THE ROLE OF ALPHA-1-ANTITRYPSIN IN THE DEVELOPMENT OF PULMONARY EMPHYSEMA

by

RAJWINDER KAUR DHAMI

B.Sc. (Honours Cell and Developmental Biology), University of British Columbia, 1993

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

December 1999

Abstract

BACKGROUND: Cigarette smoke is the most important etiologic agent in the development of emphysema (defined as airspace enlargement in the lungs) which accounts for a growing number of deaths annually worldwide. The protease-antiprotease hypothesis has been proposed to explain the pathogenesis of emphysema and states that an excessive proteolytic burden in the lung causes breakdown of connective tissue, leading to emphysema, although the specific proteases and antiproteases are not known. METHODS: Transgenic mice expressing the human alpha-1-antitrypsin (α1AT) gene were created and characterized. Markers of connective tissue breakdown, inflammatory cell numbers and elastase activities in the bronchoalveolar lavage after cigarette smoke or bacterial lipopolysaccharide (LPS) exposure were measured. Mice were given weekly intratracheal LPS and lung morphology was assessed. RESULTS: Four transgenic lines expressed human α1AT in either type II alveolar cells or type II alveolar and airway epithelial cells. Levels of human α1AT protein in the lung were low but protein was present in the pulmonary interstitium. The transgenic mice were immunologically tolerant to human α1AT. Acute cigarette smoke exposure resulted in increased elastin and collagen breakdown products in the lavage which correlated with increases in lavage PMNs. LPS instillation resulted in elastin breakdown associated with increased lavage PMN numbers, and collagen breakdown associated with an increase in macrophage numbers. Administration of AAT prevented the connective tissue breakdown in the smoke-exposed mice and prevented elastin breakdown, but not collagen breakdown in the LPS treated mice. Mice receiving weekly intratracheal instillations of LPS developed emphysema. CONCLUSIONS: α1AT expression in pulmonary cells results in delivery

of the protein to the pulmonary interstitium where connective tissue components are vulnerable to proteolytic attack. These mice, because of their tolerance to human α1AT, are useful for long-term studies on the efficacy of human α1AT augmentation therapy. Connective tissue breakdown after acute cigarette smoke exposure is mediated by PMN-derived proteases and is prevented by α1AT. LPS-mediated lung injury is associated with both PMN serine elastases and collagenolytic proteases most likely derived from macrophages. A model of chronic LPS-mediated emphysema has been developed and is available for future studies of α1AT augmentation therapy.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

α1AT. AAT-alpha-1-antitrypsin

AEC-3-amino-9-ethylcarbazole

APA-anti-PMN antibody

ARDS-adult respiratory distress syndrome

ATS-American Thoracic Society

BALF-bronchoalveolar lavage fluid

BAPN-β-amino-proprionitrile

bg-beige

CC10-Clara cell 10 kDa protein

CF-cystic fibrosis

cDNA-complimentary deoxyribonucleic acid

CMT-N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone

COPD-chronic obstructive pulmonary disease

DEAE-diethylaminoethyl

DEPC-diethylprocarbonate

DNA-deoxyribonucleic acid

dNTP-deoxynucleotide triphosphate

DTT-dithiotreitol

ECM-extracellular matrix

EDTA-ethylenediamine tetraacetic acid

ELISA- enzyme linked immunosorbant assay

ELF-epithelial lining fluid

FEV1-forced expiratory volume in the first second

FLMP-formylated methionine-leucine-phenylalanine

HCl-hydrochloric acid

Hhp-human haptoglobin

HME-human metalloelastase

HNE-human neutrophil elastase

dH_2O-distilled water

HPLC-high pressure liquid chromatography

HRP-horse radish peroxidase

IgG-immunoglobulin

IL-interleukin

i.p.-intra-peritoneal

i.t.-intra-tracheal

kDa-kilodalton

LPS-lipopolysacharide

LTB4-leukotriene B4

MME-macrophage metalloelastase

MMP-matrix metalloprotease

MOPS-3(N-morpholino)-propanesulfonic acid

NaCl-sodium chloride

NE-neutrophil elastase

PBS-phosphate buffered saline

PCR-polymerase chain reaction

PMN-polymorphonuclear cell

PPE-porcine pancreatic elastase

RNA-ribonucleic acid

SAAAN-N-succinyl-Ala-Ala-Ala-p-nitroanalide

SDS-sodium dodecyl sulphate

SLPI-secretory leukoprotease inhibitor

SPC-surfactant protein C

20X SSC-standard saline citrate (3.0 M NaCl, 0.3 M sodium citrate, pH 7.0)

TAE-Tris/Acetic acid/EDTA

TBE-Tris/Boric acid/EDTA

TE-Tris/EDTA

Tg-transgenic

TIMP-tissue inhibitor of metalloprotease

TNF-tumour necrosis factor

TRIS- TRIS (Hydroxymethyl) Aminomethane

Tsk-tight-skin

Acknowledgements

I wish to acknowledge and thank three people whose technical assistance was invaluable to my project: Dr. Susan Porter for overseeing the creation of the transgenic mice; Katalin Zay for developing the human and mouse ELISAs and for her continual willingness to assist and advise me for the last five years; Chang-shi Xie for doing all of the HPLC analyses.

I would like to thank my supervisor Dr. Blake Gilks for his support and guidance and for our reflective discussions. I also want to thank my supervisory committee members Dr. Andrew Churg and Dr. Jody Wright whose advice and guidance were essential to the completion of my project and my committee chairman Dr. Jurgen Vielkind for stepping in mid-way through my studies and taking an interest in my research.

I must give a special thanks to all of the members of the Churg laboratory, past and present, for their friendships, and for making endless days in a windowless environment not just tolerable, but sometimes even enjoyable.

Most importantly, I would like to thank all of my family for their love and support. First and foremost I am grateful to Mukay and Jas for allowing me to be and welcoming me as an integral member of their little family and to Parm and Imraj for being good friends to me despite being related. I want to acknowledge the countless small favours each one of them has done for me over the last six years.---- Last but not least, I have to thank Javin and Meric for being my most favourite distractions.

My graduate studies were funded by grants from the Medical Research Council of Canada and the Bayer/Red Cross Research and Development Fund.

1. Experimental Objectives

The first objective of this thesis was to determine if endogenous overexpression or administration of exogenous α-1-antitrypsin (α1AT) protects against development of emphysema induced by cigarette smoking or LPS-mediated lung injury. The augmentation of α1AT levels was achieved by transgenic over-expression of human α1AT in mice or administration of purified human plasma α1AT. In this way the role of the major lung antiprotease, α1AT, in lung injury, particularly cigarette smoke-induced emphysema, can be more clearly defined.

1.1 Systematic approach to gene therapy for prevention of emphysema

It has been suggested that the pulmonary manifestations of the common genetic pulmonary diseases α1AT deficiency and cystic fibrosis (CF) may be prevented by delivery of the normal genes to the lungs (Rosenfeld & Collins, 1996, Gilardi et al., 1990). Because the respiratory epithelium is easily accessible by way of the trachea, the lung has been popular as a target for somatic cell gene therapy (Glasser et al., 1994). Despite numerous attempts to achieve successful gene transfer in both animal and human studies of pulmonary disease, an understanding of the most appropriate target organ or cell type and proof of the long-term efficacy of gene therapy is lacking (Blau & Khavari, 1997). The first major objective of this thesis was to produce transgenic mice that express α1AT in specific cells of the lung and determine if local over-expression of this antiprotease is effective in preventing lung damage induced by acute or chronic injury. Cell-specific promoters were used to direct expression of the human α1AT cDNA to the

lung: the human haptoglobin (Hhp) promoter was used to direct expression to the pulmonary interstitium (D'Armiento et al.,1992), the human surfactant protein C (SPC) promoter was used to direct expression to the surfactant producing or type II epithelial cells (pneumocytes) (Glasser et al., 1991), and the rat Clara Cell 10 Protein (CC10) promoter was used to direct expression to the airway epithelium (Stripp et al., 1992).

1.2 Efficacy of purified α1AT protein in prevention of acute and chronic lung injury

The second major objective of this project was to study the efficacy of intraperitoneally administered purified human α1AT in preventing or reducing the pulmonary connective tissue breakdown associated with acute lung injury secondary to a single dose of cigarette smoke or lipopolysaccharide (LPS). Cigarette smoke and LPS induced emphysema models (see below) were used to investigate effects of exogenous α1AT on chronic lung injury.

A further aim of this project was to gain a more general understanding of the mechanisms by which lung damage associated with cigarette smoking occurs and the role that α1AT plays in this condition. A prerequisite for this is clarification of the roles of specific inflammatory cell types and proteases in pulmonary inflammation secondary to cigarette smoke. Specifically, the role of polymorphonuclear leukocytes (PMNs or neutrophils) and inflammatory cell proteases in cigarette smoke- and LPS-mediated acute lung injury was investigated.

1.3 A Model for α1AT Augmentation Therapy

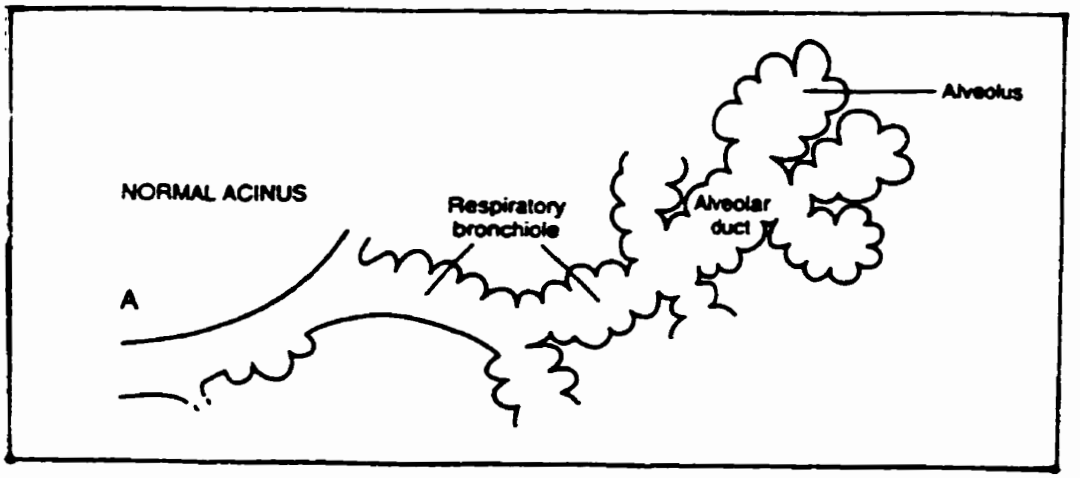
Despite the lack of conclusive proof of its efficacy, alpha-1-antitrypsin augmentation therapy is currently being used in Canada, the United States and some European countries to treat individuals with severe hereditary α1AT deficiency. The major obstacle in proving or disproving the efficacy of α1AT augmentation therapy in α1AT deficient humans with emphysema are the logistics of carrying out a randomized controlled trial in order to show that "augmentation therapy has clinical efficacy to forestall the progression of emphysema in individuals at risk" (Stoller, 1998). Past and current studies in humans are limited by lack of control groups, brevity of the follow up period (emphysema is a progressive disease that develops over many decades), and small study groups. An animal model would be ideal for such studies, and animal models of emphysema of varying degrees of relevance exist and plasma purified human α1AT is commercially available. Animals, however, cannot safely receive repeated injections of this human protein which their immune system would identify as a foreign protein. We propose that transgenic mice expressing the human α1AT gene will be able to receive repeated doses of human α1AT without developing an immune response to it. Development of a mouse model of cigarette smoke-mediated emphysema or LPS-mediated emphysema was attempted using the α1AT transgenic mice. These mice will, therefore, provide a suitable model system to determine the efficacy of α1AT augmentation therapy in preventing the development of emphysema.

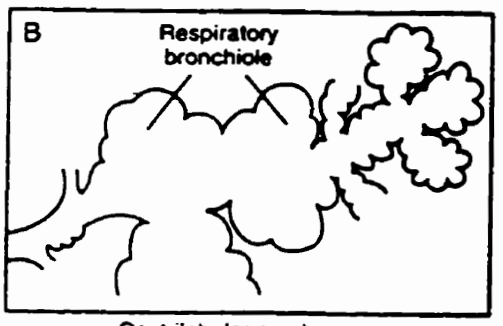
2. Introduction

2.1 Pulmonary emphysema

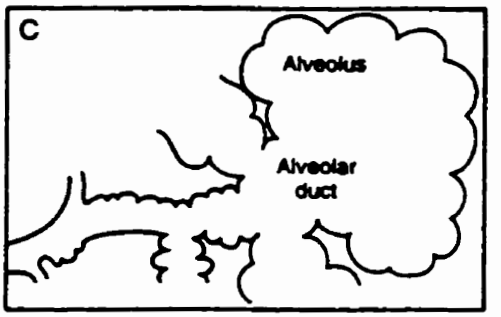
Emphysema is the irreversible enlargement of the airspaces in the lung distal to the terminal bronchioles caused by destruction of the connective tissue of the alveolar walls (Senior, 1998). This is a morphological definition and applies to both clinical and experimentally-induced emphysema. It is one of the lesions associated with chronic obstructive pulmonary disease (COPD) which was the sixth leading cause of death worldwide in 1990 (Murray and Lopez, 1997) and can be defined as a "process characterized by the presence of chronic bronchitis or emphysema that may lead to the development of airway obstruction" (Turino, 1991). Emphysema has been reported to account for approximately 18% of COPD diagnoses per year (Higgin, 1991).

