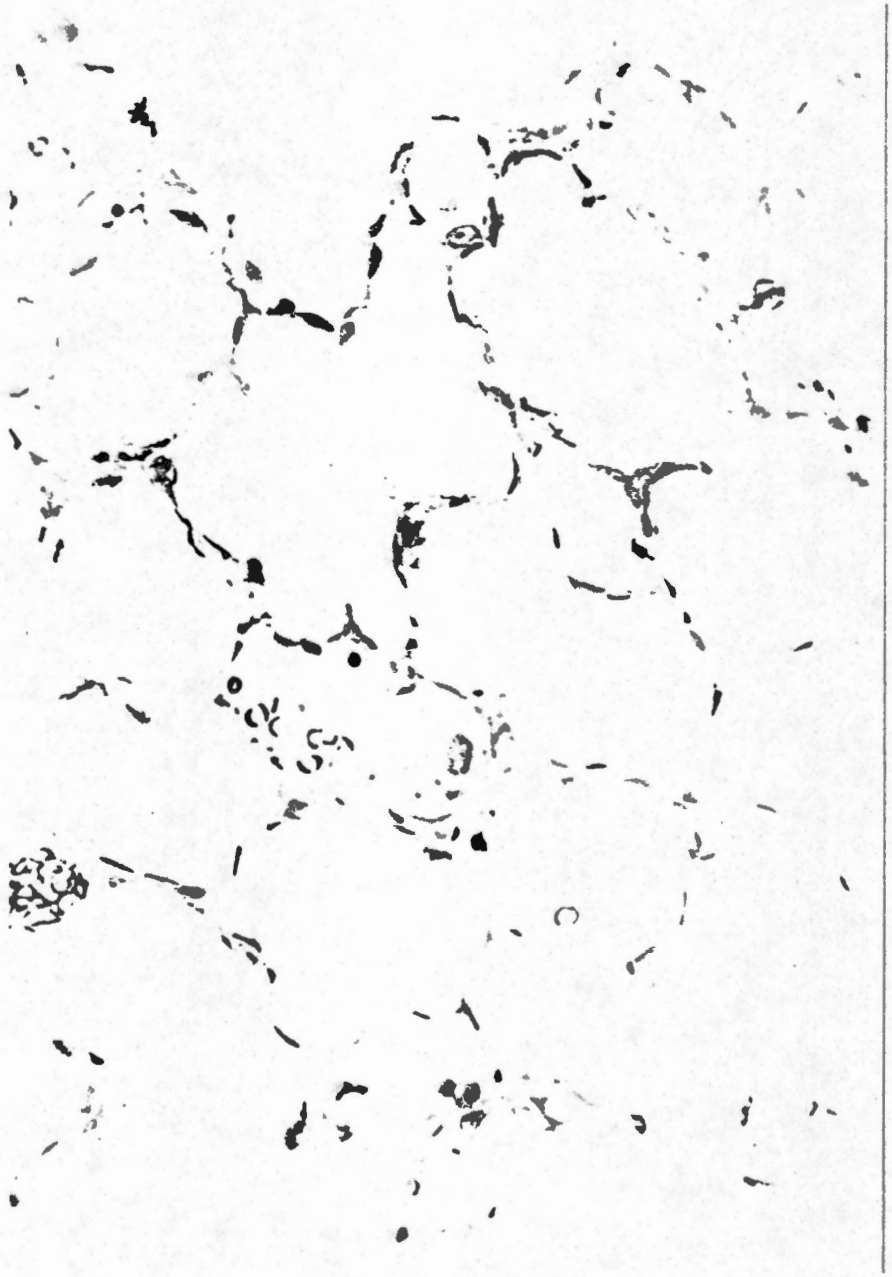

**Figure 4.15. Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lung at 24 H.** Scattered foci of increased numbers of alveolar macrophages and interstitial granulocytes are associated with small hemorrhages and mild alveolar septal thickening. Lee's methylene blue and basic fuchsin. (Magnification 200X).



**Figure 4.16. Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lung at 48 H.** Foci of macrophage and interstitial granulocyte infiltrates are larger, more intense, and more numerous than at 24 H. Scattered alveolar septa are thickened. Occasional alveoli are compressed. Lee's methylene blue and basic fuchsin. (Magnification 200X).

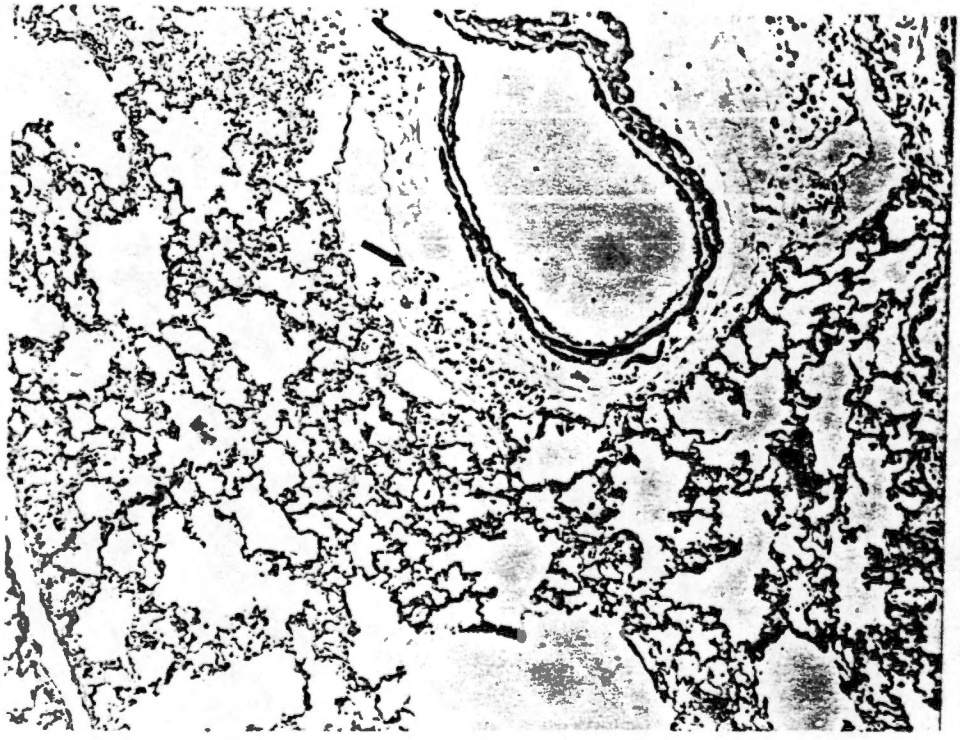

**Figure 4.17. Low power photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H.** Moderate perivascular and peribronchiolar edema is prominent; macrophages and granulocytes are scattered in the edema spaces (arrow). Lee's methylene blue and basic fuchsin. (Magnification 100X).

membranes, and necrosis (Figure 4.18, page 80). At 96 H airways and pleura were within normal limits; vessels had edematous endothelial cells as well as marked perivascular edema. Septal edema, alveolar infiltrates of macrophages and granulocytes, hemorrhages, hyaline membranes, and necrosis were similar to but more severe than those at 72 H (Figure 4.19).

## Discussion

This hamster model of acute respiratory failure demonstrates synergism of bleomycin and hyperoxia. The interaction of these two agents, bleomycin and 80% $O_2$, causes more severe pulmonary damage than either agent alone. After intratracheal bleomycin treatment, 80% $O_2$ exposure can be as short as 12 H and still potentiate the effects of bleomycin. Lesions induced are those of DAD. Fatal edema, especially alveolar air space flooding, is delayed until 72 H after bleomycin treatment.

Synergistic pulmonary damage by simultaneous bleomycin and hyperoxia in experimental animals has been described and characterized, however the length of $O_2$ exposure was usually longer than 24 H; timepoints earlier than 3 days were not examined thoroughly (Hakkinen et al., 1982; Rinaldo et al., 1982; Tryka et al., 1982; Toledo et al., 1982; Hakkinen et al., 1983; Tryka et al., 1983; Berend 1984; Moseley et al., 1984; Tryka et al., 1984). Mice given 50 to 100 mg/kg bleomycin intraperitoneally and exposed to 80% $O_2$ for 3 weeks had increased fibrosis (Hakkinen et al., 1982). Hamsters treated with 5 U/kg bleomycin intratracheally and 60% or 100% $O_2$ for 21 days developed more severe lesions of pulmonary fibrosis (Rinaldo et al.,

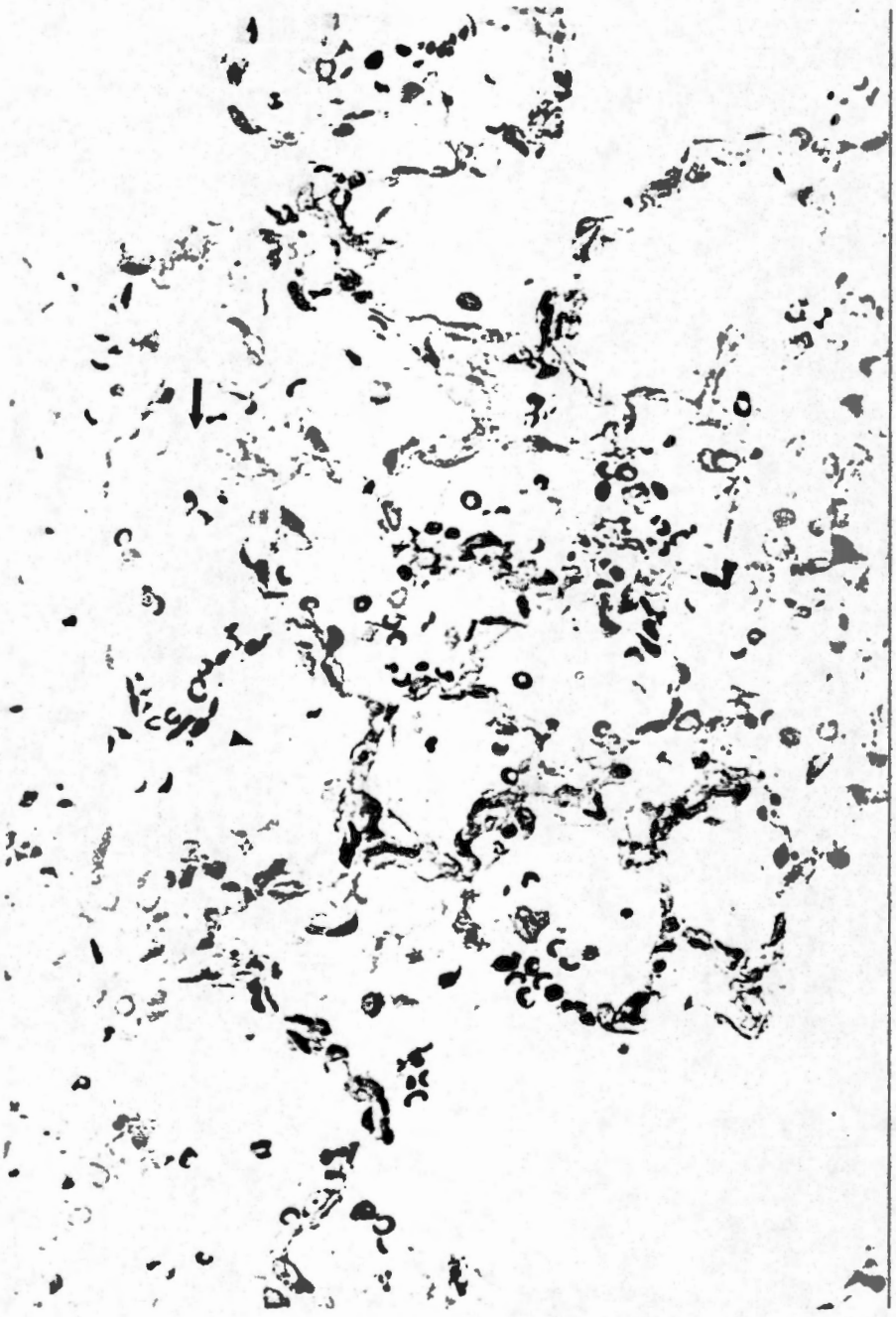

**Figure 4.18. Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H.** A moderate to marked macrophage and alveolar interstitial and air space granulocyte infiltrate is diffuse throughout the alveolar tissue. Hemorrhages, hyaline membranes (arrow), and alveolar exudates (arrowhead) are scattered through the tissues. Alveolar septa are moderately thickened. Lee's methylene blue and basic fuchsin. (Magnification 200X).

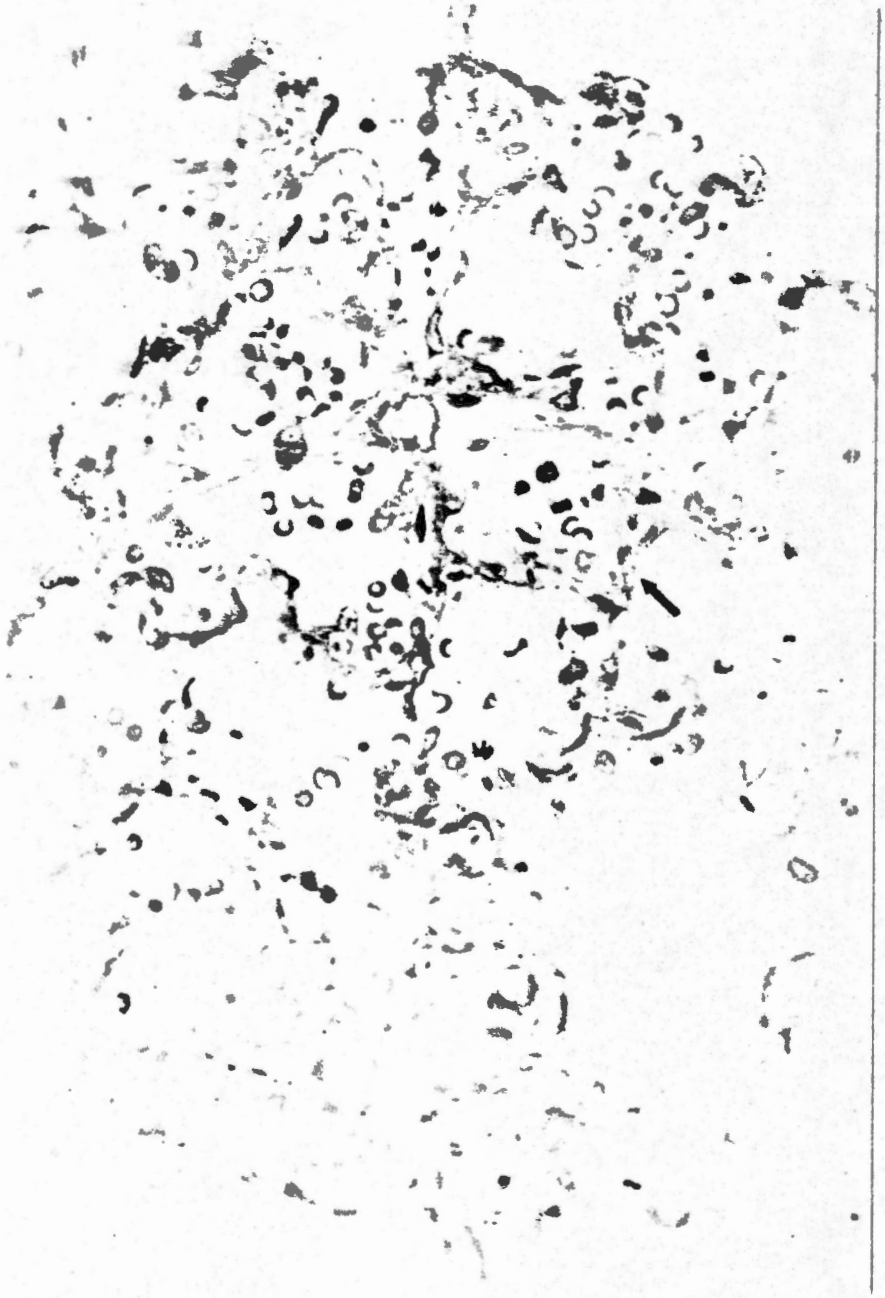

**Figure 4.19. Photomicrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 96 H.** A diffuse marked infiltrate of macrophages and interstitial and air space granulocytes is throughout the tissues. Alveolar septal edema is moderate to marked (arrow). Perivascular edema is marked (arrowhead).Lee's methylene blue and basic fuchsin. (Magnification 200X).

1982). Some of the mice given 40 mg/kg bleomycin intraperitoneally twice weekly, and exposed to 40% $O_2$ until death, died with signs of acute respiratory failure; all bleomycin and $O_2$ treated mice had decreased survival times (Toledo et al., 1982).

In the initial observations of this study, hamsters began to die at 72 H after bleomycin treatment and 80% $O_2$ exposure regardless of the length of $O_2$ exposure. Death was associated with clinical signs of pulmonary edema in moribund animals and gross and microscopic lesions of acute respiratory failure in dead animals. Signs of pulmonary edema were dyspnea, rales, and foam around the nose and mouth. Gross lesions were pulmonary congestion, edema, and hemorrhages. Histologic pulmonary lesions were typical of ARDS and were characteristic of DAD including alveolar interstitial and air space edema, mild to moderate macrophage and neutrophil infiltrates, hemorrhages, and hyaline membranes. Hamsters in all exposure groups were clinically normal until 72 H after bleomycin treatment. Pulmonary edema was evident clinically, grossly, and microscopically at 72 H, and was associated with death.

Edema, secondary to increased pulmonary microvascular permeability is delayed and occurs acutely at 72 H and later. Development of pulmonary edema showed no difference between the 493 dalton substance, $^{99m}$Tc-DTPA, and the larger substance, $^{125}$I-BSA. Prior to 72 H only mild to moderate interstitial alveolar edema was observed microscopically and none was detected by radiolabelled tracers which should be more sensitive than miroscopic observations (Effros and Mason 1983). The abrupt development of edema as detected by tracers might have indicated that normal pulmonary lymphatic drainage compensated for the edema, draining the excess fluid, until edema

was severe enough to overcome normal lymph drainage channels. It might also have indicated that vascular and epithelial damage severe enough to cause significant alveolar interstitial and subsequent alveolar air space edema did not occur until 72 H.

Inability of group BO hamster epithelium to undergo type II cell DNA synthesis prior to division and repair seems to be an important factor associated with the delayed-onset pulmonary edema and DAD. A change in type II cell metabolism may be a result of the route of bleomycin administration. Studies in mice have shown that although pulmonary damage is greater in mice treated with intratracheal versus intraperitoneal bleomycin, no qualitative difference in lung damage, as measured by hydroxyproline, autoradiography, and morphometry studies, is seen when intratracheal and intraperitoneal treatment groups are compared (Lindenschmidt et al., 1985).

Cell kinetics, as determined by $^{3}$H-TdR autoradiography studies, showed that bleomycin treatment caused increased DNA synthesis at 96 H in group BO hamsters. Cells responsible for the increase in label were primarily alveolar macrophages. Only group BA had an increase in type II epithelial cell label at 96 H. Group BO had no change in type II epithelial cell labelling; the number of labelled type II cells remained at a constant low level.

Therefore, with bleomycin damage alone, type II cells can synthesize DNA for division; with bleomycin and $O_2$ damage, type II cells do not synthesize DNA. This probably reflects type II cell damage. Type II cells are considered resistant to bleomycin or $O_2$ damage (Adamson and Bowden 1977); however, damage caused by the combination of these agents may cause severe metabolic or structural injury to type II cells. Bleomycin alone, in mice, causes

hyperplasia and metaplasia of type II cells (Adamson and Bowden, 1977). Therefore, type II cell metabolism does change with bleomycin treatment. The severe insult of bleomycin and hyperoxia may prevent DNA synthesis in type II cells at 72 and 96 H. Thus type II cells cannot divide to repair the injured alveolar epithelium. This is indirect evidence that the 72 to 96 H fatal pulmonary edema may be due to failure of type II cells to regenerate and repair the damage.

Although endothelial damage and increased microvascular permeability is considered the cause of edema in rats treated with bleomycin and hyperoxia (Berend 1984), endothelial damage reflected by endothelial cell synthesis of DNA does not seem to be a factor in hamsters treated with bleomycin and $O_2$. Endothelial cell labelling was not significantly increased in any treatment or exposure group. Increased endothelial cell labelling is an indirect indication of regeneration and repair of pulmonary vasculature following damage (Haschek et al., 1983). In this model, endothelial regeneration appears to be less important than alveolar epithelium, specifically type II epithelial cells.

Microscopic examination of the lung revealed differences in edema distribution in group BO animals. Mild to moderate perivascular and interstitial alveolar edema occurred at 24 and 48 H. Marked interstitial alveolar edema, alveolar air space edema, and hyaline membranes were seen at 72 H and 96 H. Therefore the severe, fatal edema is associated with alveolar air space flooding as well as hyaline membranes.

Potentiation of bleomycin damage by hyperoxia was suspected as the cause of ARDS and death in several human cases, therefore demonstration of

this effect in experimental animals is important (Goldiner et al., 1978). In the hamster model of intratracheal bleomycin and 80% $O_2$ synergism, the length of exposure to hyperoxia to produce ARDS is as litle as 12 H; it may be less than that. This is important for clinicians to know; that short periods of hyperoxia, in patients undergoing bleomycin therapy or with previous pulmonary insults, may induce more severe lesions in patients treated with $O_2$ therapy.

Hamsters treated with intratracheal bleomycin and 70% $O_2$ for 72 H have been shown to have markedly increased mortality (Tryka et al., 1982). Oxygen exposure has also been shown to decrease the amount of bleomycin necessary to cause the $LD_{50}$ seen with bleomycin alone (Tryka et al., 1982). In one study, bleomycin-$O_2$ treated hamster deaths, at six days or earlier, were attributed to acute respiratory failure. These animals had DAD, hyaline membranes, pulmonary hemorrhages, and scattered, prominent granulocytes (Tryka et al., 1982). Similar signs and lesions were observed in mice, given 1 to 4 U/kg bleomycin intraperitoneally, and rats, given 0.2 to 1 U/kg, and exposed to 80% $O_2$ until death (Hakkinen et al., 1983). Hamsters treated with 0.16 U/kg intratracheal bleomycin and 72 H of 70% $O_2$ also had DAD at three days (Tryka et al., 1983). Synergistic effects of 1.5 U bleomycin intratracheally and exposure to 24 or 48 H of 100% in rats also produced DAD (Berend 1984). Therefore several previous studies have shown deaths from acute respiratory failure caused by the synergism of bleomycin and $O_2$.

This study of the acute effects of bleomycin and hyperoxia showed that as little as 12 H of 80% $O_2$ can induce acute respiratory failure and death. All

hamsters, regardless of the length of $O_2$ exposure, began to die at 72 H with severe pulmonary edema and lesions of DAD if they received intratracheal bleomycin. The cause of acute-onset delayed ARDS in these hamsters could not be determined adequately by radiolabelled-tracer edema studies, cell kinetics studies, or light microscopy evaluations.

# CHAPTER V

# PULMONARY MORPHOMETRY

## Introduction

To follow development of alveolar lesions in acute respiratory failure induced by bleomycin and hyperoxia, a method more sensitive than that used to measure pulmonary edema or cell kinetics should be used to examine the progression of changes. Radiolabelled tracer studies *in vivo* are functional, but not very sensitive to minor or very early damage (Crandall et al., 1983; Effros and Mason 1983). Light microscopy can detect lesions of alveolar structure but not at cellular or subcellular levels (Dungworth, Tyler, and Plopper 1985). Autoradiography can detect changes in cell kinetics, but only measures repair, an indirect and delayed indication of cell damage (Adamson 1985). Transmission electron microscopy is sensitive enough to detect changes in cellular and subcellular structures. Therefore, ultrastructural examination of hamster lungs treated with bleomycin and hyperoxia should aid in the characterization of epithelial and endothelial damage in this model (Pinkerton and Crapo 1985).

To examine the lungs thoroughly, the study should use the techniques of morphometry. Morphometry is the study of tissues using quantitative methods to yield numbers denoting quantity and size of structures. It is an objective technique to examine tissues (Barry and Crapo 1984; Pinkerton and Crapo 1985).

To study pulmonary alveolar structures morphometrically, lungs should be fixed by vascular perfusion. This technique maintains alveolar structures the best and also maintains alveolar surface features such as macrophages and surfactant. Immersion fixation does not penetrate well and causes many artifacts. Airway instillation of fixative alters the shape and location of alveolar features, such as macrophages and surfactant, and can cause artifacts of alveolar structure (Gil 1971; Weibel 1984).

Transmission electron microscopy can also be used to observe qualitative, although more subjective morphological changes. The lung should be examined at time points earlier than the light microscopic and gross lesions are evident, as well as at the time of onset of pulmonary edema.

## Materials and Methods

### General Methods

Male LVG/LAK Syrian golden hamsters, were obtained from the same source, Charles River Breeding Laboratories, Inc., Animals were housed and screened in the same manner as previously described (Chapter IV, page 44). The bleomycin was also obtained and handled as before; 5U/kg was the dose given intratracheally to anesthetized hamsters. Oxygen exposure was also the same.

A total of 48 hamsters were divided into groups of 12 per treatment group. Treatment and exposure groups were intratracheal saline and air (SA), intratracheal saline and 24 H 80% $O_2$ (SO), intratracheal bleomycin and air

(BA), and intratracheal bleomycin and 24 H 80% $O_2$ (Table 4.2, page 48). Four animals were sacrificed from each treatment and exposure group at the end of 24, 48, and 72 H for a total of 16 hamsters per 24 H. Timepoints were designated as hours after intratracheal bleomycin or saline.

**Morphometry**

Groups for morphometry were 4 hamsters per treatment and exposure, SA, SO, BA, and BO, sacrificed at 24, 48, and 72 hours after treatment for a total of 48 animals. Hamsters were killed as for autoradiography (Chapter IV, page 50).

The lungs were fixed by vascular perfusion. Perfusion solutions used were heparanized saline followed by 2% phosphate-buffered glutaraldehyde. Heparinized saline was prepared by mixing 9 grams of NaCl with 1000 ml of distilled $H_20$ and adding 10,000 Units of heparin sodium (Fisher Scientific Co., Fair Lawn, NJ). Glutaraldehyde was prepared by mixing 3.85 g of $K_2HPO_4$, and 16 ml of distilled water with 0.66g $KH_2PO_4$, and 100 ml of 25% glutaraldehyde. The fixative solution was adjusted to ph 7.4 by addition of dilute HCl or NaOH, measured by a Corning pH meter, Corning, NY. The osmolality of the solutions (measured by an Advanced Wide Range Osmometer Model 3W, Advanced Instruments Inc., Needham Heights, MA) was 250 to 350 millosmoles or equivalent to prepared physiologic saline solution.

After sacrifice, hamsters were placed on a vertically movable stand in order to maintain the pressure of the perfusion solution. The rib cage was removed and the trachea exposed. The trachea was cannulated through the

larynx with a 4 cm long blunt metal needle which was ligated in place with 1-0 gauge silk. The pulmonary artery was cannulated with a 4 cm long blunted metal cannula, held in place with bulldog arterial clamps. The lungs were inflated with compressed air from the general laboratory supply at a constant 20 cm of pressure. The lungs were perfused with 10 to 15 ml of heparinized saline solution and then 10 to 15 ml of 2% phosphate-buffered glutaraldehyde. Lungs were left *in situ* for 10 mintues; the trachea was ligated. The trachea, major vessels, esophagus, heart, and lungs were removed from the thorax by careful dissection. The tissues were then placed in a 2% phosphate-buffered glutaraldehyde solution for a minimum of 48 hours. Lungs in glutaraldehyde were kept at 4°C until sampling.

Fixed lungs were trimmed for electron microscopy. Six 1 mm cubes, two from the center left lobe, two from the right cranial lobe, and two from the right caudal lobe, were placed in small 2% glutaraldehyde-filled glass vials. These lung samples were then post-fixed in 1% osmium tetraoxide for 3 hours. This solution was then removed by 4 rinses in 0.2 M cacodylate buffer solution. Samples in cacodylate buffer were prepared for embedding by serial passage through 50, 70, 90, and 100% ethanol, and propylene oxide. Each sample was embedded in epon (Peko Co., Tustin, CA).