Emphysema is classified into four types according to its distribution in the *acinus* which is defined as *that part of the lung supplied by one terminal bronchiole including the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveolar spaces distal to each terminal bronchiole*. Another term traditionally used to classify types of emphysema is the *lobule* which represents *a group of air spaces surrounded by fibrous septa*. There may be numerous acini within a single lobule (Pratt, 1994). The two terms are, however, often used interchangeably when describing emphysema; the term acinar will be used here to describe the two most common types of emphysema: centriacinar and panacinar emphysema (Figure 1, Thurlbeck, 1988). In **centriacinar emphysema** lung damage is restricted to the center of the acinus, involving the walls of the respiratory bronchioles early in the disease process, and eventually in severe stages of the disease, the





Centrilobular emphysema



Panacinar emphysema

Figure 1. The acinus, the fundamental unit of the lung (A) in a normal individual. Centriacinar emphysema (B) involves the respiratory bronchioles or central portion of the acinar unit. In panacinar emphysema (C) the acini are uniformly enlarged with involvement of the terminus alveoli, as well as, the respiratory bronchioles.

distal acinus is also involved. In mild centriacinar emphysema there are easily recognizable areas of lung destruction adjacent to areas of normal lung architecture. Lesions are characteristically more severe in the upper lobes in this type of emphysema. Lung damage in **panacinar emphysema**, in contrast, is evenly distributed throughout the entire acinus and is usually evenly distributed throughout the entire lung, but is sometimes more severe in the lower lung zones. The former is typically associated with cigarette smoking which is irrefutably the most important cause of emphysema and the latter with hereditary α1AT deficiency, accounting for 1-13% of all cases of emphysema (MacDonald & Johnson, 1995). Localized (also called distal acinar or paraseptal) emphysema refers to cases in which there are very few or even a single site of alveolar destruction. The fourth type of emphysema is irregular emphysema in which airspace enlargement occurs close to sites of focal lesions such as tumours. In most cases the latter two types of emphysema described are clinically insignificant.

The popular belief is that the emphysematous process begins as an increased number and size of holes in the alveolar walls and these *fenestrations* which can be observed with an electron microscope can be considered to be one of the earliest manifestations of emphysema (Pratt, 1994). The alveolar walls are composed of type I and type II pneumocytes (or alveolar cells) and an underlying connective tissue matrix (interstitium) through which the capillary network runs (Figure 2). The major elements of the connective tissue or extracellular matrix (ECM) include elastic and collagen fibres, fibronectin, proteoglycans, and the basement membrane components of endothelial and epithelial cells (Crystal & West, 1991). Progressive degradation of the components of the ECM leads to the loss of the capillary bed and alveolar attachments to small airways.

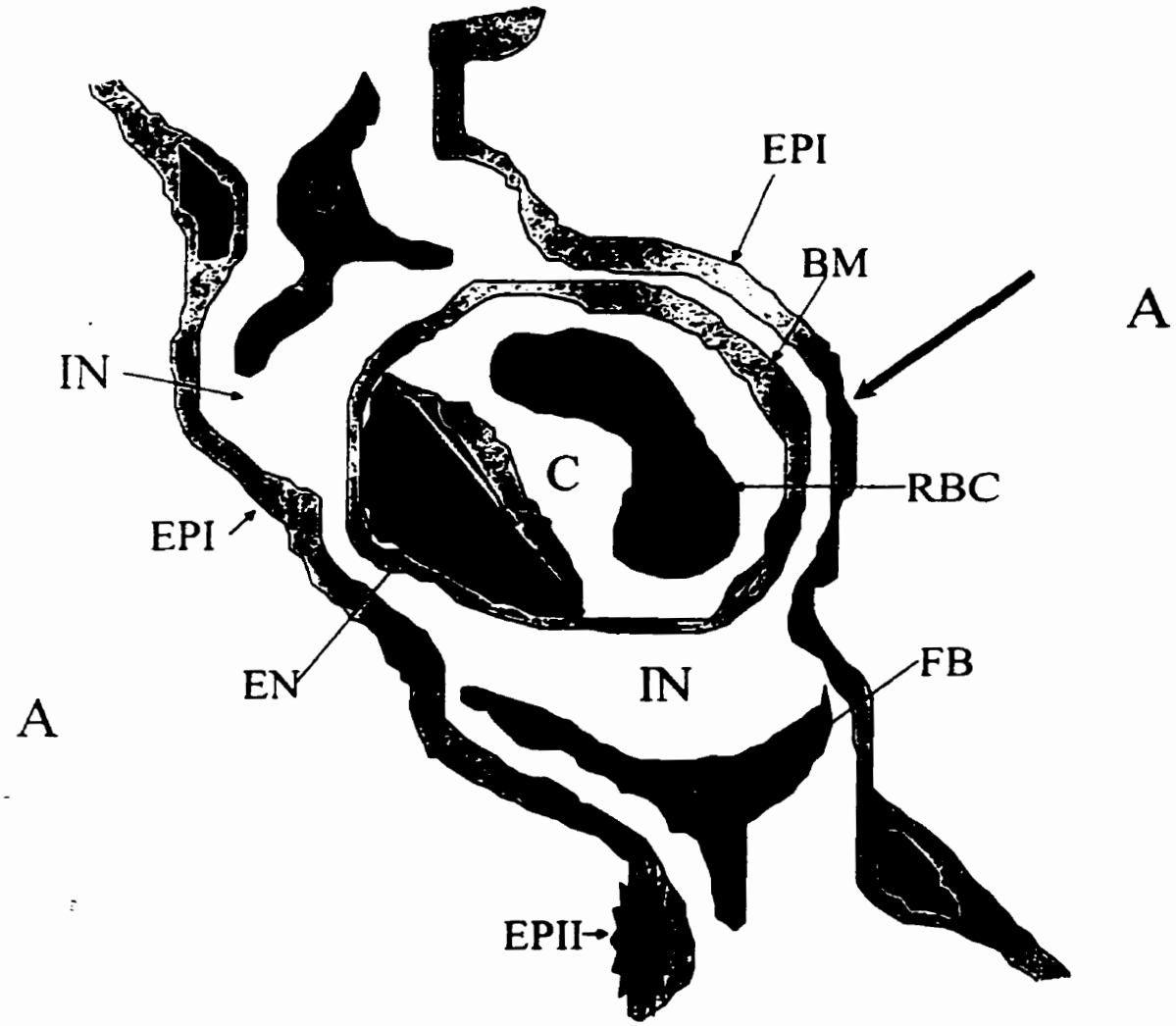


Figure 2. Illustration of an alveolar wall. A pulmonary capillary, C, type I (EPI) and Type II (EPII) epithelial cells, endothelial cells (EN), and interstitial fibroblasts (FB) are shown. Basement membranes of endothelial and epithelial cells (BM), red blood cell (RBC), interstitium (IN), and the pulmonary airspace (A) are also marked. The long arrow indicates where the epithelial lining fluid (ELF) would be present.

Significant loss of alveolar walls results in respiratory failure occurring as a result of a combination of factors including impaired gas exchange, over-inflated lungs, and airway obstruction (West, 1992).

Elastic and collagen fibers are arranged in an organized manner in the alveolar walls. Collagen probably functions in maintaining the architecture of the lungs, whereas, elastic fibres provide elastic recoil to the interstitium. The progressiveness of emphysema suggests that destruction of the entire alveolar septum occurs gradually. The development of emphysema most likely involves both destruction and synthesis of the extracellular matrix (ECM) as suggested by the increased collagen in the lungs of experimental animals with smoke-induced emphysema (Wright, et al., 1995) and by the reports of increased collagen and thickening in the alveolar walls of human emphysematous lungs (Cardoso et al., 1993). Prevention of the cross-linking of newly synthesized collagen with ß-amino-proprionitrile (BAPN) in hamsters receiving intratracheal elastase significantly worsened the emphysematous lesions in these animals (Kuhn & Starcher, 1980). Turnover of elastin in humans is extremely slow, but there is some suggestion that the elastin repair mechanism may be impaired in smokers. In vitro results indicate that lysyl oxidase, the key enzyme involved in the synthesis of elastin, is inhibited by cigarette smoke and exposure of hamsters to chronic cigarette smoke alters the incorporation of ^{14}C-lysine into desmosine (the major cross-linking amino acid in mature elastin) during the elastin repair process (Bieth,1986).

Centriacinar and panacinar emphysema rarely occur together, so it has been postulated that the underlying mechanisms of disease development of the two types of diseases are different, but based on the same principle: proteolytic degradation of the

ECM. Particulate components of cigarette smoke tend to accumulate in alveolar spaces adjacent to terminal and respiratory bronchioles where phagocytic cells are present in large numbers in an attempt to phagocytize the particles. Turnover of the cells results in release of proteolytic enzymes and free radicals that damage the alveolar walls creating lesions characteristic of **centriacinar emphysema**. In the case of individuals with α1-antitrypsin deficiency who exhibit **panacinar emphysema**, release of proteases from circulating phagocytes anywhere in the lung will leave the ECM susceptible to destruction due to a lack of plasma protease inhibitory capacity. The lower lobes receive greater perfusion of blood than the upper lobes and are therefore more exposed to elastase released by inflammatory cells circulating in that area.

2.1.1 Clinical Manifestations and Assessment of Emphysema

Symptomatic COPD is observed in only 15-20% of smokers during their lifetime depending on the length and intensity of smoking and there is substantial variation in disease susceptibility among individuals (O'Hagan, 1996). Dyspnea (shortness of breath) with physical exertion, typically in the sixth or seventh decade is the predominant symptom of COPD (WHO, 1995). Amongst individuals with α1AT deficiency, dyspnea begins at an earlier age among smokers than nonsmokers (Schwaiblmair & Vogelmeier, 1998). Weight loss and a barrel-chested appearance are seen with progression of emphysema. Many emphysema patients suffer from recurrent respiratory infections and chronic bronchitis (hence, the latter's common inclusion in the term COPD) (Senior & Anthonisen, 1998).

Prior to the development of high resolution CT scans, the most consistent method of recognizing emphysema in patients is by pulmonary function testing with the use of a spirometer and body plethysmograph (Thurlbeck, 1988). A substantial increase in total lung capacity (TLC) and functional residual capacity (FRC: volume of gas in the lung after a normal tidal volume expiration) and residual volume (RV: the volume of air remaining in the lung after a maximal expiration) are indicators of emphysema. Loss of radial traction from the surrounding parenchyma results in early airway closure and increased FRC (West, 1992). Decreased expiratory flow rate measured by a spirometer and indicated by a decrease in the forced expiratory volume (FEV_1), the volume exhaled in the first second after a maximal inspiration, is a valuable method for following the progression of disease and the efficacy of treatments (Gottlieb et al., 1996). The FEV_1 is decreased in emphysema as a result of reduced elastic recoil pressure and airway distortion. Carbon monoxide (CO) diffusing capacity measures the uptake of CO by the lung and it is determined by the diffusion properties of the blood-gas barrier which among other things depend on the thickness and area of the alveolar membrane (West, 1992). A reduced CO diffusing capacity in emphysema is an indication of the loss of alveolar walls and capillaries, but CO diffusing capacity is also reduced in interstitial fibrosis, sarcoidosis, and asbestosis, diseases in which the alveolar walls are increased in thickness. Pulmonary functions tests are useful in providing supportive information which is evaluated in conjunction with clinical history, chest radiography, and laboratory tests (West, 1992). Chest radiography can identify increased TLC indicated by a low, flat diaphragm. In α1AT deficiency overinflation is seen predominantly in the lower zones of the lung, whereas, COPD patients with normal α1AT levels show either upper zone or

uniformly distributed lung destruction (Schwaiblmair & Vogelmeier, 1998). Examination of the lung by direct inspection or high resolution CT scan is the only way to document anatomic emphysema. Morphometric measurement of the airspaces in the lungs of humans and experimental animals is used in research studies to identify emphysema. The mean linear intercept (Lm) is the most common measurement done. *The Lm is the average distance between alveolar walls* and is determined by passing a line of determined length randomly over histologic sections of the lung and counting the number of times it intercepts an alveolated surface (Thurlbeck, 1967).

Monitoring of connective tissue breakdown products in the plasma or urine of COPD patients is considered to be a feasible method of following disease progression. Elastin is the primary component of elastic fibres and lungs with emphysema contain less elastin than normal lungs (Senior & Anthonisen, 1998). Desmosine and isodesmosine are the major cross-linking amino acids present in mature elastin and their presence in the urine of experimental animals with artificially-induced emphysema correlates with degree of lung damage (Janoff, 1983a & 1985, Snider et al., 1986). Measurement of elastin-derived peptides in plasma as a test for COPD in humans has also been proposed (Akers et al., 1992) and urinary desmosine measurements were recently shown to correlate with annual FEV1 decline in smokers with COPD (Gottlieb et al., 1996).

Management of emphysema consists of a variety of supportive measures including bronchodilator use, corticosteroid administration, oxygen supplementation, the use of antibiotics for exacerbations, and education on breathing techniques and exercises (Senior & Anthonisen, 1998 and Petty, 1988). The aim of these measures is to improve the quality of life for the patient as there is no cure for this debilitating condition.