Epon-embedded lung tissue was sectioned at 80 to 100 nm (average 90 nm) and stained with uranyl acetate and lead citrate for transmision electron microscopy and placed on 200-mesh copper electron microscopy grids, Pelco Co. Grids were then examined and photographed on an Hitachi H-600 transmission electron microscope, Hitachi Ltd., Tokyo, Japan. Photographs

were taken on Kodak 4489 Electron Microscope film 3¼″ × 4″. Film was developed in Kodak D-19 Developer for 4 minutes, fixed in Kodak Fixer for 8 minutes, and rinsed in running water for 30 minutes.

A calibration grid, #606 Carbon Grating Replica Parallel Lines 54,800/inch Pelco Co., was photographed every day the electron microscope was used to determine magnification and enlargement drift and final magnification. Final magnification was calculated as:

$$\text{magnification} = \frac{\text{(cm between grid lines) (21,600 lines/cm)}}{\text{number of spaces between limiting lines}}$$

Four grids were prepared from each set of lungs. One photograph at magnification (X) 1000 was taken for each hamster; ten 2000 X photographs were taken of each grid. Photographs at 2000 X of each grid square were the upper right-hand corner. If that corner was empty or had much debris, then the lower left corner was photographed (Elias and Hyde 1980; Barry and Crapo 1985). Similarly the lower right corner and then the upper left corner were photographed if the other sites had no tissue or tissues covered by debris. Contents of each corner, tissue or air, was recorded for each corner of each grid - square photographed.

Each negative, automatically numbered by the microscope, was printed as an 8″ × 10″ black and white print with a Besseler enlarger and processed with the aid of a Kodak Ektamatic Automatic Processor.

Photographs were initially examined for qualitative changes by observation of lesions. Quantitative changes were determined by point counting. A Weibel overlay grid of 86 one cm lines evenly spaced (Elias and Hyde 1980; Weibel 1984; Barry and Crapo 1985) was made on plastic

transparencies. The grid transparencies were placed over each photograph to count where points, the ends of each 1 cm line, fell. For each photograph, 168 points were counted. Photographs of large lung vessels or airways were not used. The final number of prints used and counted from each animal varied from 30 to 45. Variation in the number of prints used was because prints with nonalveolar structures, such as bronchioles or large vessels, were rejected.

Categories of structures for counting were:

1) alveolar air spaces
2) capillary lumens
3) endothelial cells
4) type I epithelial cells
5) type II epithelial cells
6) interstitial cells
7) interstitium or interstitial space, and
8) leukocytes, to be identified separately, including alveolar macrophages, monocytes, neutrophils, eosinophils, lymphocytes, and platelets.

Air spaces were defined as any alveolar space normally filled with air, to include surfactant, exudate, debris or red cells in the space. Capillary lumens were any space lined by endothelium normally containing blood. Endothelial cells were any cells lining capillaries which frequently had pinocytotic vesicles or caveolae intracellulares as additional identifying features. Type I epithelial cells were those thin or attenuated cells lining alveoli. Type II alveolar cells were defined as cells lining alveoli with lamellar bodies and microvilli. Interstitial cells were cells in the interstitium, often with lipid-containing

vacuoles, and round to oval nuclei with irregular borders; abundant Golgi apparatus and endoplasmic reticulum was in the cytoplasm. Interstitium was any interstitial material or space, such as basement membrane, that was not part of a cell. Macrophages were mononuclear cells in alveolar lumens with large pseudopodia, lamellar bodies and various sizes and shapes of vacuoles. Monocytes were like macrophages, with fewer cytoplasmic organelles and less cytoplasm, and were in capillary lumens. Neutrophils were cells with characteristic mulilobulated nuclei and intracytoplasmic granules that were often dumbbell-shaped. Neutrophils could be located in vessels lumens, in interstitium, or in alveolar air spaces. Eosinophils had oval or football-shaped cytoplasmic granules with dark equatorial bands as well as lobulated nuclei; these could also be located in vessel, interstium or in alveolar air spaces. Lymphocytes were smaller round cells with usually central round nuclei, scant cytoplasm and few organelles. Platelets appeared as small oval to round segments of membrane-bound cytoplasm with large oval to ingot-shaped granules.

**Morphometry Calculations**

Points counted on prints derived from electron microscopy negatives were used to determine volume densities for structures of interest. The volume density of a specific structure was defined as:

$$V_v = P_i/P_T ,$$

where $V_v$ = volume fraction of the structure, $P_i$ = the number of points falling on the structure of interest, and $P_T$ = the total number of points counted (Elias and Hyde 1980; Barry and Crapo 1985). These calculations were done for each structure of interest including the categories air space, capillary lumen, endothelial cell, type I epithelial cell, type II epithelial cell, interstitial cell, interstitial space, macrophage, monocyte, neutrophil in vessel lumen, neutrophil in interstitium, neutrophil in alveolar air space, lymphocyte, platelet, eosinophil in vessel lumen, and eosinophil in alveolar air space. Calculations and statistics were performed with an IBM computer using the SAS program.

## Results

### Morphometry

For each hamster a minimum of 30 and a maximum of 45 black and white prints were counted and examined. The number varied since some prints were rejected because they were of nonalveolar structures. Counts were pooled for each animal, and then for each time point and treatment group. Resultant figures were expressed as volume fractions for each structure of interest. Volume fractions for alveolar cells and structures are summarized in Tables 5.1 through 5.7 (pages 95–101). The The group of hamsters treated with saline and room air was the control group. Values for this group were considered to be normal or a result of saline treatment.

At 24 H, Group SO had increases in volume fractions of tissue, defined as alveolar endothelium, type I epithelial cells, type II epithelial cells, and

**Table 5.1. Pooled Volume Fractions from Point Counts[a]**

Alveolar Tissues

| Treatment | Time[f] | Structure of Interest | | | |
|---|---|---|---|---|---|
| | | Endothelium | I | II | Interstitial cells |
| SA[b] | 24 | 0.0219 ±0.0020 | 0.0241 ±0.0016 | 0.0219 ±0.0031 | 0.0743 ±0.0043 |
| | 48 | 0.0134 ±0.0015 | 0.0195 ±0.0014 | 0.0104 ±0.0017 | 0.0760 ±0.0061 |
| | 72 | 0.0119 ±0.0011 | 0.0179 ±0.0012 | 0.0097 ±0.0015 | 0.0620 ±0.0047 |
| SO[c] | 24 | 0.0280 ±0.0021 | 0.0514[g] ±0.0239 | 0.0229 ±0.0023 | 0.1510[h] ±0.110 |
| | 48 | 0.0172 ±0.0013 | 0.0187 ±0.0011 | 0.0126 ±0.0016 | 0.0656 ±0.0036 |
| | 72 | 0.0151 ±0.0011 | 0.0191 ±0.0012 | 0.0124 ±0.0018 | 0.0642 ±0.0096 |
| BA[d] | 24 | 0.0185 ±0.0015 | 0.0212 ±0.0012 | 0.0140 ±0.0021 | 0.0799 ±0.0060 |
| | 48 | 0.0158 ±0.0013 | 0.0216 ±0.0015 | 0.0144 ±0.0023 | 0.0665 ±0.0041 |
| | 72 | 0.0141 ±0.0013 | 0.0172 ±0.0011 | 0.0120 ±0.0022 | 0.0738 ±0.0069 |
| BO[e] | 24 | 0.0210 ±0.0014 | 0.0203 ± 0.0011 | 0.0153 ±0.0021 | 0.0828 ±0.0057 |
| | 48 | 0.0145 ±0.0013 | 0.0196 ±0.0012 | 0.0078 ±0.0016 | 0.0828 ±0.0044 |
| | 72 | 0.0167 ±0.0013 | 0.0195 ±0.0012 | 0.0074 ±0.0015 | 0.1450[h] ±0.0100 |

[a]Volume Fraction = $\frac{\text{Points falling on structure of Interest}}{\text{Total Points Counted}}$

[b]Saline and 20% $O_2$.

[c]Saline and 24 H 80% $O_2$.

[d]Bleomycin and 20% $O_2$.

[e]Bleomycin and 24 H 80% $O_2$.

[f]Hours after treatment.

[g]Mean ± standard error.

[h]Significant at $p < 0.05$, values compared to control group Saline and Air at the same timepoints.

**Table 5.2. Pooled Volume Fractions from Point Counts[a]**

Total Alveolar Tissue

| Treatment | Time[f] | Total Tissue[i] |
|---|---|---|
| SA[b] | 24 | 0.1507 ± 0.0074[g] |
| | 48 | 0.1279 ± 0.0086 |
| | 72 | 0.1067 ± 0.0070 |
| SO[c] | 24 | 0.2771[h] ± 0.0270 |
| | 48 | 0.1264 ± 0.0062 |
| | 72 | 0.1169 ± 0.0061 |
| BA[d] | 24 | 0.1501 ± 0.0091 |
| | 48 | 0.1260 ± 0.0065 |
| | 72 | 0.1224 ± 0.0094 |
| BO[e] | 24 | 0.1545 ± 0.0084 |
| | 48 | 0.1155 ± 0.0067 |
| | 72 | 0.1999[h] ± 0.0121 |

[a]Volume Fraction =

$$\frac{\text{Points falling on structure of Interest}}{\text{Total Points Counted}}$$

[b]Saline and 20% $O_2$.

[c]Saline and 24 H 80% $O_2$.

[d]Bleomycin and 20% $O_2$.

[e]Bleomycin and 24 H 80% $O_2$.

[f]Hours after treatment.

[g]Mean ± standard error.

[h]Significant at $p < 0.05$, values compared to control group Saline and Air at the same timepoints.

[i]Tissue is defined as the total volume fraction of endothelial cells, type I epithelial cells, type II epithelial cells, and interstitial cells.

**Table 5.3. Pooled Volume Fractions from Point Counts[a]**

Noncellular Spaces

| Treatment | Time[f] | Structure of Interest | |
|---|---|---|---|
| | | Capillary lumen | Interstitial space |
| SA[b] | 24 | 0.0682 +- 0.0052[g] | 0.0083 ± 0.0010 |
| | 48 | 0.0404 ± 0.0086 | 0.0085 ± 0.0011 |
| | 72 | 0.0483 ± 0.0048 | 0.0050 ± 0.0007 |
| SO[c] | 24 | 0.0696 ± 0.0052 | 0.0234 ± 0.0034[h] |
| | 48 | 0.0528 ± 0.0046 | 0.0122 ± 0.0012 |
| | 72 | 0.0517 ± 0.0043 | 0.0060 ± 0.0008 |
| BA[d] | 24 | 0.0644 ± 0.0050 | 0.0154 ± 0.0016 |
| | 48 | 0.0430 ± 0.0035 | 0.0071 ± 0.0006 |
| | 72 | 0.0390 ± 0.0041 | 0.0052 ± 0.0007 |
| BO[e] | 24 | 0.0792 ± 0.0060 | 0.0143 ± 0.0014 |
| | 48 | 0.0468 ± 0.0043 | 0.0083 ± 0.0011 |
| | 72 | 0.0704 ± 0.0067[h] | 0.0109 ± 0.0013[h] |

[a]Volume Fraction =

$$\frac{\text{Points falling on structure of Interest}}{\text{Total Points Counted}}$$

[b]Saline and 20% $O_2$.

[c]Saline and 24 H 80% $O_2$.

[d]Bleomycin and 20% $O_2$.

[e]Bleomycin and 24 H 80% $O_2$.

[f]Hours after treatment.

[g]Mean ± standard error.

[h]Significant at $p < 0.05$, values compared to control group SA values at the same timepoints.

**Table 5.4. Pooled Volume Fractions from Point Counts[a]**

Alveolar Leukocytes-Vascular

| Treatment | Time[f] | Cell of Interest | | |
|---|---|---|---|---|
| | | Monocyte | Lymphocyte | Total |
| SA[b] | 24 | 0.0070 ±0.0011[g] | 0.0009 ±0.0001 | 0.0129 ±0.0016 |
| | 48 | 0.0060 ±0.0011 | 0.0001 ±0.0001 | 0.0075 ±0.0012 |
| | 72 | 0.0040 ±0.0007 | 0.0003 ±0.0002 | 0.0054 ±0.0008 |
| SO[c] | 24 | 0.0046 | 0.0024[h] ±0.0009 | 0.0138 ±0.0005 |
| | 48 | 0.0054 ±0.0011 | 0.0002 ±0.0001 | 0.0086 ±0.0015 |
| | 72 | 0.0051 ±0.0008 | 0.0001 ±0.0001 | 0.0072 ±0.0011 |
| BA[d] | 24 | 0.0042 ±0.0008 | 0.0005 ±0.0013 | 0.0105 ±0.0013 |
| | 48 | 0.0066 ±0.0010[h] | 0.0009 ±0.0014 | 0.0117 ±0.0014 |
| | 72 | 0.0088[h] ±0.0013 | 0.0003 ±0.0001 | 0.0082 ±0.0015 |
| BO[e] | 24 | 0.0051 ±0.0008 | 0.0005 ±0.0002 | 0.0168 ±0.0012 |
| | 48 | 0.0040 ±0.0008 | 0.0002 ±0.0001 | 0.0075 ±0.0012 |
| | 72 | 0.0125[h] ±0.0015 | 0.0006 ±0.0002 | 0.0195[h] ±0.0020 |

[a]Volume Fraction = $\frac{\text{Points falling on structure of Interest}}{\text{Total Points Counted}}$

[b]Saline and 20% $O_2$.

[c]Saline and 24 H 80% $O_2$.

[d]Bleomycin and 20% $O_2$.

[e]Bleomycin and 24 H 80% $O_2$.

[f]Hours after treatment.

[g]Mean ± standard error.

[h]Significant at $p < 0.05$.

**Table 5.5. Pooled Volume Fractions from Point Counts[a]**

Alveolar Granulocytes-Vascular

| Treatment | Time[f] | Neutrophil | Eosinophil |
|---|---|---|---|
| SA[b] | 24 | 0.0042 ± 0.0004 | 0.0003 ± 0.0002 |
| | 48 | 0.0012 ± 0.0004 | 0.0002 ± 0.0002 |
| | 72 | 0.0006 ± 0.0003 | 0.0004 ± 0.0003 |
| SO[c] | 24 | 0.0051 ± 0.0008 | 0.0007 ± 0.0003 |
| | 48 | 0.0020 ± 0.0005 | 0.0008 ± 0.0003 |
| | 72 | 0.0009 ± 0.0003 | 0.0009 ± 0.0004 |
| BA[d] | 24 | 0.0043 ± 0.0008 | 0.0004 ± 0.0003 |
| | 48 | 0.0033 ± 0.0007 | 0.0008 ± 0.0009 |
| | 72 | 0.0025 ± 0.0005[h] | 0.0005 ± 0.0002 |
| BO[e] | 24 | 0.0100 ± 0.0015[h] | 0.0007 ± 0.0003 |
| | 48 | 0.0013 ± 0.0004 | 0.0013 ± 0.0005[h] |
| | 72 | 0.0054 ± 0.00009[h] | 0.0006 ± 0.0003 |

[a]Volume Fraction =

$$\frac{\text{Points falling on structure of Interest}}{\text{Total Points Counted}}$$

[b]Saline and 20% $O_2$.
[c]Saline and 24 H 80% $O_2$.
[d]Bleomycin and 20% $O_2$.
[e]Bleomycin and 24 H 80% $O_2$.
[f]Hours after treatment.
[g]Mean ± standard error.
[h]Significant at $p < 0.05$.

**Table 5.6. Pooled Volume Fraction from Point Counts[a]**

Alveolar Leukocytes-Nonvascular

| Treatment | Time[f] | Cells of Interest | | |
|---|---|---|---|---|
| | | Macrophage | Neutrophil Interstitial | Total Nonvascular Leukocytes |
| SA[b] | 24 | 0.0041 ±0.009 | 0.0001 ±0.001 | 0.0041 ±0.0009 |
| | 48 | 0.0073 ±0.0018 | 0.0001 ±0.0001 | 0.0073 ±0.0018 |
| | 72 | 0.008 ±0.0017 | 0.0003 ±0.0002 | 0.0084 ±0.0017 |
| SO[c] | 24 | 0.0023 ±0.0008 | 0.0001 ±0.0001 | 0.0027 ±0.0008 |
| | 48 | 0.0062 ±0.0013 | 0.0011[h] ±0.0001 | 0.0064 ±0.0013 |
| | 72 | 0.0125[h] ±0.0021 | 0.0002 ±0.0001 | 0.0128 ±0.0021 |
| BA[d] | 24 | 0.0038 ±0.0010 | 0.0013[h] ±0.0004 | 0.0056 ±0.0012 |
| | 48 | 0.0074 ±0.0016 | 0.0006 ±0.0003 | 0.0080 ±0.0017 |
| | 72 | 0.0071 ±0.0014 | 0.0001 ±0.0001 | 0.0125[h] ±0.0015 |
| BO[e] | 24 | 0.0061 ±0.0012 | 0.0009[h] ±0.0004 | 0.0089[h] ±0.0016 |
| | 48 | 0.0075 ±0.0015 | 0.0001 ±0.0001 | 0.0075 ±0.0015[h] |
| | 72 | 0.0268[h] ±0.0033 | 0.0004 ±0.0002 | 0.0295 ±0.0035 |

[a]Volume Fraction = $\frac{\text{Points falling on structure of Interest}}{\text{Total Points Counted}}$

[b]Saline and 20% $O_2$.

[c]Saline and 24 H 80% $O_2$.

[d]Bleomycin and 20% $O_2$.

[e]Bleomycin and 24 H 80% $O_2$.

[f]Hours after treatment.

[g]Mean ± standard error.

[h]Significant at $p < 0.05$.

**Table 5.7. Pooled Volume Fraction from Point Counts[a]**
Alveolar Granulocytes-Air Space

| Treatment | Time[f] | Cells of Interest | |
|---|---|---|---|
| | | Neutrophil Air Space | Eosinophil Air Space |
| SA[b] | 24 | 0 | 0 |
| | 48 | 0 | 0 |
| | 72 | 0 | 0 |
| SO[c] | 24 | 0.0002 ± 0.0001 | 0.0002[h] ± 0.0001 |
| | 48 | 0.0002 ± 0.0001 | 0 |
| | 72 | 0.0001 ± 0.0001 | 0.0001[h] ± 0.0001 |
| BA[d] | 24 | 0.0018[h] ± 0/0005 | 0.0001 ± 0.0000 |
| | 48 | 0.0007[h] ± 0.0003 | 0 |
| | 72 | 0.0011 ± 0.0014 | 0 |
| BO[e] | 24 | 0.028[h] ± 0.0008 | 0 |
| | 48 | 0 | 0 |
| | 72 | 0.0027[h] ± 0.0033 | 0 |

[a]Volume Fraction = $\frac{\text{Points falling on structure of Interest}}{\text{Total Points Counted}}$

[b]Saline and 20% $O_2$.
[c]Saline and 24 H 80% $O_2$.
[d]Bleomycin and 20% $O_2$.
[e]Bleomycin and 24 H 80% $O_2$.
[f]Hours after treatment.
[g]Mean ± standard error.
[h]Significant at $p < 0.05$.

interstitial cells. Endothelial cell, type I epithelial cell, and type II epithelial cell volume fractions were not increased. The interstitial cell volume fraction was increased in group SO at 24 H. The interstitial space volume fraction was increased; capillary lumen was not increased. Group SO had an increased volume fraction of eosinophils in the alveolar air space, all other vascular and nonvascular leukocyte volume fractions did not increase. By 48 H the only increased volume fraction of group SO was interstitial neutrophils. At 72 H, only the air space eosinophil fraction was increased in group SO.

At 24 H, Group BA had increased volume fractions of neutrophils in the airspace and interstitium. At 48 H, the air space neutrophil fraction was still increased. By 72 H, the vascular neutrophils as well as the air space neutrophils were increased in group BA.

Group BO had the most changes in volume fractions of alveolar structure. At 24 H the vascular, air space, and interstitial neutrophil fractions were all increased. No other volume fraction was increased at 24 H in group BO. By 48 H, vascular eosinophils were the only increased fraction. At 72 H, however, significant increases in volume fractions of tissue, interstitial cells, capillary lumen, interstitial space, monocytes, vascular and air space neutrophils, vascular eosinophils, total vascular leukocytes, macrophages, and total nonvascular leukocytes were observed in group BO.

Therefore morphometry results showed changes from control values in groups SO, BA, and BO. Group SO had increases in tissue, intersitial cell, and interstitial space, and interstitial neutrophil and eosinophil volume fractions. Group BA had increased vascular, air space, and interstitial neutrophil

fractions. Group BO had increased leukocyte, interstitial cell, interstitial space, and capillary lumen volume fractions.

**Morphology**

The sections and photographs taken for morphometry were also examined for qualitative changes; results are summarized in Tables 5.8 and 5.9. Group SA was the control group. Alveolar and cellular structures were observed in these animals as normals for comparison with the treated hamsters. At all time points the only changes were small focal surface projections or blebs of endothelial cell and type I epithelial cell surfaces (Figure 5.1).

Group SO at 24 H had very prominent endothelial cell pinocytotic vesicles. Pinocytotic vesicles or caveolae intracellulares are normal endothelial cells, but are not usually prominent (Figure 5.2). Endothelial cells had scattered areas of mild to moderate increased cytopasmic lucency characteristic of intracellular edema. Alveoli were mildly to moderately collapsed at 24 H. At 24 and 48 H mild to moderate focal areas of increased interstitial space (Figure 5.3), compatible with interstitial edema, were scattered through the sections. The 72 H sections had changes similar to those at 24 and 48 H, although the interstitial edema was more diffuse and moderate.

Hamsters in group BA had mild diffuse increased interstitial spaces at 24 H. Scattered small aggregates of surfactant and some alveolar exudate were throughout the sections. Surfactant is normally dispersed along the alveolar epithelial surface; aggregates of surfactant are clumps of tubular myelin which look like fingerprints when viewed at high magnification (Figure 5.4). Alveolar

**Table 5.8. Alveolar Ultrastructure-Cytoplasmic Surfaces and Edema**

| Treatment | Time | Observations | | | | |
|---|---|---|---|---|---|---|
| | | Blebs[i] | | Edema | | |
| | | Endo[j] | Epi[l] | Endo | Epi | Inter[l] |
| SA[a] | 24 | -[e] | | | | |
| | 48 | - | - | - | - | - |
| | 72 | sl[f] to min[g] | sl to min | - | - | - |
| SO[b] | 24 | min to mod[h] | min to mod | min | min | min to mod |
| | 48 | min | min | min | min | min to mod |
| | 72 | min | min | min | min | min to mod |
| BA | 24 | min | min | min | min | min |
| | 48 | min | min | min | min | min |
| | 72 | min | min | min | min | min |
| BO | 24 | min | min | sl | sl | sl |
| | 48 | min | min | min | min | min |
| | 72 | min to mod | min to mod | sl | sl | sl |

[a]Saline and air.
[b]Saline and 80% $0_2$.
[c]Bleomycin and air.
[d]Bleomycin and 80% $O_2$.
[e]None observed or normal.
[f]Slight.
[g]Minimal.
[h]Moderate.
[i]Blebs = small surface projections of cell membrane and cytoplasm.
[j]Endo = endothelial cell.
[k]Epi = epithelial cell.
[l]Inter = interstitial cell




**Figure 5.1. Electron micrograph of Saline and Air treated lungs at 24 H.** Normal alveolar structures include type I epithelial cell (i), type II epithelial cell (ii), endothelial cell (e), air space (s), and capillary lumen (c). The epithelial and endothelial surfaces have small surface blebs of membrane and cytoplasm (arrowhead). Uranyl acetate and lead citrate. (Magnification 4000X).

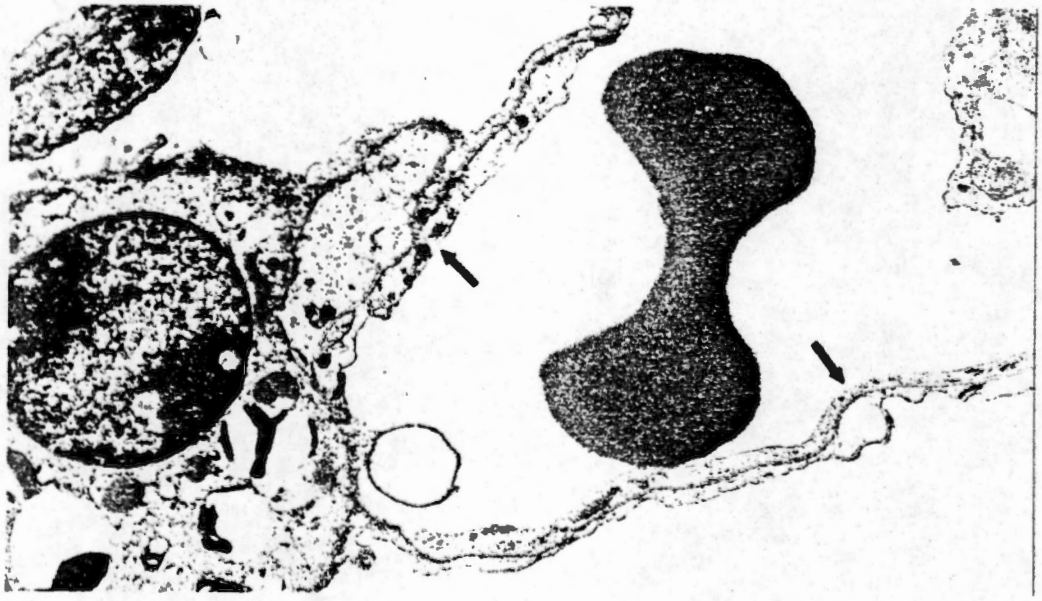

**Figure 5.2. Electron micrograph of Saline and 24 H 80% $O_2$ treated lungs at 24 H.** Pinocytotic vesicles are prominent in the endothelial cells (arrows). Uranyl acetate and lead citrate. (Magnification 4000X).

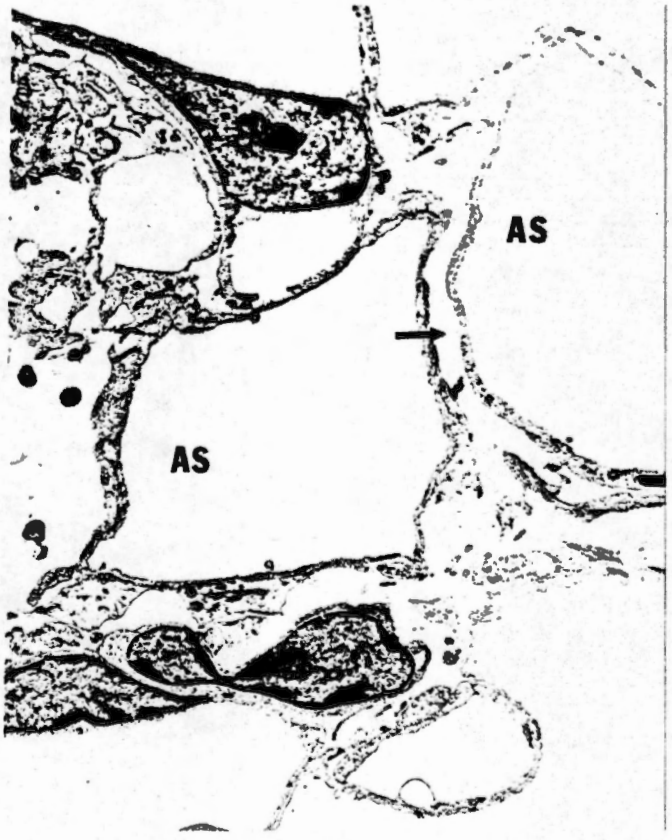


**Figure 5.3. Electron micrograph of Saline and 24 H 80% $O_2$ treated lungs at 24 H.** Interstitial edema (arrow) is moderate. Air spaces (AS) are mildly compressed. Uranyl acetate and lead citrate. (Magnification 4000X).