Unquestionably, the cessation of cigarette smoking is the most effective preventative measure against the development of COPD or for halting disease progression (O'Hagan, 1996). Lung reduction surgery, and lung transplantations are proposed for patients with end-stage disease (Roche & Huchon, 1997, Zenati et al., 1998). In recent years, α1AT replacement therapy has been proposed for the treatment of individuals with severe α1AT deficiency and clinical trials to test the efficacy of this treatment have been carried out or are in progress (Schwaiblmair & Vogelmeier, 1998).

2.2 Protease-antiprotease hypothesis for the pathogenesis of emphysema

A major part of the extracellular matrix (ECM) destruction associated with COPD is believed to be caused by normal inflammatory processes. Components of ECM in the lungs are vulnerable to an extensive arsenal of degradative enzymes. A significant cause of pulmonary connective tissue breakdown is the action of several proteolytic enzymes (proteases), most of which are transported to the lungs by inflammatory cells including PMNs, macrophages, T lymphocytes, eosinophils, and mast cells which have all been shown to accumulate in the lungs of patients with COPD (Hiemstra et al., 1998). In a normal, healthy lung proteolytic activity is held in check by plasma and lung-derived antiproteases.

The **protease-antiprotease theory** is widely accepted as the basis for the pathogenesis of emphysema. This theory states that **excessive protease burden in the lower respiratory tract results in connective tissue breakdown and subsequent airspace enlargement**. *The imbalance in proteases and antiproteases can occur due to either an increase in local protease activity or a decrease in antiproteases, which may be*

either functional or quantitative. Emphysema results when the balance shifts in favour of unchecked proteolytic activity. This theory originated based on two important observations. The first was a study by Laurell and Eriksson showing early-onset pulmonary emphysema in patients with hereditary deficiency in α1AT (Laurell & Eriksson, 1963); this was supported by studies showing that emphysematous lesions could be induced in animals by instilling elastolytic enzymes into their lungs (Gross, 1964, Janoff, 1983).

2.2.1 Proteases in the lung

Proteases are enzymes that break peptide bonds in the central region of proteins and they play a role in important physiological processes involving extracellular protein degradation such as tissue remodeling and repair, wound healing, and coagulation, as well as facilitating the migration of leukocytes from the vasculature into tissues (Owen & Campbell, 1999). However, excessive proteolytic activity is also involved in the pathogenesis of various diseases including emphysema. *The two main categories of proteases of potential relevance in the development of COPD are serine and metalloproteases*, classified according to the chemical nature of their catalytic centre. Serine proteases have a serine residue at their active site which is essential to their activity and metalloproteases are dependent on the metallic ions Zn^{2+} and Ca^{2+} for their action. Both types of proteases play a major role in the degradation of extracellular proteins and are produced by both PMNs and macrophages. Three of the serine proteases PMNs synthesize are neutrophil elastase (NE), cathepsin G, and proteinase 3 which are stored in an active form within azurophilic granules and have broad substrate specificities

including elastin and collagen. PMNs also produce the metalloproteases PMN collagenase (MMP-8) and 92-kDa gelatinase (MMP-9) which are stored in different cytoplasmic granules and, as the nomenclature implies, can degrade collagens. Macrophages produce primarily matrix metalloproteases (MMPs) including those with collagenolytic activity (MMP-1,-2,-3,-9,-10) and elastolytic activity (MMP-12, also called macrophage metalloelastase (MME)) (Murphy & Docherty, 1992). Although macrophages have lost their ability to produce serine proteases (Campbell et al, 1991), they are able to internalize NE and cathepsin G present in the extracellular space by virtue of surface receptors for these serine proteases and their phagocytic functions which enable them to engulf apoptotic PMNs.

PMN serine proteases are freely released in response to phagocytosis, oposonized substrates or immune complexes, and from dead or dying cells. Proteases can also be expressed on the surface of PMNs upon exposure to cytokines and chemoattractants (Owen et al., 1997); these membrane-bound proteases are fully active against their substrates. PMNs also secrete MMPs in response to various stimuli (Owen & Campbell, 1999). Unlike PMNs, macrophages do not store MMPs, but rather synthesize and rapidly secrete the proteases in response to growth factors and cytokines (Niewoehner, 1988). All MMPs are secreted in the form of proenzymes which necessitates their extracellular activation by other proteases (Shapiro, 1995).

There are several proposed mechanisms by which proteases are able to degrade the ECM under conditions where their activity should be tightly regulated by extracellular protease inhibitors. Serine protease inhibitors are susceptible to proteolytic inactivation by several proteases: α1AT is inactivated by its major substrate NE (Travis, 1988),

macrophage metalloelastase (Shapiro, 1995), cathepsin B (a cysteine protease) and several bacterial proteases (Owen & Campbell, 1999); secretory leukoprotease inhibitor (SLPI) is inactivated by bacterial proteases; and in vitro studies suggest that α1AT, the major lung antiprotease, and other antiproteases including SLPI, α1-antichymotrypsin, and α2-macroglobulin can be inactivated by reactive oxygen species released by activated leukocytes. Tight binding of proteases to their substrates, and perhaps to the surface of inflammatory cells, prevents high molecular weight inhibitors (such as α1AT and α2-macroglobulin) from gaining access to their protease substrates due to steric hindrance (Owen & Campbell, 1999, Owen et al., 1997). This exclusion of inhibitors from the matrix region in direct contact with the proteases could allow localized ECM destruction. In certain situations, the occurrence of a large number of activated or dying inflammatory cells simultaneously liberating their proteolytic contents can overwhelm the inhibitor activity. Another mechanism by which inflammatory cells can overwhelm the protease inhibitors in a localized micro-environment is with the release of extremely high concentrations of proteases from individual cells, locally exceeding the concentration of inhibitors. In this case, ECM destruction can occur virtually unimpeded until diffusion of the proteolytic activity away from the site of release allows the ratio of protease to antiprotease to decrease to below 1:1 (Owen & Campbell, 1999).

2.2.1.1 Neutrophil elastase

Circulating polymorphonuclear cells (PMNs) that have marginated in the pulmonary vasculature can migrate in large numbers into the alveolar spaces in response to chemotactic gradients and release their granular products in response to many stimuli

(Parkas, 1997). Degranulation can occur at any point during the movement of PMNs from the capillaries, through the interstitium, into the pulmonary airspaces. One of these products, neutrophil elastase (NE), is a 29 kDa serine protease that is synthesized while the cell is in the bone marrow and stored in azurophilic granules in very high concentrations (approximately 3 pg per cell) (Turino, 1991). Because of this high concentration and its relatively high elastolytic activity compared to other elastases, NE is considered to be the major protease in the lung (Senior & Anthonisen, 1998) and has historically been considered the main effector agent in the pathogenesis of emphysema. Neutrophil elastase is secreted into tissues when the PMNs are stimulated and encounter objects to be phagocytized, or after they die. The predominant role of NE is bacterial digestion in the phagolysosomes , but it is capable of attacking all the major components of the ECM including elastin, proteoglycans, collagen type III and IV, and fibronectin (Turino, 1991). Instillation of purified NE into the lungs of experimental animals leads to destruction of the lower respiratory tract (instillation of papain, a plant protease with high elastolytic activity, and purified pancreatic elastase also result in similar damage (Janoff, 1985 & Snider et al., 1986)). NE has a great avidity for α1AT (its major inhibitor), but is also inhibited by the serum protease inhibitor, α-2-macroglobulin, and by the locally produced secretory leukoprotease inhibitor (SLPI). The association rate constant of α1AT and NE is extremely rapid and the binding of the proteins is essentially an irreversible process with a particularly slow dissociation rate. Under normal conditions, the burden of PMNs and, therefore, NE in the lungs is low and the concentration of α1AT in the lungs exceeds that of NE, thereby effectively abrogating its destructive potential. α1AT is not, however, able to inhibit NE which is already bound to its substrate (Kramps et al., 1991).

Besides the classical intratracheal instillation experiments, investigators have sought other lines of evidence to demonstrate the involvement of NE in the pathogenesis of emphysema: they have shown a correlation between BAL fluid PMN elastolytic activity and NE-α1AT complexes with increased BAL fluid elastin degradation products (Betsuyaku et al., 1996), and excessive NE in the BAL fluid from patients with subclinical emphysema (Betsuyaku et al., 1995). Others have used immunohistochemistry to demonstrate the presence of neutrophil elastase in association with elastic fibres in human emphysematous lungs and in lungs of mice that spontaneously develop emphysema (Damiano et al., 1986, de Santi et al. 1995, Cavarra et al., 1996).

2.2.1.2 Macrophage-derived proteases

In recent years there have been numerous reports suggesting that macrophages and their proteases, particularly macrophage metalloelastase (MME, also known as MMP-12), are the principal effector agents in the pathogenesis of cigarette smoke-induced emphysema. MME is synthesized as a 53 kDa proenzyme and is secreted into the extracellular space where it is activated to form a 22 kDa elastase (Shapiro, 1994). Activation is achieved by cleavage of the proenzyme by serine proteases, cathepsins, or plasmin (Janoff, 1985). Metalloprotease activity of macrophages has been shown to have the capability of degrading the elastin component of ECM (Senior et al., 1989) and it has been suggested that up to half of the elastolytic activity in BAL fluid from smokers is attributable to metalloproteases (Janoff, 1983). In a guinea pig model, macrophages from the airspaces and the pulmonary interstitium displayed significantly higher elastolytic

potential after smoke exposure (Sansores et al., 1997) and in humans, macrophages from emphysematous lungs produced elevated levels of both elastolytic and collagenolytic metalloproteases (Finlay et al., 1997). Hautamaki et al., (1997) have shown that mice lacking the gene for MME do not develop emphysema after long-term cigarette smoke exposure while their normal counterparts do. Morphological evidence from human studies also points to macrophages as important agents of connective tissue degradation: resected lungs from chronic smokers show a direct relationship between alveolar wall destruction and numbers of macrophages in the lung parenchyma (Eidelman et al., 1990, Finkelstein et al., 1995). A recent study reports that macrophage-derived elastolytic activity is more closely associated with the time course of emphysema progression in rats exposed to chronic cigarette smoke than PMN associated elastolytic activity (Ofulue et al.. 1998).

2.2.1.3 Cysteine Proteases

Cysteine (or thiol) proteases include a large group of plant and animal enzymes that all contain a cysteine residue at their active sites. They have a very broad substrate range including elastin and collagen. Papain, a plant cysteine protease, was used for the original demonstration of emphysema development after intratracheal instillation (Gross 1964). Cathepsins L and S have been shown to have more activity against insoluble elastin under optimal conditions than neutrophil elastase (Chapman et al., 1994). There are four cysteine cathepsins, B, H, L, and S (cathepsin G is a serine protease) and all four are expressed in alveolar and interstitial macrophages. Cathepsin B and L-like activities have been detected in bronchoalveolar lavage fluid and some studies suggest that levels

of these cysteine proteases are elevated in the BALF and alveolar macrophages of smokers (Reilly et al., 1991, Takahashi et al., 1993). Doubts remain about the true significance of cysteine proteases in the pathogenesis of lung injury, however, because they are normally tightly sequestered in the lysosomes. For these enzymes to be active macrophages must be in close contact with the substrate (making them less susceptible to the effects of soluble inhibitors) (Chapman et al., 1984). Another putative role for cysteine proteases is the activation of proenzymes as demonstrated by the ability of cathepsin B to process prorenin, procollagenase, and prourokinase. Cathepsin B is also capable of proteolytically cleaving α1AT (Burnett et al., 1983).

2.2.2 Protease inhibitors in the lung

The lung contains a number of protease inhibitors that play the consequential role of hindering the destructive actions of proteases. Most protease inhibitors are active against a specific class of proteases. Alpha-1-antitrypsin, secretory leukoprotease inhibitor (SLPI), and elafin specifically inhibit serine proteases. **Alpha-1-antitrypsin** is a serum protein produced primarily by hepatocytes, accounts for the highest serum, as well as lung, antiprotease concentrations, and will be discussed in detail below. **SLPI** is a 12 kDa protein produced by large airway epithelium and type II pneumocytes and its substrates include NE and cathepsin G. SLPI is the major anti-NE enzyme in the upper respiratory tract and it is capable of inhibiting NE to the same degree as α1AT (McElvaney & Crystal, 1997b). In vitro studies suggest that SLPI may theoretically be a more efficient inhibitor of NE than α1AT (it can inhibit elastin-bound NE and it has some activity even when oxidized, Bingle & Tetley, 1996) and has the interesting ability to also

increase the antioxidant screen of the lung (Vogelmeier et al., 1996). As a result of its low molecular mass, it may be able to diffuse through the interstitium more readily than the larger inhibitors (Bieth, 1986). Due to these characteristics, SLPI, in conjunction with α1AT, has been proposed as a therapy for the treatment of NE-mediated diseases (Bingle & Tetley, 1996). **Elafin** is a 6 kDa protein also produced by airway epithelium and inhibits NE and proteinase-3, but its contribution to the protection against alveolar destruction is not known (Senior & Anthonisen, 1998). **Alpha-2-macroglobulin** is a large (720 kDa) molecule that has the broadest substrate spectrum and has inhibitory activity against all classes of proteases. It is synthesized primarily by hepatocytes and fibroblasts and, to a lesser extent, by macrophages (Senior & Anthonisen, 1998). It is an effective inhibitor of NE and cathepsin G, but it has been postulated that it plays its primary role is as an inhibitor in the circulation rather than the tissues (Travis, 1988). Tissue inhibitors of matrix metalloproteases (**TIMPs**) are the fourth important inhibitors in the lower respiratory tract. They are the major natural inhibitors of MMPs and are produced by various cell types including macrophages and lung parenchymal cells (Murphy & Docherty, 1992). TIMP-1 and -2 (30 kDa and 23 kDa respectively) have been characterized and recently TIMP-2 (along with MMP-2) has been shown to be expressed at high levels in human pulmonary emphysema (Ohnishi et al., 1998). Recent reports implicating MMPs in the pathogenesis of emphysema suggest that TIMPs may prove to be important protective agents in this process (Senior & Anthonisen, 1998, Hautamaki et al., 1997, Finlay et al., 1997).