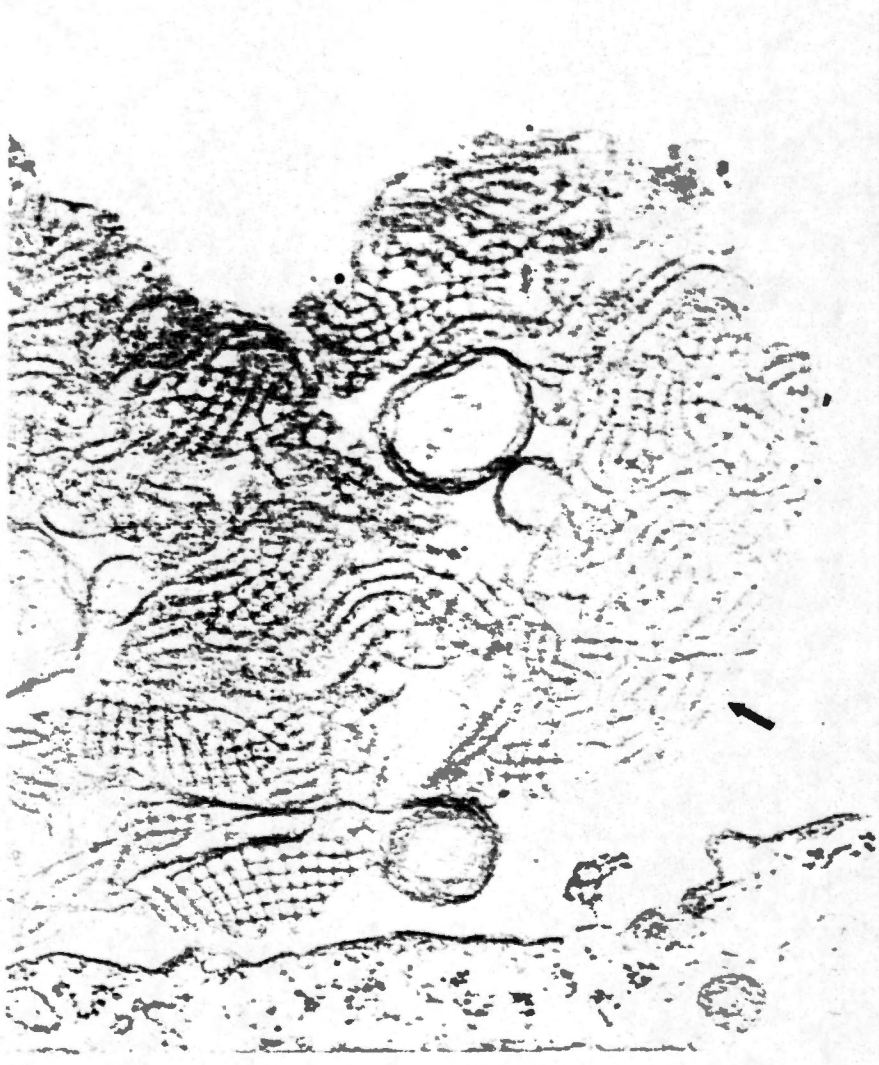

**Figure 5.4. Electron micrograph of surfactant in Bleomycin and Air treated lungs.** Surfactant aggregate (arrow) in the air space along the alveolar epithelium (e). Uranyl acetate and lead citrate. (Magnification 20,000X).

**Table 5.9. Alveolar Ultrastructure-Leukocytes and Exudates**

| Treament | Time | Observations | |
|---|---|---|---|
| | | Leukocytes | Exudate |
| SA[a] | 24 | -[e] | - |
| | 48 | - | some erythrocytes |
| | 72 | some macrophages | slight |
| SO[b] | 24 | eosinophils prominent | minimal to moderate |
| | 48 | some macrophages | - |
| | 72 | increased macrophages | some erythrocytes |
| BA[c] | 24 | alveolar neutrophils | minimal to moderate |
| | 48 | interstitial neutrophils<br>some macrophages | some erythrocytes<br>surfactant prominent |
| | 72 | increased macrophages | - |
| BO[d] | 24 | alveolar neutrophils<br>eosinophils prominent | minimal |
| | 48 | interstitial neutorphils<br>some macrophages | moderate |
| | 72 | marked increased macrophages | moderate |

[a]Saline and air.
[b]Saline and 24 H 80% $0_2$.
[c]Bleomycin and air.
[d]Bleomycin and 24 H 80% $O_2$.
[e]None observed or normal.

exudate is a granular amorphous material found along the alveolar epithelial surfaces. A few small foci of cytoplasmic luceny, indicative of intracellular edema, were in epithelial and some endothelial cells. Neutrophils were increased in number in alveolar air spaces, interstitial spaces, and in vessels (Figure 5.5). Only one degranulated neutrophil, in an alveolar air space, was seen. All cells were intact; however, small scattered cytoplasmic surface




**Figure 5.5. Electron micrograph of Bleomycin and Air treated lungs at 24 H.** Neutrophils (n) are prominent in the air spaces. Uranyl acetate and lead citrate. (Magnification 4000X).

projections, as seen in group SA, were slightly increased in size and number in group BA. At 48 H, more foci of surfactant aggregates were prominent. Fewer neutrophils were present. At 72 H, epithelial and endothelial cell cytoplasmic lucency was increased, compared to 48 H, as were interstitial cell spaces, indicative of mild to moderate intracellular and interstitial edema.

Group BO hamsters had variable areas of mild to moderate epithelial and endothelial cell lucency and increased interstitial spaces at 24 H. Occasional endothelial cells were seen with portions of cytoplasm elevated from the basement membranes. Alveolar exudate was scattered and mild with small foci of prominent surfactant aggregates. Neutrophils were increased in air spaces; two degranulated neutrophils were observed in air spaces (Figure 5.6). Lungs from animals at 48 H were similar to those at 24 H. In some areas alveolar exudate was moderate (Figure 5.7). By 72 H the alveolar exudate was diffuse and marked. Numerous large macrophages, some fibrin strands, erythrocytes, and neutrophils as well as surfactant aggregates, were in the exudate (Figure 5.8, page 114). Epithelial cells were intact and had scattered small foci of mild to moderate intracellular edema. Endothelial cells were not necrotic or edematous. Endothelial cell junctions were often larger and more prominent then normal with overlapping (Figure 5.9, page 115). Endothelial cell cytoplasm had several areas of marked attenuation (Figure 5.10, page 116). Some areas of endothelial cell basement membrane were covered by a very thin layer of cytoplasm or debris. Gaps in endothelial cells, exposing basement membrane, were occasionally seen (Figure 5.11, page 117). A few scattered small areas of focal segmental dissolution of endothelial cell cytoplasm were observed, frequently associated with intravascular leukocytes




**Figure 5.6. Electron micrograph of Bleomycin and 24 H 80% treated lungs at 24 H.** Neutrophils (n) are in air spaces (s) and capillaries (c). A large macrophage (m) is also in the air space. Uranyl acetate and lead citrate. (Magnification 4000X).




**Figure 5.7. Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 48 H.** Alveolar exudate (X) is mild. Surfactant aggregates (a) are prominent. An eosinophil (e) is in the air space exudate. Uranyl acetate and lead citrate. (Magnification 4000X).




**Figure 5.8. Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H.** Marked exudate (X) and macrophages (m) are in the air spaces. Type II cells (t) and capillary lumens (c) are relatively normal. Uranyl acetate and lead citrate. (Magnification 4000X).

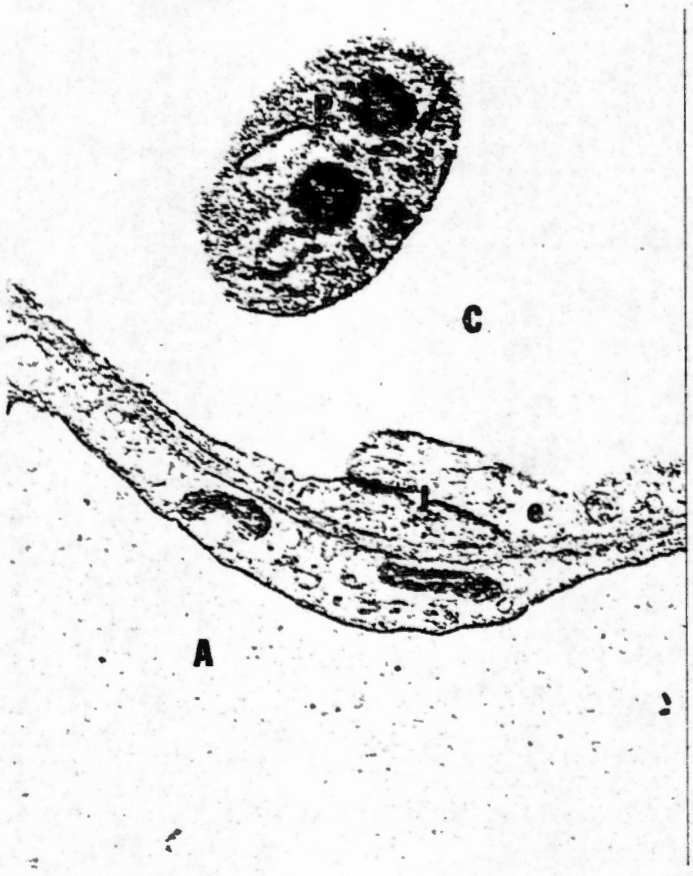


**Figure 5.9. Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H.** The endothelial cell junction (J) is prominent and overlapping. (Capillary lumen = C, platelet = P, endothelial cell = e, air space = A with exudate). Uranyl acetate and lead citrate. (Magnification 15,000X).

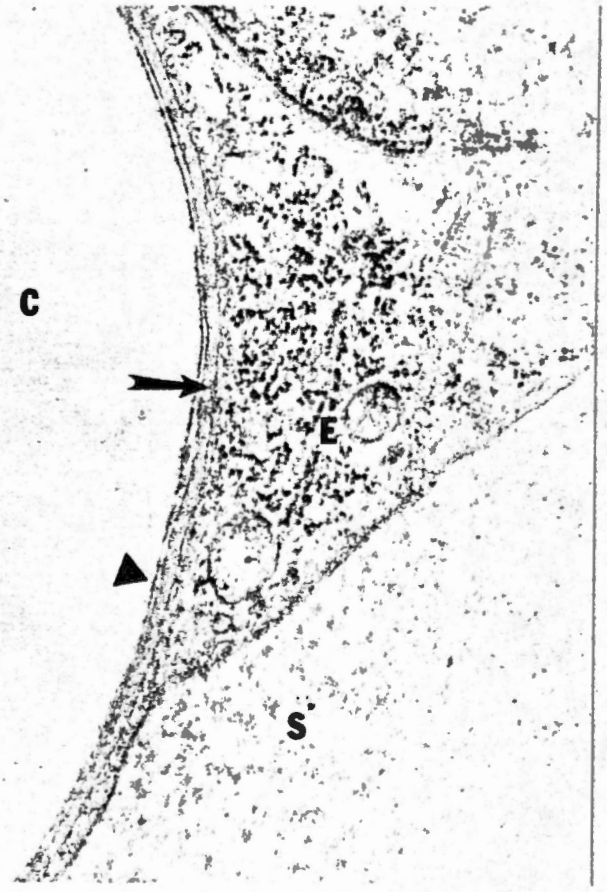


**Figure 5.10. Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H.** Endothelial cell (arrowhead) is markedly attenuated. Capillary lumen = C, epithelial cell = E, air space = S. Basement membrane at arrow. Uranyl acetate and lead citrate. (Magnification 17,000X).

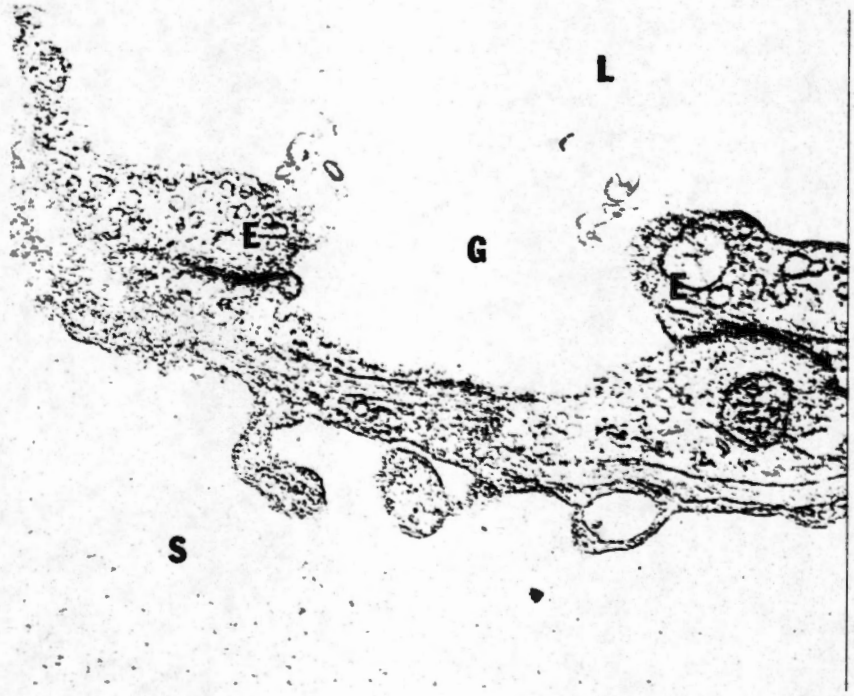


**Figure 5.11. Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H.** A gap (G) between two segments of endothelial cells (E). Capillary lumen = L, air space = S. Uranyl acetate and lead citrate. (Magnification 17,000X).

(Figure 5.12, page 000). Major morphologic changes included moderate interstitial edema and prominent endothelial cell pinocytotic vesicles in group SO, increased neutrophils and mild to moderate intracellular and interstitial edema in group BA, and marked alveolar exudate in group BO. Groups BA and BO had prominent surfactant aggregates. Increased neutrophils were in group BA; group BO had increased macrophages. Endothelial cell alterations in group BO at 72 H, not seen in the other treatment groups, included large, overlapping cell junctions, focal cytoplasmic attenuation, and segmental cytoplasmic dissolution.

## Discussion

Previous ultrastructural morphometry and morphology studies have examined animals treated with either hyperoxia or bleomycin. These studies have concentrated on subacute and chronic lesions; acute lesions were observed but usually not on a day-by-day basis. Ultrastructural examination of human biopsy or necropsy samples has been done at several time points during the course of the disease. This study of bleomycin-$O_2$ treated hamsters examined samples every 24 H for three days after bleomycin treatment and 24 H 80% $O_2$ exposure. A systematic ultrastructural study of pulmonary changes observed at 24, 48, and 72 H in rodents treated with bleomycin and hyperoxia has not been previously reported.

Human cases of acute bleomycin pulmonary toxicity have ultrastructural changes of DAD. These lesions including smaller alveolar spaces, a result of atclectasis, increased size and numbers of macrophages;




**Figure 5.12. Electron micrograph of Bleomycin and 24 H 80% $O_2$ treated lungs at 72 H.** Segmental focal dissolution of an endothelial cell (E) associated with a monocyte (M) in the capillary lumen. Uranyl acetate and lead citrate. (Magnification 17,000X).

desquamated type II epithelial cells with bizarre morphology; damaged, necrotic, and absent type I cells; and marked interstitial edema (Bedrossian et al., 1973).

In rodents exposed to continuous hyperoxia, 80% $O_2$ or higher for 6 days or longer, the earliest lesions observed in dead animals were type I epithelial cell edema and type I cell necrosis. At 3 to 5 days, surviving animals developed acute interstitial edema without type I cell damage (Adamson et al., 1970). These changes are similar to those seen in group SO hamsters in this study of bleomycin and hyperoxia in hamsters. However lungs from killed but not dead animals were examined in this study.

In other rodent studies, animals treated with bleomycin have increased number of macrophages by day three. These samples also had type I epithelial cell and endothelial cell edema. Endothelial cells also had areas of discontinuity and cytoplasmic ballooning. Type I epithelial cells were intact and type II cells were normal until 1 to 2 weeks after treatment (Aso et al., 1976). These acute changes are similar to those observed in group BA and BO, reported here.

The cause of the marked alveolar air space edema in group BO hamsters was probably increased microvascular permeability due to endothelial as well as epithelial cell alterations. Endothelial cell nuclei and most of the cytoplasm was intact. Pinocytotic vesicles were not prominent, as they were in group SO hamsters. The gaps or discontinuities and areas of segmental cell dissolution were adjacent to normal cell cytoplasm. The gaps and attenuated areas may represent greatly enlarged cell pores or may be areas resulting from segmental cell disruption. The epithelium appeared normal and only focal areas

of individual endothelial cells seem affected associated with alveolar edema.

A possible cause of endothelial cell damage is free radical formation. The source of free radicals could be bleomycin, hyperoxia, or a combination of these two agents. Leukocytes, especially neutrophils which were increased in group BO, may also be involved in free radical production. Free radicals cause lipid peroxidation of cell membranes resulting in cell damage and necrosis. This would correlate with the morphology of the endothelial cells, including the segmental cell dissolution and membrane alterations.

Another possible cause of pulmonary edema may be hyperactivtion or alteration of cellular transport mechanisms. Both epithelial and endothelial cells transfer fluids and particles through the cytoplasm by active transport mechanisms (Effros and Mason 1983). An increase in cAMP or other alteration in messenger activity may cause enhanced transport into the alveolar interstitium or air space, or decreased transport from the alveolar air space into the vasculature and interstitium. (Crandall et al., 1983; Effros and Mason 1983; Ryan et al., 1985).

Alveolar edema and interstitial edema can be due to structural or functional changes in alveolar epithelial or alveolar capillary endothelial surfaces. Many investigators consider the endothelial barrier to be the more important surface that regulates alveolar flooding (Brigham 1977; Brigham 1982; Staub 1981; Crandall et al., 1983). The epithelial surface, however, is more impermeable and has tighter cell junctions and smaller pores than the endothelium (DeFouw 1983; Pietra 1984). In this study, the endothelial cells have structural alterations; however the cells are not necrotic. The epithelial cells have changes of intracellular edema, but also are not necrotic. The

pulmonary edema, therefore, is probably a result of epithelial and endothelial interaction and is not reliant on one surface alone to restrict it or to cause it.

This study defines the ultrastructural lesions of bleomycin and hyperoxia synergism of hamster lungs at the acute stage of damage. In this model, ultrastructural studies are more sensitive than functional methods or light microscopy to evaluate edema. This study does not answer whether endothelial or epithelial surfaces are more important in alveolar fluid regulation, although group BO animals do have definite endothelial cell changes. Further studies on bleomycin and $O_2$-induced lung damage should include leukocyte activity studies, free radical measurements, and examination of endothelial cell changes at time points between those examined.

# CHAPTER VI

# PULMONARY SURFACTANT

## Introduction

Pulmonary surfactant in the lining layer of the alveoli is a complex phospholipid mixture responsible for decreasing suface tension so that the alveoli do not collapse. Since it is in the lining layer, surfactant is exposed to all inhaled gases and substances. Any gas, liquid, or small particulate agent entering through the airways first comes in contact with the surfactant lining of the alveoli, then the epithelial and endothelial tissues. Toxic agents may have several effects on the surfactant layer. The layer may be physically disrupted. The phospholipids may react with the agents such that the normal composition is altered. The surfactant layer may be removed by the toxic substance, or by a subsequent pulmonary reaction. Surfactant production may be altered by action of the substances on the type II epithelial cells, which produce surfactant. Therefore, alterations or damage to type II cells may be reflected in the surfactant produced (Kikkawa and Smith 1983).

The importance of an intact, normal surfactant layer is emphasized by the disease process in newborns called hyaline membrane disease (HMD) or respiratory distress syndrome (RDS). The amount of surfactant or the phospholipid composition of surfactant is altered in HMD such that the alveoli collapse easily (Farrell and Avery 1975; Brown and Duck-Chong 1982). Therefore, both the amount of surfactant as well as its composition must be

normal in order to maintain normal low alveolar surface tension (Morgan 1971; Clements 1977; Kikkawa and Smith 1983).

In adult respiratory failure, studies have been done in selected cases to determine changes in surfactant quantity and quality. Unfortunately neither the methods, the sampling technique, nor the results have been consistent. Quality of surfactant has been determined as the ability to adequately decrease surface tension rather than by the more quantitative phospholipid assays, few of which have been done (Petty et al., 1977; Petty et al., 1979).

The adult respiratory distress syndrome is characterized by lesions of DAD, including pulmonary edema. The alveolar edema fluid, generally considered due to a change in pulmonary vascular permeability, must pass through alveolar endothelium, interstitium, epithelium, and finally the surfactant lining layer. High-protein content alveolar fluid is believed to react with and to "wash-out" the lining layer. Therefore, surfactant content of ARDS patients' bronchoalveolar lavage may be increased. The quality of this surfactant is altered as measured by decreased ability to maintain surface tension. The quality may be altered because of interactions with the initial toxin or pulmonary insult, reactions with the high-protein content edema fluid, which may de-activate the phospholipids, or because of changes in type II epithelial cell metabolism (Petty et al., 1979). Altered type II cells, secondary to damage or toxic interactions, may increase or decrease surfactant production, or may be unable to produce the normal phospholipid complexes (Kikkawa and Smith 1983). Experimental studies of disease models in animals would be useful to determine surfactant alterations.

Assays of surfactant quantity and quality can be done on broncho-alveolar lavage fluid, fractionated lung samples, or any combination of pulmonary fluids or tissues. Assays of surfactant are frequently performed in newborn infants to determine lung status by obtaining and assaying samples of amniotic fluid. These assays of surfactant in amniotic fluid are routinely done on infants that are premature or delivered by caesarean section. Samples for surfactant assays from older patients are usually obtained by bronchoalveolar lavage. In animals, many technique to assay surfactant can be used, including culture of type II pneumocytes (Brown and Duck-Chong 1982; Kikkawa and Smith 1983).

Both quantity and quality of the surfactant samples should be assessed. Quantity can be asessed by measurement of total phosphorous content, however this is not accurate for very small quantities such as may be present in bronchoalveolar lavages (Kikkawa and Smith 1983). Quantity can also be roughly measured by density gradient centrifugation and direct visualization with electron microscopy. Quality and quantity of phospholipid content can both be assessed with various methods based on thin-layer chromatography techniques (Brown and Duck-Chong 1982).

To determine if surfactant alterations occur in the bleomycin-hyperoxia model of ARDS in hamsters the quantity and quality of surfactant was measured. Surfactant sampling was performed by bronchoalveolar lavage. This method was chosen because it was simple and was comparable to samples obtained from adult human cases of lung damage. To determine quantity and quality of surfactant, extracted phospholipid material was separated by high-

performance thin-layer chromatography (HPTLC). This technique yields good phospholipid separation in a short time. Because of rapid chemical reactions of phospholipids, rapid processes are essential. Also, HPTLC is sensitive enough to give very good results with small amounts of sample. (Touchstone et al., 1980).

## Materials and Methods

### General Methods

Hamsters were obtained and handled as in the previous studies (Chapter IV, page 44). Bleomycin was prepared and administered as for previous experiments.

### Surfactant Measurement Methods

Hamsters were divided six per cage into groups of 24 per treatment group, and 24 per day for four days for a total of 96 animals. The treatment and exposure groups were SA, SO, BA, and BO (Table 4.2, page 48). Six animals were sacrificed from each treatment and exposure group at the end of 24, 48, 72, and 96 H for a total of 24 hamsters per 24 H. Timepoints were designated as hours after intratracheal bleomcyin or saline.

Hamsters were anesthetized with intraperitoneal Na pentobarbital prior to sample collection. Anesthetized animals were placed on their backs; the diaphragm was penetrated and the trachea exposed. A 22 gauge needle covered by a 3 cm sleeve of 19 gauge Teflon$^{R}$ tubing was placed and ligated with 2-0

gauge cotton sutures into the trachea. Four ml of 0.9% sterile saline was slowly instilled into the lungs, alllowed to remain 30 to 45 seconds, and then rapidly withdrawn by a 5 ml plastic syringe. Additional lavages were not done because diseased animals would have large amounts of blood contaminating the subsequent samples. The volume of recovered lavage fluid was noted. Lavage fluid was placed in 15 ml plastic screw-capped slanted test tubes. Animals were then killed by abdominal aorta laceration.

Fluid samples in slanted, capped plastic tubes were centrifuged for 5 minutes at 250 g. The supernatant fluid was collected, leaving approximately 0.5 ml above the cell pellet, and placed in a glass test tube. Two ml of 100% methanol was added; this was vortex-mixed for 10 seconds. Two ml of 100% chloroform was added; the mixture was vortex-mixed for one minute. The capped tubes were then centrifuged at 950 g for 10 minutes. The lower chloroform layer was collected and placed in another glass test tube. This fluid was evaporated to dryness with a gentle stream of nitrogen or argon at 37 to 40°C. Samples were resolubilized in 200 ul of chloroform and vortex-mixed (Touchstone et al., 1980; Brown and Duck-Chong 1982).

Plates used were nonfluorescent high-performance thin-layer chromatography (HPTLC) plates with Silica Gel 60, F-254 for nano-TLC, E. Merck, Darmstadt, W. Germany. Prior to use, plates were washed in methanol and dried in an oven at 145 to 150°C. Ten ul of each sample were applied to the plates, six per plate, with a Camag Linomat III, Muttenz, Switzerland.

Standards used were L-alpha-lecithin and sphingomyelin 1 mg/ml, L-alpha-phosphatidylethanolamine 1 mg/ml, DL-alpha-phosphatidyl-DL-glycerol

dipalmitoyl 1 mg/ml, L-alpha-phosphatidyl-L-serine 1 mg/ml, and L-alpha-phosphatidylinositol 1 mg/ml (Sigma Chemical Company, St. Louis, MO). Compounds were supplied in chloroform solutions. Standards were applied to plates in varying known amounts, from 1 to 30 ug, to obtain standard curves. Samples and standards were allowed to dry after application on plates.

Prepared plates were saturated by placing in a chamber for 5 to 7 minutes with evaporating solvent. Solvent used was 15 ml chloroform, 4.5 ml methanol, 12.5 ml 2-propanol, 3 ml 0.25% KCl in $H_20$, and 9 ml trimethylamine (Touchstone et al., 1980). Plates were run with the above solvent in a Camag HPTLC Linear 28510 chamber for 35 to 40 minutes. Plates were dried and then sprayed thoroughly with cupric acetate spray reagent (3% cupric acetate and 15% phosphoric acid in ethanol, (Sigma Chemical Company, St. Louis, MO). Sprayed plates were placed in an oven at 145 to 150°C for a minimum of 2 hours (Touchstone et al., 1980).

Charred HPTLC plates were scanned for reacted phospholipid bands in a densitometer, a Camag TLC-Scanner, at 525 nm with a tungsten visible light source. The slit width of the light source was 12 nm; zero was set to 0.27, span to 0.91 and sensitivity to 12. Tracings were made at 0.5 mm/second. Densitometry tracings were analyzed for areas-under-the-curve with the morphometry measurement program (MOP) of a Zeiss Videoplanner.

Standard curves for values of dipalmitoyl phosphatidylcholine (DPPC) and sphingomyelin (SM) were determined by use of a Zeiss Videoplanner. Densitometric tracings of HPTLC plates yielded areas-under-the-curve relative to the amount of material. Areas under the curve were measured on the

computer screen and converted to a standard curve. Correlation between known values of DPPC and standard curve from areas-under-the-curve was 0.78; for SM correlation was 0.72. To correct areas-under-the-curve to ug, DPPC values were multiplied by 0.28934 and SM values by 0.14962. These figures were determined from the slopes of the standard curves.

Phospholipids deteriorate rapidly and must be processed and analyzed rapidly or the correlations will be less than those observed. Variables, other than the extremely rapid deterioration, include differences in processing times of HPTLC development, spray reagent reactions, heating time and temperature for plate charring, and densitometric scanning. These procedures were all undertaken as rapidly and as uniformly as possible to avoid variables in measurement (Brown and Duck-Chong 1982).

## Results

The results of the surfactant analysis are summarized in Table 6.1 and Figures 6.1 through 6.3. Hamsters treated with saline and air were the control animals. Values measured were DPPC, SM, and L/S ratios. The values from groups SO, BA, and BO were compared to group SA values which were defined as the control values. Measurements were compared as ug amounts and as deviations from control values.

Group SO had decreased DPPC at 48 and 72 H. The DPPC and SM values of group SO were normal at all other timepoints. The L/S ratio for SO hamsters was the same as SA at 24, 48, and 96 H, but significantly decreased at 72 H.