The major physiologic inhibitor of the cathepsins (cysteine proteases) is cystatin C which is also expressed in macrophages (Chapman et al., 1994) and the major serum inhibitor of cysteine proteases is α-2-macroglobulin (Burnett et al., 1983).

Table 1: Cells considered to be important in the pathogenesis of emphysema, the major proteases they release, their respective substrates, and inhibitors. α1AT = α1-antitrypsin, α2MG = α2-macroglobulin, SLPI = secretory leukoprotease inhibitor, MMP = matrix metalloprotease

Cell	Protease	Class	Substrates	Inhibitors
PMN	Neutrophil Elastase	Serine	Elastin, collagen	α1AT, α2MG, SLPI, Elafin
	Cathepsin G	Serine	Elastin, collagen	α1AT, α2MG, SLPI, Elafin
	Proteinase 3	Serine	Elastin, collagen	α1AT, α2MG, SLPI, Elafin
	MMP-8	Metallo-	Collagen, gelatin	TIMPs, α2MG
	MMP-9	Metallo-	Gelatin, collagen, elastin	TIMPs, α2MG
Macrophage	MMP-1	Metallo-	Collagen, gelatin	TIMPs, α2MG
	MMP-2	Metallo-	Gelatin, collagen, gelatin	TIMPs, α2MG
	MMP-12	Metallo-	Elastin, collagen	TIMPs, α2MG
	Cathepsin S, L, B, H	Cysteine	Elastin, collagen	Cystatin, α2MG
Fibroblast	Cathepsin D	Cysteine	Elastin, collagen	Cystatin, α2MG
	MMPs	Metallo-	Collagen	TIMPs, α2MG

Commonly used names:
MMP-1 = Fibroblast type or interstitial collagenase
MMP-2 = Gelatinase A
MMP-8 = Collagenase 2 or Neutrophil collagenase
MMP-9 = Gelatinase B
MMP-12 = Macrophage metalloelastase

2.2.2.1 Alpha-1-antitrypsin

Human α1AT is a 52 kDa glycoprotein, made of 394 amino acids, produced mainly by hepatocytes and released into the circulation (Colau et al., 1984). It is a globular protein with three carbohydrate side chains. Differences in these carbohydrate sidechains are seen as two major bands and three minor bands on isoelectric focusing analyses of normal serum (Crystal & West, 1991). It is a member of the 'serpin' or serine protease inhibitor family, so called because inhibitors in this group all have a serine residue at their reactive centre. The α1AT gene is 12.2 kb and a 1.4 kb transcript is expressed in hepatocytes (Brantly et al., 1988) and a 1.3 kb transcript is expressed in macrophages (Perlmutter et al., 1985). The mouse α1AT gene is a 413 amino acid polypeptide with a molecular mass of 54 kDa. There is a 70% identity between the mouse and human α1AT nucleic acid sequences and a 65% identity between mouse and human α1AT peptide sequences (Sifers et al., 1990). Both proteins share similar carbohydrate attachment sites and an active site Met-Ser sequence, as well as, similarity in their signal peptide sequences suggesting possible similarities in protein processing and transporting. The kidney, intestines, salivary glands, islet cells, and megakaryocytes are all possible additional sites of expression of both mouse and human α1AT as suggested by transgenic studies using the complete human α1AT gene (Koopman et al., 1989, Ruther et al, 1987, and Carlsson et al., 1988). Like many other serum proteins, α1AT is an acute phase reactant and serum levels of the protein can increase several fold in response to stressors, but the exact stimuli responsible for upregulation are not known (Sanford et al., 1997). The plasma half-life of α1AT is approximately 4-5 days and deglycosylation of the

protein significantly reduces the half-life (Crystal, 1990) as in the case of recombinant human α1AT protein.

α1AT is capable of inhibiting the proteolytic action of several proteases including trypsin, chymotrypsin, pancreatic elastase, cathepsin G, plasmin, and thrombin (McElvaney & Crystal, 1997a). Despite the fact that it has a broad spectrum of substrate specificity, inhibition of neutrophil elastase (NE) is considered to be its major function in vivo, and α1AT accounts for greater than 90 percent of the anti-NE activity in the lower respiratory tract (Crystal,1990). The reactive site of α1AT contains the Met358 residue which is essential for its enzymatic activity, and is very sensitive to oxidation, which results in inactivation of the antiprotease.

2.3 Pulmonary inflammatory response

An acute and chronic inflammatory response is believed to be fundamental in the pathogenesis of cigarette smoke-induced emphysema. Acute pulmonary inflammation is characterized by large numbers of PMNs in the vasculature, interstitium, and intra-alveolar spaces of the lung (Downey et al., 1993). This response is important in host defense functions, but in many situations it is integral to the frequently accompanying tissue injury that occurs. The production of oxidants and release of proteases by PMNs is critical to both conflicting roles (i.e. anti-microbial and tissue destruction) these cells play in the inflammatory response. PMNs are capable of generating reactive oxygen species by virtue of a plasma membrane-associated NADPH oxidase system. Upon stimulation, PMNs actively generate superoxide anion, hydrogen peroxide (H_2O_2) and the hydroxyl radical which react with many important biological substrates (Weiss, 1989). Moreover,

PMN granules store the enzyme myeloperoxidase which, when released into the extracellular environment, reacts with H_2O_2 and halides to generate large quantities of reactive oxygen species (Weiss, 1989). Reactive oxygen species can directly damage the ECM and cells of the lung or they can mediate reactions that impair antiproteases or that activate proteases (Gadek, 1992). The respiratory epithelium is ordinarily protected against damaging effects of oxidants by a sufficient antioxidant screen. The enzymes superoxide dismutase (SOD), catalase, glutathione reductase, and glutathione peroxidase are intracellular antioxidants (Buhl et al., 1996). The extracellular environment is protected from oxidants by albumin, ceruloplasmin, vitamins E, A, and C, as well as, SOD and catalase. The molecule GSH (reduced glutathione methionine) is a very important antioxidant both intracellularly and in the epithelial lining fluid. Proteases produced by inflammatory cells can inactivate antioxidants (Buhl et al., 1996).

Fluids recovered from sites of inflammation contain free, active PMN proteases that have escaped the antiprotease defense (Weiss, 1986). Release of proteases in an unregulated manner results in the haphazard destruction of host cells. It is likely that the oxidative and proteolytic capabilities of the PMNs act in concert or synergistically to give rise to acute inflammatory lung injury (Gadek & Pacht, 1996) and a further recruitment of inflammatory cells occurs after release of cytokines from the cells initially recruited. Because of this destructive potential, PMNs are implicated in a number of inflammatory lung disorders including COPD, cystic fibrosis, adult respiratory distress syndrome (ARDS), chronic bronchitis, and asthma (Hiemstra et al., 1998).

2.3.1 Proinflammatory mediators

The hallmark of the inflammatory response is the influx of leukocytes into the affected area. PMNs account for a large percentage of these cells in acute inflammatory reactions and are recruited into the lungs by the cooperative actions of chemoattractants and cytokines produced by lung cells. Cytokines are a large group of non-enzymatic protein hormones that influence the migration and activation of lymphocytes and phagocytic cells during the inflammatory response by allowing cell-to-cell communication or by inducing chemotactic responses of leukocytes (Kelso, 1998, Lukacs & Ward, 1998). The term chemokine refers to small chemotactic peptides (Johnston et al., 1998). The expression of these molecules occurs in a coordinated manner and influences the intensity of the PMN response (Figure 3) (Hiemstra et al, 1998).

IL-1 and TNFα are early-response cytokines and are critical for initiation of cytokine production and PMN recruitment into tissues by stimulating the expression of adhesion molecules on the surfaces of endothelial cells and PMNs (Streiter et al., 1993). They are produced early in the inflammatory response following the initial insult although all of the cellular sources of these cytokines are uncertain (Strieter et al., 1993). In response to LPS, however, it is known that alveolar macrophages produce these two early inflammatory mediators (Lukacs & Ward, 1998).

TNFα and IL-1 are two of the known factors responsible for the expression of IL-8 (also called PMN-activating peptide, NAP) which is a powerful, specific PMN chemoattractant and activating agent (Kunkel et al., 1991). IL-8 over-expression has been implicated in a number of acute inflammatory pulmonary diseases. It is produced by alveolar macrophages, endothelial cells, lung fibroblasts and type II-like epithelial

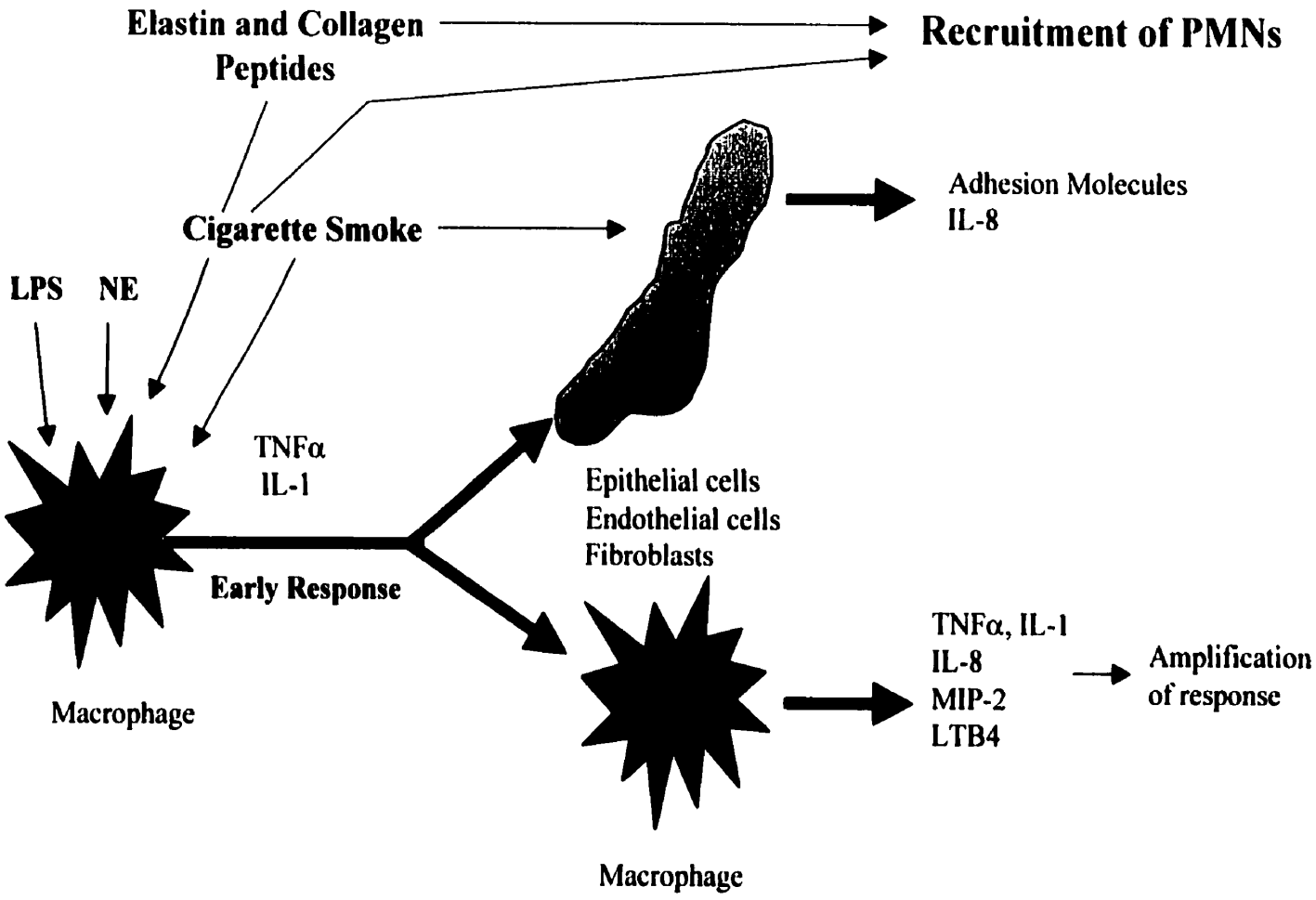


Figure 3: A variety of stimuli have been shown to induce release of early inflammatory mediators from macrophages which stimulate lung cells to release cytokines that are chemotactic for PMNs or induce the expression of adhesion molecules required for PMN migration. Cigarette smoke and elastin and collagen peptides may be directly chemotactic for PMNs.

cells (A549, Kunkel, et al., 1991) in response to IL-1 and TNFα (Carolan & Casale, 1996). Elevated levels of IL-8 in the lavage of COPD patients and smokers with chronic bronchitis correlated with increased numbers of PMNs (Pesci, et al., 1998, Sun, et al., 1998). Components of cigarette smoke may also be responsible for the increase in IL-8 production (Mio et al., 1997, Pesci et al., 1998). Elevated levels of this cytokine were also demonstrated in the lavage of cystic fibrosis patients (Khan et al., 1995, Canonico et al., 1996), as well as, in the lungs of ARDS patients (Strieter & Kunkel, 1994).

IL-8 appears to be critical for the PMN response, but other unidentified chemokines are probably also involved in directing PMN migration into the lung (Strieter & Kunkel, 1994). Mice do not express IL-8, but they express MIP-2 which belongs to the IL-8 supergene family and is produced by LPS-stimulated murine macrophages. MIP-1α, a PMN and macrophage chemoattractant, has also been implicated in the LPS-mediated pulmonary inflammatory response (Huang et al., 1992, Standiford et al., 1995). Furthermore, bacterial components such as FMLP, and immune complexes and complement fragments such as C5a are directly chemoattractant for PMNs (Carolan & Casale, 1996). The presence of complex cytokine networks between immune and nonimmune cells of the lung serve to direct the resolution of the inflammatory response, but in many instances may lead to irreversible injury to the affected organ.

In addition to cytokines, other factors present in the lungs as a result of the initial inflammatory influx serve as PMN chemoattractants. Free NE attaches to surface receptors on macrophages inducing the release of leukotriene B4 (LTB4), and it has been shown that macrophages recovered from smokers' lungs release more LTB4 than those from nonsmokers' lungs (Hubbard et al., 1991). Membrane-bound NE has also been

shown to induce the secretion of a number of other proinflammatory mediators including IL-8, IL-6, GM-CSF, and PAF (platelet-activating factor) from epithelial cells, endothelial cells and macrophages (Owen et al., 1997). In addition, the complex between NE and α1AT is a PMN chemoattractant (Banda et al., 1988) and so are the breakdown products of the connective tissue components elastin and collagen (Riley et al., 1988, Gipson, et al., 1999).

2.4 Cigarette smoke-mediated emphysema

As discussed above, cigarette smoking is indisputably the major factor linked to the development of emphysema (Senior & Anthonisen, 1998). The populations of alveolar macrophages and PMNs (protease-containing cells) in the lung are increased in response to cigarette smoke exposure (Hunninghake et al., 1983, Ludwig et al., 1985, Finkelstein et al., 1995). Because these cells are both significant sources of elastases, they are thought to be the most likely candidates contributing to excess elastolytic activity in smokers. Some studies suggest that cigarette smoke delays PMN retention in the lungs and this contributes to a localized proteolytic imbalance within the vascular space (Brumwell et al., 1991). The activation of the marginated PMNs causes release of PMN proteases and generation of oxygen radicals that inactivate α1AT. The excessive proteases resulting from the imbalance then pass into the interstitium causing alveolar wall destruction. This could explain the greater severity of lesions in the upper lung regions of emphysema patients where transit times of blood cells are slower (Hogg, 1987). Furthermore, PMN retention was shown to be significantly higher in patients

experiencing an acute exacerbation of COPD compared to patients with stable COPD or normal subjects (Selby et al., 1991).

A compilation of evidence acquired over the past thirty years strongly favours the protease-antiprotease hypothesis as an explanation for the pathogenesis of cigarette smoke-induced emphysema, and states that excessive elastolytic burden in the lungs is a direct cause of the destruction of connective tissue components. Neutrophil elastase (NE) has historically been considered the main culprit, since a deficiency in the major inhibitor of NE, α1AT, is associated with early-onset pulmonary emphysema (Eriksson, 1964). PMNs produce ten times as much elastase per cell as macrophages (Chapman & Stone, 1984) and NE has been shown to increase significantly in the plasma immediately after smoking (Weitz, 1987). In vitro studies have shown that components of cigarette smoke are able to activate PMNs and cause them to release their elastolytic contents and generate oxidants (Blue & Janoff, 1978). Experiments with intratracheal administration of human NE into experimental animals confirmed that this protease is capable of causing lung airspace enlargement (Janoff, 1985). More recently, a direct correlation between elastase burden and emphysema in humans has been demonstrated (Fujita et al., 1990). The presence of desmosine, a breakdown product of enzymatic degradation of elastin, is increased in smokers, with the highest levels present in those individuals with the most rapid decline in pulmonary function (Gottlieb et al., 1996). Excessive amounts of NE are present in the airspaces of smokers' lungs (Janoff, 1983b), but have been shown to be efficiently inhibited by functional α1AT (Yoshioka et al, 1995, Jochum et al., 1985).

Nevertheless, it is believed that loss of antiproteolytic activity can also contribute to the protease/antiprotease imbalance in the lungs and oxidation of α1AT directly or

indirectly by cigarette smoke results in its inactivation (Evans & Pryor, 1994). The issue of inactivation of α1AT by cigarette smoke is controversial, but is a crucial point in determining whether serine elastases are most important in the pathogenesis of cigarette smoke-induced emphysema and whether anti-serine protease therapy could be of benefit in smokers. Early studies showed that α1AT in lungs of smokers had a two-fold reduction in anti-elastase activity compared to that from normal lungs (Gadek et al., 1979) which may result from a reduced association rate constant (Ogushi et al., 1991). This reduction in activity may result from direct inactivation of α1AT by oxidants present in cigarette smoke or by oxidants released by PMNs recruited to the lungs (Weiss, 1989). Oxidized methionine residues in α1AT were detected in BALF from smokers, but not in BALF from non-smokers (Janoff, 1983a). Oxidized residues were identified in α1AT purified from the lungs of cigarette smokers and these interfered with the association between α1AT and NE (Carp et al., 1982). Oxidation of the α1AT reactive methionine results in the loss of over 95% of its anti-elastase activity (Johnson & Travis, 1979). It would appear from a number of investigations, therefore, that smokers have reduced defenses against NE activity and an "acquired" local α1AT deficiency, but other studies have not reproduced these results (Stone et al., 1983, Abboud et al., 1985).

In recent years, the principal role of PMNs in cigarette smoke-mediated emphysema has been challenged with the view advanced that macrophages and macrophage elastases are more important mediators of lung damage (Chapman & Stone, 1984, Shapiro, 1994). Metalloproteases present in macrophages are also capable of degrading mature elastin in the ECM (Senior et al., 1989) and as a metalloelastase, MME is not inhibited by α1AT and, in fact, is capable of degrading it (Senior & Anthonisen,

1998). Since the vast majority of inflammatory cells in smokers' lungs are macrophages, they may prove to be important mediators of chronic lung injury in long-term cigarette smokers.

In addition to cigarette smoke, there is evidence that occupational exposure to mineral dusts such as coal and silica is associated with the development of emphysema (Oxman, et al., 1993). Elastin and collagen breakdown has been demonstrated acutely in the lungs of rats after intratracheal instillation of silica (Li, et al., 1996), with subsequent development of morphological evidence of airspace enlargement (Churg, et al., 1989). An acute pulmonary inflammatory response has been implicated in the pathogenesis of these changes (Churg, et al., 1999, DiMatteo, et al., 1996). Silica has been shown to cause oxidative inactivation of α1AT in vitro and this may play a role in dust-induced emphysema (Zay, et al., 1999).

2.5 Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin deficiency is a hereditary disorder characterized by a quantitative (and occasionally, functional) reduction in serum α1AT levels. It was first identified by Laurell and Eriksson (1963) when they noticed that the serum protein electrophoresis pattern of some individuals lacked an alpha-1 peak and several of these individuals had pulmonary emphysema. Since the majority of the alpha-1 fraction consists of α1AT, they identified the association between α1AT deficiency and early-onset pulmonary emphysema (now more commonly referred to as hereditary α1AT deficiency associated emphysema). It is a common genetic disorder that is most prevalent among Caucasians and occurs in 1 in 2000 to 1 in 7000 individuals in Europe and North

America (WHO, 1995). The clinical manifestations of COPD among α1AT deficient individuals are highly variable with some displaying no symptoms at all and others demonstrating severe pulmonary function abnormalities as early as the third decade of life (Eriksson, 1996). There are various allelic forms (approximately 75) of α1AT which are expressed in an autosomal codominant pattern in an individual. The M allele is normal α1AT, the S allele is associated with protein levels 30-50% of normal levels, and the Z allele leaves the individual with as little as 10% of normal serum α1AT levels (Table 2). Complete absence of serum α1AT is associated with the "null" allele. S and Z are referred to as "at risk alleles" and inheritance of one Z allele with either another Z allele or an S allele confers risk for early-onset pulmonary emphysema (McElvaney & Crystal, 1997b). Even mild protease inhibitor deficiency (resulting from inheritance of one "at risk" allele with a normal allele) can result in chronic pulmonary symptoms (Huet-Duvillier et al., 1995, Sanford et al., 1999). S and Z mutations are the result of a single base substitution leading to a change in an amino acid in the final protein product. In the case of the Z mutation there is a glutamine to lysine substitution at position 342. The molecule is still synthesized in normal amounts, but for unknown reasons the hepatocytes fail to secrete all of the synthesized product. The majority of the protein accumulates in the rough endoplasmic reticulum, a phenomenon probably responsible for the liver disease associated with α1AT deficiency (Crystal, 1990). Other ailments reported to be associated with α1AT deficiency are asthma and panniculitis (Eriksson, 1996).

Table 2: α1AT Phenotypic Classification (Adapted from Macdonald & Johnson, 1995)

Alleles	%Normal Serum α1AT Level*	Risk for Emphysema**
MM	100	None
SS	40-70	Low
ZZ	10-15	High
Null/Null	0	Very High

* Normal serum α1AT levels=20-53 μM
** Cigarette smoking significantly increases risk for emphysema regardless of phenotype

Individuals who are homozygous for the Z allele have a high risk of developing emphysema at a relatively early age. Normal serum levels of α1AT are approximately 20-53 μM (150-350 mg/dl; Brantly et al., 1988) and ZZ individuals have as little 10% of this concentration in their blood (Schwaiblmair & Vogelmeier, 1998). Movement of α1AT into the lungs occurs by passive diffusion, therefore insufficient serum levels result in interstitial and epithelial lining fluid levels that are inadequate to protect the lung from the continual, low level presence of NE. In a normal individual, interstitial levels are 50-70% (10-40 μM) of plasma levels and epithelial lining fluid (ELF) levels are approximately 10% (2-5 μM) of plasma levels (Crystal, 1990). In addition to having extremely low levels of the major lung anti-proteolytic enzyme, individuals with the Z allele have a protein that is unable to inhibit NE as efficiently as the normal protein, thereby accentuating the susceptibility of α1AT-deficient patients to the development of emphysema (Llewellyn-Jones et al, 1994).

There is some evidence that α1AT deficient individuals have a higher number of PMNs in their lungs compared to normal individuals. One mechanism suggested for this accumulation involves the release of chemoattractants by macrophages resulting in PMN recruitment into the lungs. Alveolar macrophages have surface receptors for Neutrophil elastase. Upon binding free NE alveolar macrophages from α1AT-deficient individuals release threefold more PMN chemotactic activity than alveolar macrophages from normal individuals (Hubbard et al., 1991). The bulk of this chemotactic activity has been identified to be due to leukotriene B4 (LTB4). These findings suggest that the presence of uninhibited NE in α1AT deficient people leaves it free to bind to the surface of macrophages and stimulates them to release LTB4, thereby recruiting more PMNs to the lungs and further enhancing the protease-antiprotease imbalance already present.

2.6 Strategies for α1AT therapy

Pulmonary emphysema occurring in individuals with α1AT deficiency is believed to be a result of reduced anti-elastolytic potential in the lungs. Restoring the anti-elastolytic screen (that is α1AT levels) is a rational therapeutic strategy for preventing the development of emphysema in individuals with this condition. Theoretically, there are two methods that could be used to elevate serum and lung concentrations of α1AT:

1) administration of purified human plasma α1AT or recombinant α1AT protein and

2) increase in production of α1AT with gene therapy.

2.6.1 Augmentation of α1AT levels with exogenous protein

A direct approach to treating α1AT deficiency is to inject the protein intravenously or provide it as an aerosol to patients. Purified α1AT from pooled human plasma (Protease® from Bayer Inc.) has been commercially available for over a decade as a therapeutic agent. The safety and feasibility of this therapy over a long period has been demonstrated (Wencker et al., 1998). Weekly i.v. administration of 60 mg/kg of purified α1AT is sufficient to increase ELF levels and has been shown to possess significant anti-Neutrophil elastase activity (Gadek & Pacht, 1996a). Several clinical studies have been carried out in an attempt to prove the efficacy of augmentation therapy for α1AT deficiency (Schwaiblmair,et al., 1997, The α1AT Deficiency Registry Study Group, 1998). The potential effectiveness of this therapy has been shown as a decrease in the rate of decline of expiratory flow rate (FEV_1) in treated patients, but proof of true efficacy (i.e. halting of the development of emphysema) remains elusive. In a hamster model of emphysema, a single instillation of α1AT was shown to prevent human NE-induced alveolar wall destruction occurring over a period of three weeks (Rudolphus et al., 1994). Currently, α1AT augmentation therapy is not considered for treatment of cigarette smokers with signs of emphysema because of the debate over the principal protease involved in this pulmonary disease and absence of experimental data supporting its efficacy in this setting.