**Table 6.1. Surfactant Values of Bronchoalveolar Lavage Samples**

| Treatment Group | Hours After Treatment | DPPC (ug)[c] | SM (ug)[d] | L/S Ratio[e] |
|---|---|---|---|---|
| SA | 24 | 19.0 ± 3.9[a] | 1.1 ± 0.1 | 18.4 ± 4.2 |
| | 48 | 37.2 ± 5.5 | 1.7 ± 0.3 | 22.6 ± 2.6 |
| | 72 | 22.7 ± 3.2 | 1.0 ± 0.1 | 24.0 ± 3.5 |
| | 96 | 13.7 ± 0.9 | 1.2 ± 0.2 | 12.3 ± 1.5 |
| SO | 24 | 10.4 ± 0.3 | 1.1 ± 0.3 | 11.1 ± 1.4 |
| | 48 | 19.8[b] ± 0.8 | 1.1 ± 0.1 | 18.1 ± 1.9 |
| | 72 | 7.16[b] ± 0.6 | 0.6 ± 0.1 | 12.5[b] ± 1.4 |
| | 96 | 11.5 ± 1.2 | 1.1 ± 0.1 | 10.5 ± 1.4 |
| BA | 24 | 12.0 ± 0.9 | 1.0 ± 0.1 | 12.6 ± 1.0 |
| | 48 | 13.1[b] ± 1.5 | 1.3 ± 0.2 | 10.6[b] ± 0.9 |
| | 72 | 21.2[b] ± 1.6 | 1.5 ± 0.1 | 15.3[b] ± 1.8 |
| | 96 | 12.8 ± 1.2 | 1.5 ± 0.4 | 10.5 ± 1.7 |
| BO | 24 | 18.5 ± 1.9 | 2.5 ± 1.1 | 11.2 ± 2.4 |
| | 48 | 12.6[b] ± 2.0 | 1.7 ± 0.2 | 7.3[b] ± 0.50 |
| | 72 | 7.5[b] ± 0.7 | 1.8 ± 0.2 | 4.5[b] ± 0.8 |
| | 96 | 16.6 ± 2.4 | 1.7 ± 0.5 | 11.5 ± 1.4 |

[a]Mean ± standard error.

[b]Significant at $p < 0.05$, compared to control values, Saline and Air, at the same timepoints.

[c]DPPC = dipalmitoylphosphatidylcholine = lecithin.

[d]SM = sphingomyelin = S.

[e]L/S = lecithin/sphingomyelin.

Group BA hamsters had decreased DPPC at 24 and 48 H; values were the same as SA hamsters at 72 and 96 H. Values for SM were not increased or decreased at any timepoint. Group BA L/S ratios were decreased at 48 H and 72 H.

Hamsters in group BO had the most significant differences in DPPC, SM, and L/S ratio values. At 24 H DPPC was equal to SA; at 48 and 72 H it

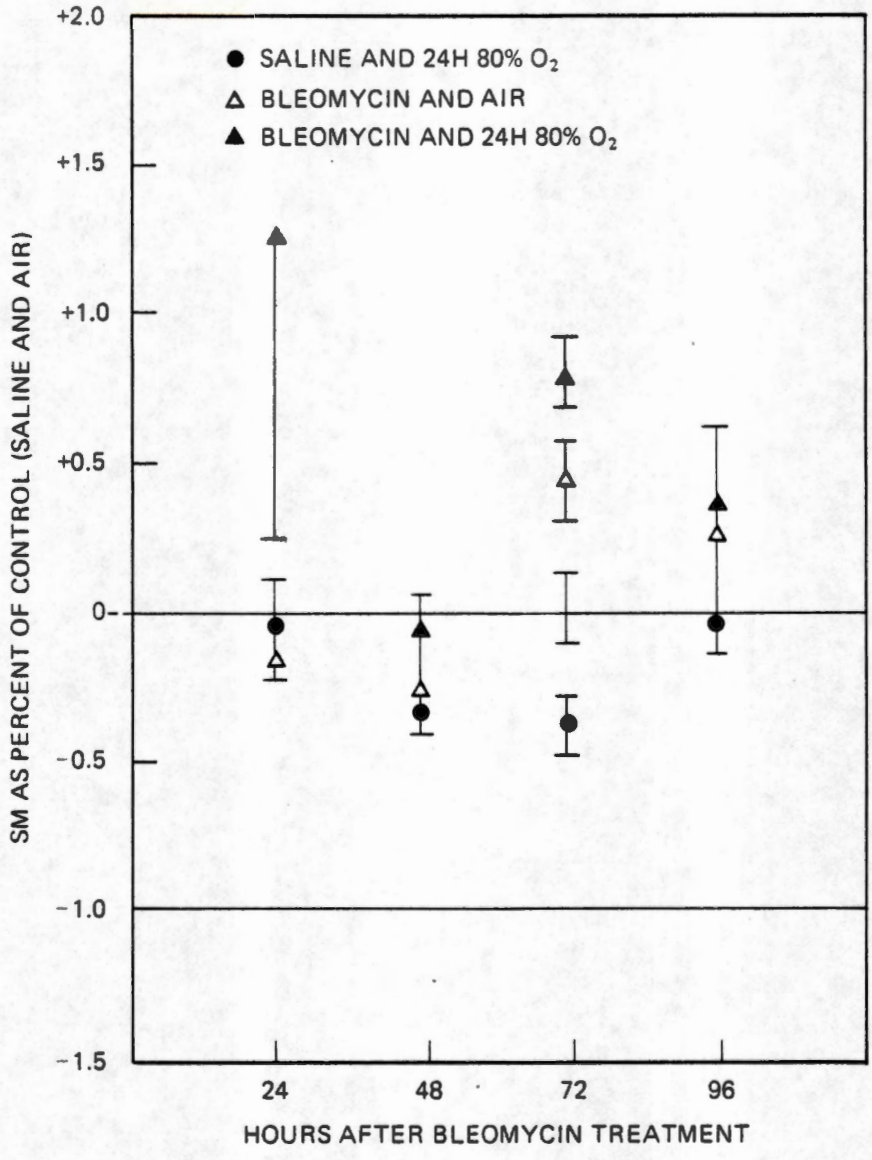


**Figure 6.1. DPPC values expressed as percent of control. Control is denoted group SA.** Significant decreases in DPPC are seen at 48 and 72 H in groups SO, BA, and BO.

[1]Mean ± standard error.

*Significant at $p < 0.05$.

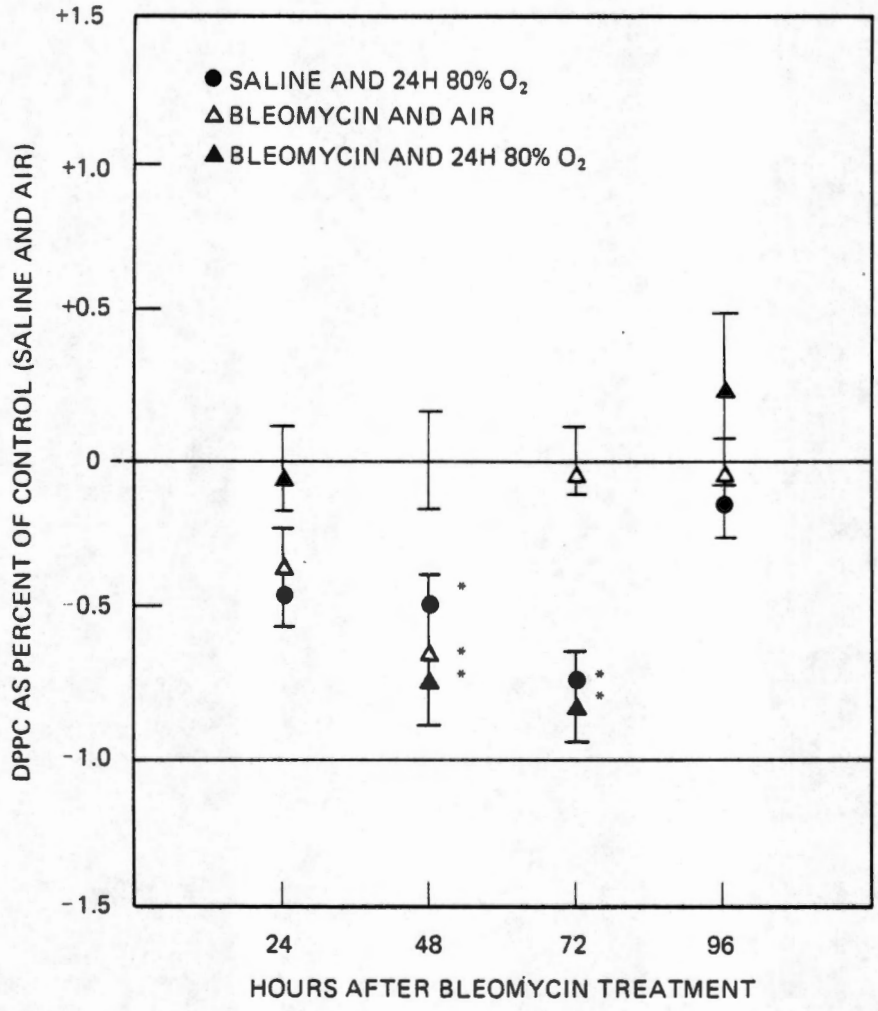


**Figure 6.2. SM values expressed as percent of control, denoted group SA.** No statistically significant differences are noted.

[1]Mean ± standard error.

*Significant at $p < 0.05$.

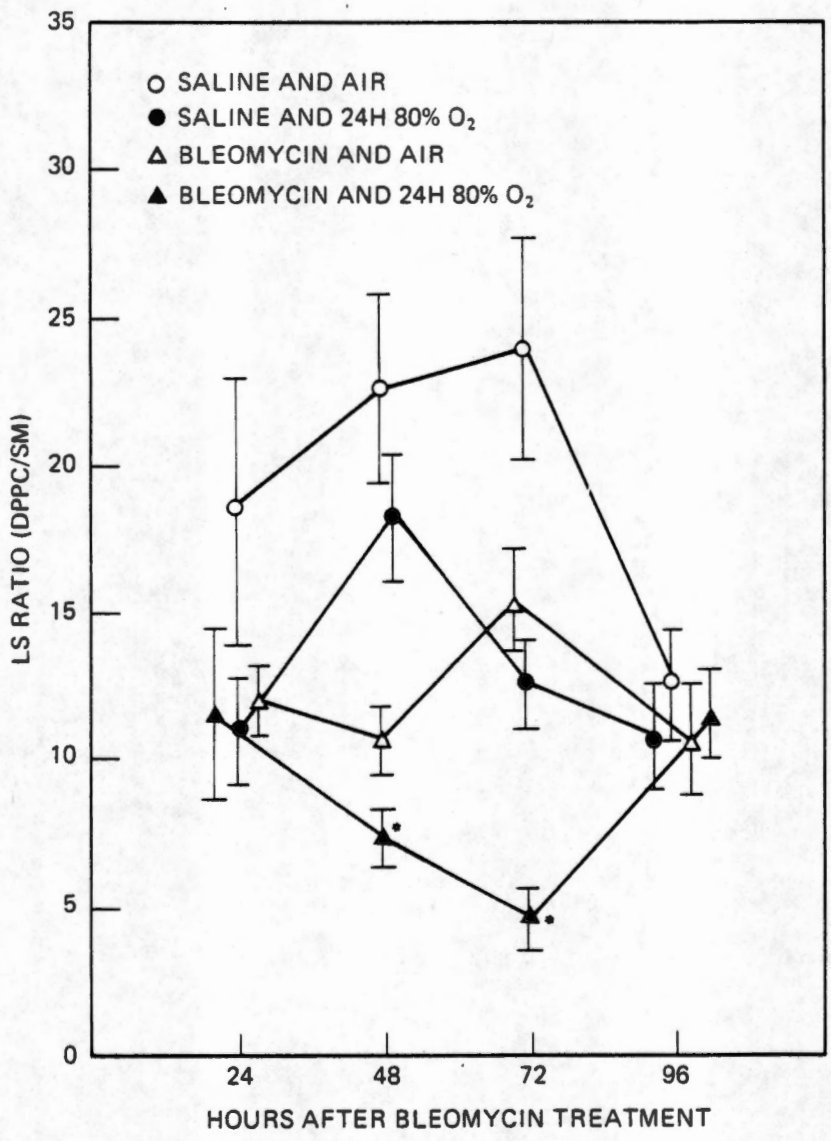


**Figure 6.3. DPPC/SM ratios (lecithin/sphingomyelin ratios) indicate changes in amounts and quality of phospholipids.** The DPPC/SM ratio is significantly decreased at 48 and 72 H in group BO only.

[1]Mean ± standard error.

*Significant at $p < 0.05$.

was significantly decreased. By 96 H, DPPC was again the same as SA values. The SM values were significantly increased at 24 H and were the same controls at 48. The L/S ratio of group BO was significantly decreased at 48 and 72 H. By 96 H the L/S ratio was the same as control.

Therefore, significant changes were a decreased DPPC ratio in groups SO, BA, and BO at 48 and 72 H. Group BO L/S ratios at 48 and 72 H were significantly lower than groups SO and BA.

Gross examination of bronchoalveolar lavage samples is usually not informative, however at 72 and 96 H group BO hamster samples were blood-tinged. This color was removed when the samples were centrifuged. Centrifugation probably removed most of the erythrocytes and larger particles contaminating the sample, however some serum may have not been removed. Blood contamination of surfactant samples may interfere with HPTLC (Touchstone et al., 1980; Brown and Duck-Chong 1982).

## Discussion

The major change measured in pulmonary surfactant in hamsters treated with bleomycin and 24 H 80% $O_2$ is a marked decrease in L/S ratio at 48 and 72 H which is mainly due to a marked decrease in DPPC. Dipalmitoylphosphatidyl choline (DPPC) is the phospholipid unique to pulmonary surfactant and is considered the active component. Sphingomyelin (SM) is a component of surfactant and is also found in cell membranes and serum (Kakkawa and Smith 1983). The L/S (DPPC/SM) ratio allows

comparison of changes in lung surfactant by comparing the amount of pulmonary phospholipids (Brown and Duck-Chong 1982).

The decrease in DPPC and L/S ratio in group BO hamsters may reflect a change in pulmonary type II cell metabolism. In autoradiograph studies of hamsters treated with bleomycin and 80% $O_2$, type II cells are not labelled in response to alveolar damage (Chapter IV). Alveolar damage occurs as early as 24 H and is severe and diffuse by 72 H. Bleomycin is known to inhibit normal type II cell division and DNA synthesis in acute phase damage and to cause hyperplasia and metaplasia in subacute phases (Adamson 1976). Lungs injured by butylated hydroxytoluene also have altered type II cell metabolism and acute decreases in surfactant (Smith 1983). The additional insult of 24 H exposure to 80% $O_2$ increases the damage to type II cells. Type II cells are then less able to divide to repair damage and to synthesize surfactant.