2.6.2 Gene Therapy

Gene therapy directed towards pulmonary disorders involves the genetic manipulation of cells in the lung or elsewhere in the body with the ultimate purpose of

modifying the pulmonary environment in order to prevent, or slow down the progression of disease (Crystal, 1992). α1AT deficiency is an ideal candidate for gene therapy because it is the result of a single gene mutation which can, theoretically, be corrected by the replacement of the defective gene. The goal of gene therapy for α1AT deficiency is to increase the lower respiratory tract levels of anti-elastolytic activity. As for all gene therapy protocols, four criteria must be realized. These are: 1) the α1AT gene must be isolated and characterized, 2) the gene must be delivered to the appropriate target cell(s), 3) an appropriate level of expression must be achieved, and 4) the procedure must be safe (Curiel, 1997). The first criterion has been successfully met. The normal and mutant α1AT genes have been isolated and characterized (Crystal, 1990) and their normal sites of expression determined (discussed previously). To fulfill the second consideration, the inhibitor can be locally produced by pulmonary cells or in distant sites that would allow secretion into the serum, thereby elevating lung levels as well. The second and third criteria of achieving therapeutic levels of protein expression are major challenges for α1AT gene therapy. Maintenance of ELF α1AT levels of 1.0-1.5 μM is considered essential for achieving a sufficient anti-NE screen (McElvaney & Crystal, 1997a). Different cells targeted in an attempt to achieve this goal have been hepatocytes, endothelial cells, lymphocytes, fibroblasts, respiratory epithelial cells, macrophages, and peritoneal cells (Crystal, 1990 & 1992, Ferkol et al., 1998, Setoguchi et al., 1994, Kay et al., 1995, Crespo et al., 1996, Rosenfeld et al., 1991) using various gene therapy delivery systems, including retrovirus, adenovirus, adeno-associated virus, and liposomes. The human α1AT gene has been delivered to lung epithelial cells of rats with adenovirus vectors (Rosenfeld, et al., 1991) with small and transient elevations in lavage protein

levels. Cationic liposome-DNA complexes have similarly been used for this purpose with short-term (1 week) expression of α1AT mRNA and protein in airway and alveolar epithelial cells, but the resulting protein levels were not documented (Canonico et al., 1997). Anionic and cationic liposomes were used to deliver the α1AT gene to mice with an associated partial hepatectomy and resulted in expression of protein in the plasma for 2-3 weeks (Crespo et al., 1996). Setoguchi et al (1994) reported that small but detectable serum levels of human α1AT could be produced by intraperitoneal administration of a viral vector containing the human α1AT cDNA. More recently, Ferkol et al. (1998) showed that intravenous injection of a mannnose receptor-DNA conjugate leads to expression of human α1AT in pulmonary macrophages in about 50% of rats, but lavage protein levels were again extremely low. The feasibility of α1AT gene transfer to skeletal muscle with adeno-associated viral vector and bone marrow with a retroviral vector have recently been demonstrated. In the first instance, high level expression of α1AT was maintained for 15 weeks, but immune responses were reported in some cases (Song et al., 1998) and in the latter study the results were unimpressive with only low level expression for less than six weeks (Saylors et al., 1998). To date, varying degrees of success have been observed as far as amount of α1AT produced and duration of gene expression. Finding the optimal combination of target cell and gene delivery system remains an important consideration. Safety concerns with the use of viral vectors also remains an unresolved issue with this form of treatment. The major safety issues are associated with the effect of the transferred genetic material on the target cells, particularly with the possibility of insertional mutagenesis using retroviral vectors, that is, the insertion of the transferred DNA into a site of the host cell genome that inactivates a tumour suppressor

gene or activates an oncogene. Recombination of viral particles inside the host to make an active virus is also a concern (Culver, 1994).

2.6.3 Augmentation therapy with other antiprotease inhibitors

As previously discussed, there are several naturally occurring antiprotease inhibitors contributing to the pulmonary antiproteolytic screen which are potential therapeutic agents for the prevention of NE-mediated lung injury with SLPI receiving the most attention. Recombinant SLPI can augment the anti-NE capacity of the pulmonary interstitium and epithelial lining fluid in sheep when administered intravenously (Birrer et al., 1992). Experimentally, SLPI has been shown to effectively reduce lesions in the lungs of hamsters receiving intratracheal LPS (Stolk et al., 1991) and truncated SLPI, which is more specific for elastase compared to native SLPI, can attenuate Neutrophil elastase-mediated lung injury induced by LPS and FMLP (Mitsuhashi et al., 1997).

Based on the assumption that the elastase-antielastase balance is important in preventing tissue damage associated with various pulmonary inflammatory diseases and that PMNs are the primary source of the effector agent, synthetic inhibitors of NE have been studied. The objective of these efforts has been to develop a low molecular weight, efficient inhibitor of elastases that is non-toxic and relatively stable in vivo. There are three general types of synthesized inhibitors based on their mechanism of action: 1) active site-directed, irreversible inhibitors, 2) enzyme activated, irreversible inhibitors and 3) tight-binding, reversible inhibitors (See Figure 4). In vitro studies have demonstrated a new family of ketone-based synthetic inhibitors that restrain NE activity without interfering with the physiological activities of the PMN (Huang et al., 1998). These

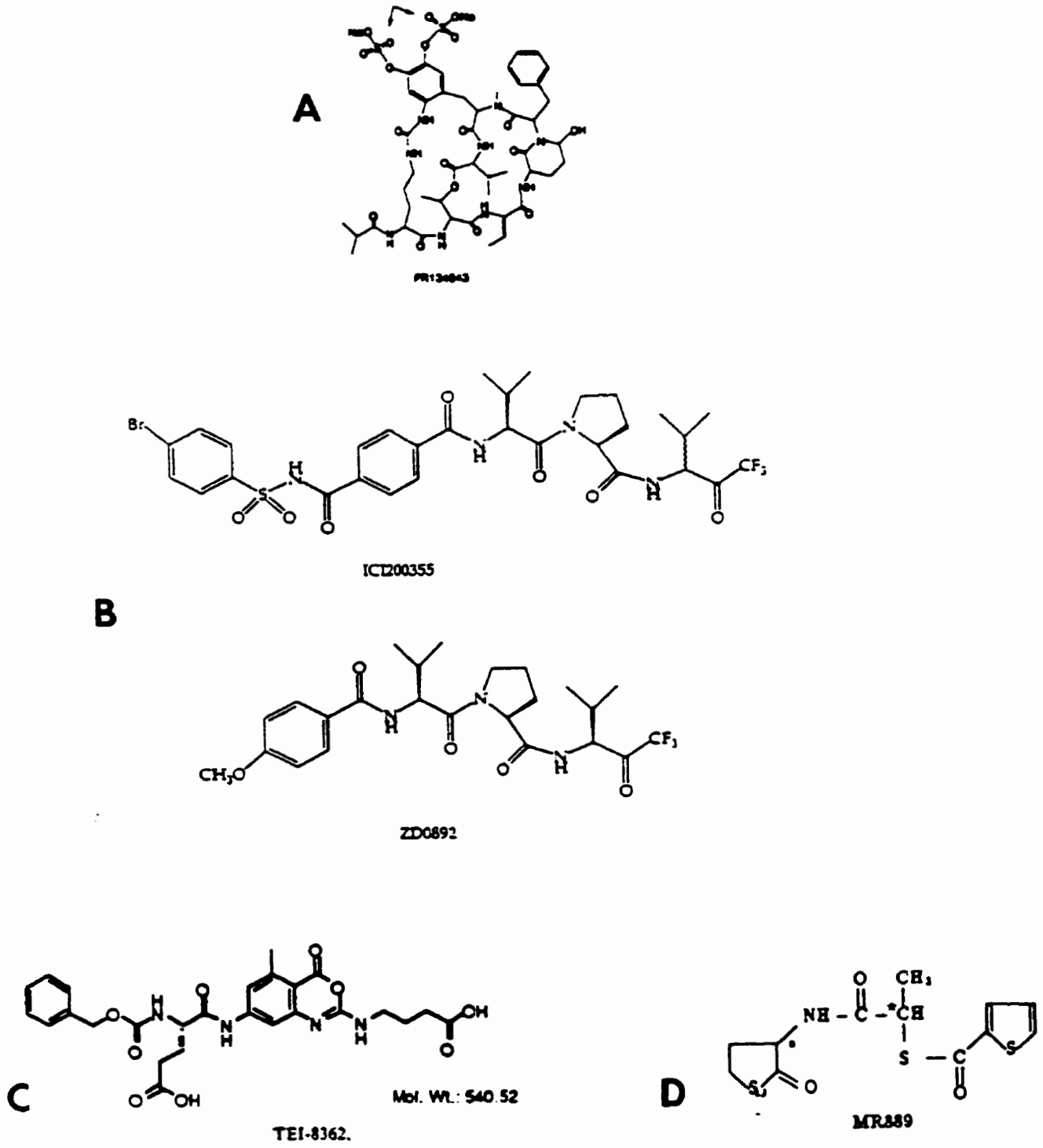


Figure 4. Synthetic elastase inhibitors. (A) an enzyme-activated, irreversible inhibitor (Shinguh et al, 1998). (B,C,D) tight binding, reversible inhibitors (Huang et al., 1998, Mitsuhashi et al., 1999, and Baici et al., 1990, respectively).

inhibitors were resistant to various oxidizing substances including superoxide and hydrogen peroxide and were shown not to interfere with the ability of PMNs to phagocytose and to kill *S. auerus*. Another synthetic inhibitor has been developed and shown to attenuate LPS and human NE-induced lung injury in hamsters when administered intratracheally, intravenously, and as an inhalant (Mitsuhashi et al., 1999), while another group has developed a type 2, water soluble form of an elastase inhibitor isolated from *S. resistomycificus* and it has been shown to inhibit human NE, porcine pancreatic elastase, and LPS-induced lung injury, as well as, human NE-induced paw edema (Shinguh et al., 1998). There has only been one human study with a synthetic NE inhibitor demonstrating the safety of the drug although its effectiveness in reducing biochemical markers of elastin breakdown in COPD patients was not convincingly shown (Luisetti et al., 1996). See Figure 4 for examples of these synthetic inhibitors.

2.7 Alpha-1-antitrypsin augmentation therapy for other pulmonary disorders

PMNs have been implicated in the tissue destruction associated with pulmonary disorders other than emphysema. Because of their presence and the known destructive potential of PMN granule contents, particularly NE, PMNs have been suggested as culprits in tissue damage during the acute inflammatory response occurring in the following disorders.

2.7.1 Cystic fibrosis

Cystic fibrosis (CF) is a pulmonary disease for which α1AT augmentation therapy is considered potentially useful. This genetic disease is characterized by the presence of a

faulty chloride channel (cystic fibrosis transmembrane regulator-CFTR) that leads to abnormal mucus accumulation in the small airways. Patients with CF are unusually susceptible to secondary bacterial infections and it is believed that the host response to infection ultimately leads to respiratory insufficiency and death (Allen, 1996). The inflammatory process in adult CF patients results in 95% PMNs in the BAL fluid compared to less than 5% in normal adults and excessive quantities of NE are present in the airways of CF patients (Allen, 1996, Canonico et al., 1996). It has also been demonstrated that inflammation is present in infants with CF prior to the occurrence of infections and it is characterized by free BALF elastase activity and elevated α1AT-NE complexes (Khan et al., 1995). Proteases released from PMNs have considerable destructive potential including reduction of ciliary beat, stimulation of mucus production, degradation of elastin, and cleavage of fibronectin (Mahadeva, 1998). As a consequence of the large proteolytic burden in CF lungs, normal anti-proteolytic defenses are overwhelmed and NE is able to directly injure airway and parenchymal tissues and exacerbate the inflammatory response by recruiting more PMNs. In vitro studies demonstrate that a compound secreted by *Pseudomonas aeruginosa* (a common infection in CF airways), pyocyanin, is able to induce cells to generate reactive oxygen species which decrease the ability of α1AT to inhibit serine proteases (Britigan et al., 1999) and the polysaccharide, alginate, released by *P. aeruginosa* is capable of reducing the association rate between NE and α1AT (Ying et al., 1996). Therapeutic attempts using intravenously delivered α1AT and direct delivery to the lungs of an aerosol form have elicited varying degrees of success as far as significantly suppressing and maintaining depressed levels of the NE in cystic fibrosis lungs (Allen, 1996, Vogelmeier et al., 1997).

Recombinant secretory leukoprotease inhibitor (SLPI) by itself or in combination with α1AT, has also been considered and tested as a means of increasing the anti-NE screen in the lungs of CF patients (Vogelmeier et al., 1996, Bingle & Tetley, 1996). Although preliminary efforts are promising , longer term studies are required for determination of efficacy. Somewhat surprisingly, a recent report has confirmed earlier studies that α1AT deficiency is not associated with more severe pulmonary disease in cystic fibrosis and may actually be linked with milder lung disease (Mahadeva et al., 1998). It is possible, therefore, that the role of NE in cystic fibrosis has been over-estimated and this should be more thoroughly investigated before further studies designed to test the efficacy of α1AT and SLPI augmentation therapy for patients with CF are carried out.

2.7.2 Adult respiratory distress syndrome (ARDS)

ARDS refers to the acute lung injury that occurs following catastrophic events such as hypotension, trauma, sepsis, or pancreatitis (Donnelly et al, 1995). The survival rate of individuals with ARDS is less than 50% (Petty, 1991). Inflammatory mediators, present as a result of the predisposing condition, cause inflammatory cells to become abnormally isolated in the vasculature of the lungs. It has been postulated that the products from these cells significantly contribute to the pulmonary epithelial and endothelial cell damage observed in individuals with ARDS. The exact mechanism of tissue destruction remains difficult to elucidate, but it is consistently indicated by large numbers of PMNs in the lung interstitium and airspaces and high levels of free Neutrophil elastase, as well as, elastase-α1AT complexes in the bronchoalveolar lavage (Donnelly et al., 1995). The major cause of death in patients who succumb to ARDS is

respiratory failure, subsequent to the endothelial and epithelial injury which is consistently present in these patients. It has been suggested that early restraint of the inflammatory cell-mediated injury may be beneficial in ARDS (Gadek & Pacht, 1996b). The number of PMNs in the BAL of patients with ARDS correlates with degree of lung damage, but, interestingly, ARDS has also been reported in neutropenic patients (Petty, 1991). There is controversy over the degree of free NE or NE-α1AT complexes in ARDS lavage which must be clarified before it can be determined whether these factors can be used as predictors for the onset of ARDS (Gadek, 1992 and Gadek & Pacht, 1996b). If NE is shown indisputably to be a significant effector in ARDS, this would argue strongly for further study of α1AT therapy in this life-threatening condition.

2.8 Animal Models of Emphysema

Animal models of emphysema are important for gaining a complete understanding of the pathogenesis of human disease. The most important criteria for the demonstration of emphysema in animals are based on anatomic changes in the lungs, particularly increases in Lm. (Snider et al. 1986). An animal model of emphysema has been described as one in which there is an abnormal state of the lungs with enlargement of the air spaces distal to the terminal bronchioles (Snider, et al., 1985). In this discussion, models will be classified into two major types: 1) animals that are genetically predisposed to the development of emphysema and 2) animals with experimentally induced emphysema resulting from an artificially generated shift in the protease-antiprotease balance.

There are at least three known C57-BL/6J mouse strain mutants that develop spontaneous emphysema and investigations have focused on determining whether there is an inherent elastase-antielastase imbalance in these mice as an explanation for the lung destruction. The beige (*bg*) mouse is a mutant that displays diluted pigmentation and characteristics similar to that of humans with Chediak-Higashi syndrome, that is, platelet storage pool deficiency, increased susceptibility to infection, and presence of giant lysosomes (Brandt et al., 1981, Nowak et al., 1984). Beige mice develop pulmonary airspace enlargement by one month of age, but they are believed to lack the proteases neutrophil elastase and cathepsin G and have high α1AT serum levels compared to their parent strain, C57-BL/6J, so a primary role for an elastolytic process in the pathogenesis of emphysema in these mice is most unlikely (Starcher & Williams, 1989, Keil et al., 1996). An inability to form proper alveolar walls due to a defect in connective tissue components may be responsible for the observed lung abnormalities. However, recent studies have demonstrated that PMNs of *bg* mice have a processing defect that inhibits their ability to release active elastase, but they do indeed produce cathepsin G (Gardi et al., 1994) that can be released in normal quantities in response to proinflammatory stimuli and they release a proelastase that can be activated by a protease-dependent mechanism (Cavarra et al., 1997). Furthermore, a single intratracheal instillation of FMLP in *bg* mice results in an influx of PMNs into the alveoli, an increase in elastase burden, an increase in mean linear intercept, and a decrease in total lung elastin content after 21 days (Cavarra et al., 1999). These results are completely contrary to an earlier study concluding that intratracheal instillation of endotoxin into *bg* mice does not produce emphysema (Starcher & Williams, 1989). Further studies are needed to identify the cause of the

pulmonary abnormalities in the *bg* mouse but the relevance to human emphysema is, at the present time, doubtful.

Mice that are heterozygous for the tight-skin (*Tsk*) gene display many connective tissue abnormalities, one of which results in a tightness of the skin (Rossi et al., 1984). They show a rapid development of emphysema which is evident at one month (Keil, 1996) and persistent low levels of inflammatory cells in the alveoli before and during the development of emphysema (Rossi et al., 1984). They have also been reported to have decreased elastase inhibitory capacity (EIC), increased alveolar elastolytic burden and increased lung elastin degradation. Also, *Tsk* mice have reduced serum concentration of α1AT, and abnormally high amounts of PMN-derived neutrophil elastase and cathepsin G activities (compared to other strains) (Gardi, 1994). Based on this collective data, it is possible that an inborn protease-antiprotease imbalance in these mice is the cause of emphysema.

The third, most recently discovered, C57-BL/6J mutant is the pallid (*pa*) mouse. These mice have a mutation in their band 4.2 gene that affects the formation and/or function of intracellular storage granules in melanocytes, platelets, and lysosomes in the kidney (Korsgren & Cohen, 1994, White et al., 1992) and they are characterized by a deficiency in serum α1AT with levels, that are 54% lower than C57-BL/6J control mice and emphysema appearing late in life (beginning at 12 months) (Martorana et al., 1993). A decrease in lung elastin content and immunolocalization of NE with elastin in the alveolar walls correlates with the appearance of emphysematous lesions (de Santi et al., 1995). Of note, the elastolytic burden in the *pa* mice determined by EM-immunohistochemistry does not correlate with BAL fluid levels of PMNs suggesting that

release of elastase into the pulmonary interstitium is of more consequence to lung destruction than ELF elastase levels (de Santi et al., 1995). Challenge with intratracheally instilled FMLP results in a faster progression of and a more marked emphysema in *pa* mice than normal C57-BL/6J. This occurs despite the fact that *pa* PMN lysosomes secrete only 50% of their lysosomal enzymes. These observations strongly suggest that the genetic deficiency of the serum anti-elastase screen in these mice contributes to the elastolytic lesions observed in the lungs of *pa* mice and, therefore, this mutant can be used as a model of genetic α1AT deficiency.

A very recently identified mouse model of premature aging with a mutation in the *klotho* gene has been identified (Kuro-o et al., 1997). The original genetic background of these mice was a mixture of C57BL/6J and C3H/J, but backcrossing into the BALB/c background produced identical phenotypes. Preliminary characterization shows that, in addition to a number of aging related attributes, homozygous *klotho* mice have enlarged airspaces distal to the terminal bronchioles and destruction of alveolar walls identical to that found in human emphysema. Further investigation of this mouse model is required to determine the exact nature of the lesions and the cause of the alveolar destruction. These mice have a very short life span of not more than 8-9 weeks. This and the myriad of other phenotypes similar to those seen during human aging that are present in these mice may complicate its application as a model of emphysema, but future studies will help to determine its usefulness.

Many studies have concentrated on attempting to understand the workings of or to test the protease-antiprotease hypothesis by manipulating the balance of these enzymes in the lungs. A number of experiments have established that exposure of the lung to

proteolytic, specifically elastolytic, enzymes (by intratracheal instillation or aerosol) results in the development of emphysema in a dose dependent manner. Degradation of lung elastin following intratracheal instillation of elastases such as papain, porcine pancreatic elastase (PPE) or human neutrophil elastase (HNE) has been reported in rabbits, hamsters, rats, and mice (reviewed in Snider et al., 1986). It has been shown that elastase instilled into the trachea enters the interstitium and persists there for several days thereby temporarily shifting the balance between elastases and antielastases in favour of elastases (Kueppers, 1985). Airspace enlargement induced by elastase instillation occurs rapidly (within weeks) and is accompanied by mild inflammation (influx of PMNs and macrophages), edema, and hemorrhage in some animals. In a model of elastase-induced emphysema in strain A/J mice, however, pulmonary edema, and inflammation were absent, and hemorrhage was completely resolved by 5 weeks after intratracheal instillation (Valentine et al., 1983). In this model and the hamster model, there was a suggestion that resynthesis of connective tissue (both collagen and elastin components) is also present (Snider et al., 1986).

Investigators have also attempted to test the protease-antiprotease hypothesis by experimentally reducing the level of the major circulating elastase inhibitor, α1AT. An early successful effort used chloramine T, an oxidizing agent that may act by oxidizing the active site methionine of α1AT, to reduce the amount of active α1AT in the serum of dogs (Abrams, 1981). This treatment resulted in evidence of mild emphysema after several weeks, but the logistics of using dogs and the uncertainty of other effects of the treatment limit the usefulness of this model. Administration of galactosamine has also

been used to reduce α1AT levels, but the same limitations apply to this model as to the chloramine T model (Kleinerman & Rynbrant, 1974).

A review by Thomas and Vigerstad in 1989 concluded that there was not yet any demonstration of a laboratory animal model of cigarette smoke-induced emphysema. In the subsequent decade, however, there have been reports of the development of emphysema in animals exposed to cigarette smoke. Quantitative morphological evidence of emphysema has been demonstrated in guinea pigs exposed to cigarette smoke for 1 to 12 months (Wright & Churg, 1995). Development of emphysema, determined by increased mean linear intercept and pulmonary function testing, has been demonstrated in rats exposed to chronic tobacco smoke for at least 45 days (Cendon et al., 1997, Ofulue et al., 1998). Strain 129/svJ mice have also recently been shown to develop airspace enlargement after exposure to cigarette smoke for six months (Hautamaki et al., 1997).

3. Experimental Rationale

The pathogenesis of pulmonary emphysema is thought to be based on protease-antiprotease imbalance. Evidence for this hypothesis is based largely on two main types of studies. The first are clinical studies connecting development of emphysema with a genetic condition resulting in severe reduction in serum α1AT levels. The second series of experiments involve the generation of morphometric changes in the lungs of animals after instillation of elastolytic proteases into the lungs (Gross, 1964, Snider, 1986). It is believed that the protease-antiprotease imbalance hypothesis can also explain the development of emphysema in individuals who are not genetically deficient in the major

lung antiprotease and who comprise the majority of emphysema sufferers. Most of these people are cigarette smokers and a link has been sought between cigarette smoke and increased lung elastase burden and/or depressed elastase inhibitor function. The last twenty years have seen descriptive studies of various proteases and antiproteases in the lower respiratory tract and their respective contributions to the protease-antiprotease balance in smokers. The mechanisms by which the balance is upset to result in lung damage after other insults to the lungs have also been extensively investigated. Despite these efforts, definitive proof of the protease-antiprotease hypothesis, and particularly, the importance of any specific proteases/antiproteases remains to be determined.

The connection between severe α1AT deficiency and emphysema is undeniable and makes a potent case for a vital role for α1AT in protecting against pulmonary connective tissue breakdown leading to airspace enlargement. Whether this antiprotease plays a similarly important role in emphysema associated with cigarette smoking is an important question. Finding a protective role for α1AT in this form of the disease could lead to a therapy for an important disease currently treated with supportive measures only. A more complete understanding of the biochemical factors important in the etiology of cigarette smoke-induced emphysema will allow a realistic approach to determining the feasibility of therapeutic intervention with α1AT augmentation. Even for α1AT deficiency, there are still questions about the efficacy and rationale of augmentation therapy. A recent report by the Canadian Medical Association recommended that individuals with α1AT deficiency continue treatment with supportive measures only and not receive α1AT augmentation therapy until such time that its efficacy be proven by randomized controlled trials (CMA, 1992). Furthermore, the

staggering cost of α1AT replacement therapy makes it imperative that the true value of the treatment over the long-term be demonstrated unquestionably (MacDonald & Johnson, 1995). The cost of a human trial to make such a clear-cut assessment and ethical concerns about having a no treatment control arm, given the results of studies to date suggesting that this treatment is efficacious, however, makes it virtually impossible that this will occur in the near future. **The use of animal models is an important, alternative approach to such investigations.** Ideally, a model of cigarette smoke-mediated emphysema or α1AT deficiency is needed to determine the efficacy of α1AT augmentation in preventing the development of emphysema. Large scale preparations of species-specific α1AT protein are not commercially available, making an animal model tolerant to human α1AT highly desirable.

Many investigations are currently focused on implicating classes of proteases other than serine proteases in the pathogenesis of emphysema (Shapiro, 1994, Chapman, 1994, Ohnishi, 1998). Rather than definitively proving the significance of any one type of protease in the pathogenesis of emphysema, these studies emphasize the complexity of the proteolytic destruction of the ECM in the lungs and, as a whole, question the status α1AT has traditionally been given as the most important anti-proteolytic molecule in the lungs. Despite the reservations raised by these inquiries about the preeminence of α1AT, the fact that it plays a protective role in the pathogenesis of emphysema and other pulmonary diseases (by contributing at least in part and probably more than any other single antiprotease, to the antiproteolytic screen in the lungs) cannot be abandoned. The preponderance of evidence suggesting that α1AT therapy is feasible for the treatment of emphysema, cystic fibrosis and perhaps even ARDS, makes it imperative that the

therapeutic potentials of this antiprotease be fully explored. Even if the substrate enzyme/s of α1AT (that is, neutrophil elastase) is only partially contributing to the ECM destruction during the pulmonary inflammatory response, α1AT therapy may have a significant impact on disease progression.