By ultrastructural studies, surfactant aggregates are prominent in groups BA and BO at 24 H and 48 H. Prominent surfactant aggregates may indicate protein-surfactant interactions or deactivation of surfactant (Kikkawa and Smith 1983). Few aggregates are observed at 72 H in group BO animals. Therefore, decreased surfactant in group BO hamsters may be due to "wash-out" by alveolar edema fluid at 72 H as well as aggregation and deactivation by protein interactions.

Surfactant alterations in bleomycin and hyperoxia-induced acute respiratory failure may be caused by alterations of type II cell metabolism or may be secondary to surfactant interactions with high-protein edema fluid. Regardless of the cause, surfactant amounts and composition, reflected in

DPPC content and L/S ratios, are altered in the bleomycin and hyperoxia-induced ARDS of hamsters. The decrease in surfactant is more likely a reflection of ARDS and pulmonary damage than a direct type II epithelial cell effect. Measurements of surfactant quantity and quality are probably good additional tests in the diagnosis and characterization of acute respiratory failure.

# CHAPTER VII

# CONCLUSIONS

## Bleomycin and Hyperoxia in Hamsters

Hamsters treated with 5 U/kg bleomycin intratracheally and exposed to as little as 12 H 80% $O_2$ develop acute-onset pulmonary edema by 72 H after treatment. Hamsters dying with pulmonary edema show signs of dyspnea, tachypnea, lethargy, and nasal and oral foamy exudates. Gross lesions observed are diffuse pulmonary edema and congestion, and focal to diffuse hemorrhages. The interaction of bleomycin and $O_2$, given at doses which alone cause 0 to 15% mortality induces fatal delayed-onset acute pulmonary edema.

Microscopic lesions progress from mild change at 24 H to severe lesions at 72 H. At 24 H, lungs have scattered focal increases in alveolar macrophages, and mild perivascular and peribronchial edema. Changes at 48 H are mild to moderate perivascular edema, larger foci of alveolar macrophages, and some interstitial and alveolar polymorphonuclear leukocytes. Scattered alveoli, associated with the infiltrates, have mild interstitial edema and hemorrhages. Lesions seen at 72 H are more diffuse and severe than those of 48 H. The perivascular and peribronchial edema is moderate to marked. By light microscopy large areas of alveolar tissue which are almost but not quite confluent have marked interstitial edema, numerous alveolar macrophages, some alveolar and interstitial polymorphonuclear cells, hemorrhages, proteinaceous exudate, and scattered hyaline membranes. Similar but more severe lesions are seen at 96 H. The observed clinical signs and gross and microscopic lesions are characteristic of ARDS and DAD.

Pulmonary edema development, followed with a large molecular weight substance, $^{125}$I-BSA (60,000 daltons) and a smaller substance, $^{99m}$Tc-DTPA (493 daltons), occurred at 72 and 96 H. Although mild to moderate perivascular, peribronchial, and focal alveolar interstitial edema was evident by light microscopy at 24 and 48 H, the degree of edema was not enough to be detected by the radiolabelled tracers. Therefore radiolabelled tracer studies are not sensitive enough to detect early changes of edema. Clinically the lesions and edema were not severe enough to result in death, which occurred at 72 H and later.

Cellular kinetics, measured by autoradiography, showed a highly significant increase in alveolar macrophage label at 96 H in bleomycin-$O_2$ treated hamsters. The type II epithelial cell label, considered a marker of type I epithelial cell injury and repair, did not change but remained at a constant low level. This indicated a lack of type II cell response to that damage. Interstitial and endothelial cell responses were not different from those of control animals. Therefore the major observations of cell kinetics studies was the large increase in macrophage cell labeling at 96 H, and the lack of type II epithelial cell response.

Morphometry studies at the electron microscope level focused on alveolar structure and alterations. Changes detected by objective examination and comparison of bleomycin and $O_2$ animals with controls were seen at 24, 48, and 72 H. At 24 H the volume fractions of the interstitial spaces, alveolar neutrophils, interstitial neutrophils, vascular neutrophils, and vascular eosinophils were increased. At 48 H, all neutrophils and vascular eosinophil fractions were increased. By 72 H, total tissue, vascular lumen, endothelial cell,

interstitial cell, interstitial space, macrophage, alveolar neutrophil, interstitial neutrophil, and total vascular and air space leukocytes all had increased volume fractions. At no time point was a change in type I or type II epithelial cell volume observed. Therefore at 24 H the interstitial space volume indicated interstitial edema. By 72 H, both intracellular and interstitial edema were detected, but not in epithelial cells. At all time points the leukocytes were increased, most notably all categories of neutrophils and, at 72 H, macrophages. Morphometry at the electron microscope level, therefore, is sensitive to early small changes in alveolar structure as well as later, larger changes.

Subjective evaluation of alveolar ultrastructure correlated well with morphometrical analyses. Lesions observed at 24 H were variable mild to moderate epithelial cell, endothelial cell, and interstitial space edema. Alveolar exudate was mild to moderate and had prominent surfactant aggregates. At 48 H, the edema was less prominent, the alveolar exudate was mild to moderate. By 72 H the exudate was marked and diffuse. The air spaces had numerous macrophages, some neutrophils, fibrin strands, erythrocytes, and surfactant aggregates in the exudate. Ultrastructural interstitial alveolar edema at 72 H was morphometrically significant, but morphologically mild and focal. Endothelial cells had overlapping cell junctions, foci of attenuated cytoplasm, variably-sized gaps, and some discontinuities consistent with small foci of segmental cell dissolution. Abnormalities of endothelial cell nuclei and organelles were not detectable. The major lesions at 72 H were the endothelial cell cytoplasmic changes and the alveolar air space edema.

Measurement of pulmonary surfactant, as a reflection of pulmonary edema and type II cell metabolism, also had significant changes at 72 H. Bleomycin-$O_2$ treated hamsters had decreased DPPC, SM and L/S ratios at 72 H. Levels returned to control values at 96 H. Changes in surfactant parameters may have been caused by an alteration of type II cell metabolism and surfactant production, or by surfactant alteration secondary to interation with high-protein content edema fluid.

**Significance**

Changes observed in bleomycin-treated 80% $O_2$-exposed hamsters included clinical signs of ARDS, gross, light and electron microscopic lesions of acute phase DAD, electron miroscopic lesions of endothelial cells, and alteration of pulmonary surfactant. Therefore hamsters treated with bleomycin and exposed to $O_2$ are a good model of ARDS. This model also shows a definite synergism of two agents, bleomycin and $O_2$, demonstrating a greater additive effect than the effects seen with either agent alone. It is a good model of oxygen potentiation of acute pulmonary damage. The development of pulmonary edema and damage; intracellular, interstitial, and alveolar; is characterized by several techniques. The results from these techniques correlate well. This model is predictable, reproducible, and also easy to work with because of the delayed-onset of edema.

Although the model of bleomycin and hyperoxia in hamsters may be criticized since it requires two insults to induce ARDS, it is still comparable to human ARDS since most patients with acute respiratory failure are placed on

$O_2$ therapy. In fact, many studies indicate that $O_2$ therapy may enhance acute lung damage or may be the more important factor in prognosis (Katzenstein et al., 1976; Pratt et al., 1979; Cheney et al., 1980; Hackney et al., 1981; Witschi et al., 1982). Oxygen alone, at concentrations of 90% and higher, causes ARDS in laboratory animals (Kistler et al., 1967; Kapanci et al., 1969; Adamson et al., 1970; Bowden and Adamson 1971; Adamson and Bowden 1974; Bowden and Adamson 1974; Crapo et al., 1980).

Hyperoxia, from 40 to 100%, has been shown to enhance the damage caused by other pulmonary toxins. Interaction of $O_2$ therapy and acute respiratory failure in human cases of ARDS was suggested when lesions correlated better with $O_2$ therapy than with the duration of ARDS (Pratt et al., 1979). Hyperoxia slowed the regenerative response of ozone-damaged mouse lungs (Hackney et al., 1981). Mouse lungs damaged by butylated hydroxytoluene had increased damage and fibrosis after $O_2$ exposure (Witschi et al., 1981; Haschek et al., 1983). Hyperoxia also potentiated pulmonary damage induced by methylcyclopentadienyl manganese tricarbonyl, cadmium chloride, oleic acid, and chemotherapeutic drugs (Hakkinen et al., 1983). Therefore hyperoxia has been shown to potentiate simultaneous or previous lung injury.

Bleomycin alone induces acute phase DAD and chronic pulmonary interstitial fibrosis in man. Human patients treated with bleomycin and exposed to hyperoxia as therapy or during surgery have been observed to die with signs of ARDS and lesions of DAD. The two factors considered most contributory to these fatalities were hyperoxia and excess fluids. Excess fluids were considered less significant as a cause of pulmonary damage. Therefore

bleomycin and hyperoxia synergism was suspected as a cause of fatal respiratory failure in human patients (Goldiner et al., 1978). This observation was followed by experimental animal studies, most of which concentrated on the potentiation effects of $O_2$ on subacute and chronic bleomycin pulmonary toxicity.

Hyperoxia has been shown to potentiate bleomycin-induced pulmonary fibrosis in mice (Hakkinen et al., 1982; Toledo et al., 1982) and hamsters (Rinaldo et al., 1982; Tryka et al., 1982; Tryka et al., 1984). Acute lesions of bleomycin and hyperoxia were observed in hamsters (Tryka et al., 1982), mice (Hakkinen et al., 1983), and rats (Hakkinen et al., 1983; Berend 1984). Gross and light microscopic lesions and clinical signs observed in those animals were similar to those observed in this study. This study differs in that early time points, 24, 48, 72 and 96 H after bleomycin treatment, are characterized by several techniques. Findings from these techniques correlate well with each other.

Pulmonary edema, in this model, is by definition due to increased microvascular permeability (Sprung et al., 1981; Staub 1981). It is high-protein content fluid, since substances the size of albumin, measured by $^{125}$I-BSA, leak into the interstitium and alveolar air spaces. The onset of marked acute edema is at 72 H; mild to moderate focal edema is present at 24 and 48 H. Severe acute diffuse edema is delayed until 72 H. Lesions of alveolar capillaries, detected by electron microscopy, are mild focal intracellular edema and endothelial cell changes. Alterations of endothelial cells include overlapping cell junctions, focal cytoplasmic attenuations, gaps in the cell membranes, and focal segmental cell necrosis. Epithelial cells have

mild intracellular edema. Therefore the acute delayed-onset alveolar edema at 72 H is associated with observable endothelial cell changes and not with observable epithelial cell changes. Similar endothelial cell changes have been observed in previous studies (Hurley 1978; Hurley 1982). Unfortunately these results do not rule out functional epithelial or endothelial alterations which are usually undetectable by light or electron microscopy.

Other changes leading to pulmonary edema can include functional alterations in fluid, electrolyte, and protein transport mechanisms of epithelial and endothelial surfaces. The transport mechanisms, in healthy and damaged lungs, are active transport, cell pores, pinocytosis and exocytosis, cell junctions, and diffusion (Cotrell et al., 1967; Robin et al., 1973; Gorin and Stewart 1979; Brigham 1982; Crandall et al., 1983). Cyclic AMP may be one substance that is highly significant in transport mechanism regulation (Ryan et al., 1985).

Numerous proposed mechanisms of ARDS and pulmonary edema formation have been investigated. Factors studied, as substances directly or indirectly related to ARDS and pulmonary edema, include cells, such as neutrophils, and humoral substances and local hormones, such as complement fragments and prostaglandins. Specific factors studied in the pathogenesis of bleomycin and bleomycin-$O_2$ pulmonary damage are superoxide radicals (Ishida and Takahashi 1975); pulmonary calcium, pulmonary cAMP, and pulmonary cGMP (Giri et al., 1980), prostaglandins, angiotensin-converting enzyme (Chandler and Giri 1983), superoxide dismutase, and pulmonary prolyl hydroxylase (Cunningham et al., 1983; Giri et al., 1983); and granulocyte chemotaxis (Moselely et al., 1984; Wesselius et al., 1984). *In vitro* damage of DNA by bleomycin is enhanced by superoxide radicals (Ishida and Takahashi

1975). In hamsters treated with intratracheal bleomycin, pulmonary calcium levels, and cAMP were acutely increased; cGMP was decreased (Giri et al., 1980). Intratracheal bleomycin in hamsters also acutely increased pulmonary prostaglandins $PGE_2$, $TxB_2$, and 6-keto-$PGF_{1alpha}$; angiotensin converting enzyme levels were decreased (Chandler and Giri 1983). As early as two days after bleomycin, hamster lungs had increased levels of prolyl hydroxylase and superoxide dismutase (Giri et al., 1983). Cytotoxicity of bleomycin *in vitro* can be partially reversed with superoxide dismutase (Cunningham et al., 1983). Rat pulmonary granulocytes and lung tissue exposed to bleomycin had increased granulocyte chemotactic activity (Moseley et al., 1984; Wesselius et al., 1984).

In the bleomycin-$O_2$ model of pulmonary damage, the most likely mechanism of injury is enhanced superoxide radical production. The other cellular and humoral factors associated with bleomycin and $O_2$, especially granulocyte activity, are also important factors to be considered. More detailed studies of the pathogenesis of bleomycin damage should be studied in this model. Specific factors to be included should be neutrophil activity, superoxide radical production, and effects of antioxidants.

Intratracheal bleomycin and 24 H 80% $O_2$ in adult male hamsters induces delayed-onset acute respiratory failure with pulmonary edema. Animals die at 72 H to 96 H after bleomycin with as little as 12 H 80% $O_2$ exposure. Mild edema and alveolar lesions are seen at 24 and 48 H. Severe alveolar edema and variable mild to moderate endothelial cell alterations are detectable by leakage of large and small molecular weight intravenous tracers

and by light and electron microscopy. Macrophages increase in number, size, and DNA synthesis; type II cells have no significant changes. Surfactant levels decrease at 72 H.

Endothelial cells seem to have the greatest detectable changes, in hamsters treated with bleomycin and $O_2$; however, epithelial surface changes may have gone undetected by the methods used. Future studies of this model should look at pathogenesis and mechanisms of pulmonary edema and alveolar transport of substances across endothelial and epithelial surfaces. Changes in granulocyte activity, superoxide radical formation and antioxidants are also important factors to examine.

# LIST OF REFERENCES

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