4. Hypothesis

Polymorphonuclear leukocytes (PMNs or neutrophils) are major contributors to tissue damage associated with inflammatory pulmonary diseases such as emphysema, cystic fibrosis, and adult respiratory distress syndrome. Alpha-1-antitrypsin (α1AT) is the major inhibitor of neutrophil elastase, the most potent of the PMN proteolytic enzymes. PMNs are recruited into the lungs with resulting lung injury, in response to inflammatory stimuli, specifically, cigarette smoke and lipopolysaccharide. **Our hypothesis is that endogenous over-expression of human α1AT in the lungs of transgenic mice, and augmentation of mouse serum levels with purified human α1AT reduces connective tissue destruction and airspace enlargement in a mouse models of cigarette smoke and LPS-induced emphysema.**

5. Materials and Methods

5.1 Technical Protocols

Creation and Characterization of Transgenic mice

5.1.1 Transgene Construction

All transgene constructs contain a 1.4 kb human α1AT cDNA fragment with a 3' flanking SV40 polyadenylation sequence in the *Hind*III/*Xho*I site of pBluescript SK + plasmid (Stratagene) (which will be referred to as pα1AT). This 1.4 kb human α1AT cDNA was kindly provided as an expression cassette (MLPα1AT) by M. Rosenfeld (Rosenfeld et al., 1991). The *Hind*III and *Xho*I restriction enzyme sites flanking the transcriptional start site of the cDNA and the SV40 polyadenylation sequences, respectively, were added to the ends by polymerase chain reaction (PCR). The complete PCR product was sequenced by the dideoxy sequencing method using the Sequenase kit (USB) to ensure the fidelity of the amplification. The 2.3 kb rat Clara cell 10 kDa protein (CC10) promoter fragment (kindly provided by J. Whitsett, University of Cincinnati) was inserted into the *Hind*III site of pα1AT and orientation of the promoter was determined by digesting with restriction enzymes having unique sites within the promoter region. The 3.7 kb SPC promoter (also provided by J. Whitsett) was inserted into *Sac*I and *Pst*I sites of pα1AT. The 0.9 kb human haptoglobin (Hhp) promoter fragment (PCR amplified by Edmond Wong) was inserted into the *Xba*I/*Hind*III sites of pα1AT. All transgene constructs were liberated from the plasmid by cutting with the enzyme *BssH*II. The constructs (Figure 5) were then isolated on a 2% agarose gel, purified with the

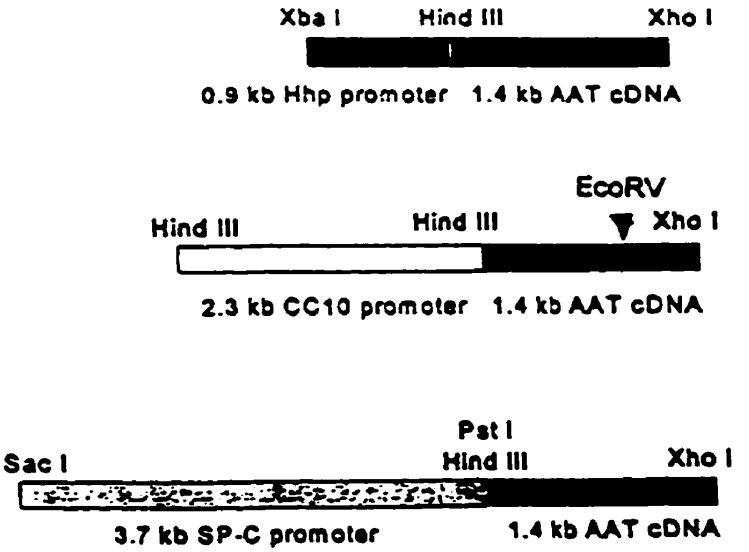


Figure 5. The transgene constructs used for creation of transgenic mice. The 1.4 kb human α1AT cDNA with approximately 150 bp of the SV40 polyadenylation signal sequence at the 3' end is ligated to the 0.9 kb human haptoglobin promoter (Hhp), 2.3 kb rat CC10 promoter and the human SP-C promoter fragments. Relevant restriction enzyme sites are indicated (see text).

GeneClean extraction kit (Bio101, Vista, CA; see below) and resuspended in 10:1 TE buffer.

Polymerase chain reaction (PCR)

The following oligonucleotide primers were used to facilitate subcloning of the human α1AT cDNA: 5' primer with *Hind*III site 5'ATCAAGCTTTGCCGTCTTCTGTCTCG 3'

3' primer with *Xho*I site 5'CGCTCGAGGGGATCCAGACATGATAAGA 3'

A 50 µl reaction mixture was made up with 29.5 µl dH_2O, 5 µl of 10X PCR buffer, 4 µl of 2.5 mM dNTPs, 100-200 ng of cDNA (1µl) , and 5 µl of each primer. The mixture was overlayed with 50 µl of mineral oil and heated to 92° for 5 minutes before adding 0.5 µl of *Taq* polymerase enzyme. A standard Perkin-Elmer thermocycler was used for the reaction. The program used consists of 4 minutes at 92°C (denaturation step), 1 minute at 92°C (*Taq* added at this point), 1 minute at 55°C (annealing step), 1 minute at 72°C (elongation step). This was repeated 30 times. The amplification product was electrophoresed on a 2% agarose gel and the 1.4 kb cDNA fragment excised and purified using GeneClean (Bio101) and subcloned into pBluescript SK.

DNA Sequencing

α1AT was transformed into DH5α library efficiency competent cells (Gibco/BRL) (as described in Ausubel, et al., 1994) and the recombinant plasmid was isolated using a standard DNA mini preparation technique with polyethylene glycol (PEG) precipitation (Maniatis et al., 1989). The plasmid DNA was denatured prior to sequencing with 2N sodium hydroxide and 5M ammonium acetate followed by ethanol precipitation. Sequencing reactions were done using the Sequenase (version 2.0)

sequencing kit (USB). The manufacturer's instructions were followed using 5 μCi of 800 Ci/mmol ^{35}S-dATP (Dupont-NEN) and T7 and T3 primers. The reactions were separated on a 6%, 6M polyacrylamide denaturing gel with 1X TBE running buffer at 1600 V for 3-4 hours. Gels were transferred to Whatman 3M filter paper, dried on a gel dryer (Model 583, BioRad), and exposed to Kodak XOMAT AR autoradiograph film overnight at room temperature.

Preparation of transgenes for microinjection

Each of the three recombinant plasmids containing the transgenes were prepared in larger scale using a plasmid midi-preparation protocol (Sambrook et al., 1989). The DNA was digested with the appropriate restriction endonucleases for several hours in order to remove the transgene from the plasmid. The restriction digest reaction was electrophoresed on a 2% TAE agarose gel at 40V overnight. The transgene was excised from the gel and purified using the GeneClean DNA purification kit (Bio101, Inc.). Briefly, the agarose gel containing the DNA was incubated for 5 minutes in 3 volumes of sodium iodide solution at 55°C. After the agarose was completely melted, the solution was incubated overnight with 20 μl of Glassmilk®. The Glassmilk/DNA complex was pelleted by centrifugation and washed several times with New Wash® buffer. The DNA was eluted into buffer (5mM Tris, 0.1mM EDTA) and a DNA solution of approximately 2 ng/ml was prepared for microinjection.

5.1.2 Creation of Transgenic Mice (this procedure was done by Dr. Susan Porter)

CD-1 mice were used for all transgenic work. Preparation of pseudopregnant females, vasectomization of males, and collecting, explanting, and transplanting of eggs was done according to standard practice as outlined in Manipulating the Mouse Embryo (Hogan, Costantini, & Lacy, 1986). Eggs microinjected with the transgenes were transplanted into the uteri of pseudopregnant females and the offspring were screened for the presence of the transgene. Stable transmission was confirmed with Southern analysis and subsequent positive offspring were identified by dot blot analysis of tail DNA. All lines were bred to homozygosity for the transgene.

5.1.3 Screening of Transgenic Mice

DNA Isolation from Tails

One to two centimetres of tail was excised from approximately 14 day old pups and incubated overnight at 55°C with constant mixing in 500 µl of digestion buffer (50mM EDTA, 50mM Tris-Cl pH 8, 0.5% SDS, 0.5 mg/ml Proteinase K). The genomic DNA was extracted with phenol/chloroform and precipitated with 0.3M sodium acetate and two and a half volumes of ethanol. The DNA was removed with a glass rod, washed with 70% ethanol and dissolved in 100 µl TE (10mM Tris-Cl, 1 mM EDTA, pH 7.6).

Southern Analysis of Mouse DNA

Ten micrograms of genomic DNA from each offspring was digested with 30 units of the appropriate restriction endonuclease in a 100 µl reaction mixture containing 10 µl of the complementary 10X restriction buffer, 10 µl of 20mM spermidine, 1 µl of

10mg/ml BSA, and distilled water. All digestions were carried out overnight at the appropriate temperature. The following morning, DNA was precipitated with 10 µl 3M sodium acetate and 250 µl ethanol at -20°C for half an hour. The DNA was completely redissolved in 20 µl of TE and separated by electrophoresis overnight at 60V in a 0.8% TBE agarose gel. After electrophoresis the gel was stained with ethidium bromide for approximately 30 minutes, washed with dH_2O, and photographed. In preparation for Southern blotting, the DNA was partially hydrolyzed in the gel with 0.25N HCl, followed by denaturing solution (1.5M NaCl, 0.5M NaOH), and two incubations with neutralization solution (1M Tris, 1.5M NaCl, HCl). Each wash was for thirty minutes and was separated by a rinse with dH_20. The DNA was then transferred to a nylon membrane (either Hybond; Amersham Life Science or Nytran Plus; Schleicher & Schuell) via capillary transfer overnight with 10X SSC (pH 7.0). The membrane was rinsed with 1X SSC (pH 7.0), dried with filter paper, and the DNA was UV cross-linked to the membrane by exposure to 254 nm ultra-violet radiation in a Strata-linker (Stratagene).

DNA Probe Synthesis

Positive transgenic offspring were identified on the basis of hybridization to the 1.4 kb *Hind*III/*Xho*I α1AT cDNA fragment. The recombinant plasmid pα1AT was digested with the enzymes *Hind*III and *Xho*I and the fragments were separated on a 2% agarose gel. The 1.4 kb human α1AT cDNA fragment was excised and purified using the GeneClean DNA extraction kit. The fragment was dissolved in TE to a final concentration of 12ng/µl. For probe synthesis, 12-25 ng of denatured DNA (by heating to

100°C for 7 minutes prior to reaction) was labeled with a random-primed labeling kit (Pharmacia) according to the manufacturer's directions. The reaction mix consisted of the DNA, reaction mix, dH_2O, 50 μCi of 3000 ci/mmole $^{\alpha-32}$P-dCTP (Dupont-NEN), and approximately 10 U of Klenow enzyme. The reaction was carried out at 37°C for 30-60 minutes and the probe was denatured by boiling for two minutes before adding to the hybridization mixture.

Probe Hybridization

Prehybridization and hybridization of blots was done according to protocols supplied by the manufacturer of the Nytran membrane (Schleicher & Schuell). Both steps were carried out at 42°C with constant shaking. Prehybridization was for 2-4 hours using approximately 150 μl of prehybridization solution per square centimetre in 50% formamide, 6X SSPE , 10X Denhardt's reagant, 2% SDS, and 150 μg/ml of denatured herring sperm DNA. Hybridization was carried out overnight in the same solution with the addition of the labeled probe. Following hybridization, the blots were washed in 2X SSC, 0.1% SDS at room temperature for 30 minutes and then at 65°C for 45 minutes. This was followed by two 45 minute washes in 0.1X SSC, 0.1% SDS at 65°C. The blots were dried and left overnight at -80°C in contact with Kodak XOMAT-AR autoradiograph film in between two intensifying screens.

5.1.4 Northern Blot Analysis

Total Cellular RNA Isolation

RNA was isolated from the liver, lung, and trachea of mice from each transgenic line using a standard guanidine thiocyanate isolation method. Tissues were homogenized in Wheaton vials with approximately 1ml Solution D (4M guanidine thiocyanate, 25mM sodium citrate pH 7.0, 0.5% Sarkosyl, 0.36 ml mercaptoethanol) per 100mg of tissue. A 1/10 volume of sodium citrate (all solutions used for RNA work were DEPC treated) was added to the homogenate, shaken vigorously, followed by 1.0 volume of water saturated phenol and 0.2 volume of chloroform:isoamyl alcohol (23:1) and left on ice for 15 minutes. The mixture was centrifuged at 10000g for 20 minutes at 4°C and the aqueous, RNA containing, phase transferred to a new tube and precipitated with isopropanol at -20°C. The RNA pellet was redissolved in Solution D and precipitated once again with isopropanol, washed with 75% ethanol, and finally dissolved in DEPC treated dH_2O.

Northern Blotting

Ten micrograms of the RNA samples was prepared in a 20 µl solution containing 10 µl formamide, 3.5 µl formaldehyde, 2.0 µl 10X MOPS, and the remaining volume of DEPC treated dH_2O. The samples were heated for 15 minutes at 65°C before loading in duplicate onto a denaturing RNA gel (1 % agarose, 18% formaldehyde, 10% MOPS). The gel was run at 60V for 4 hours in 1X MOPS running buffer. After completion of electrophoresis, half of the gel containing each of the samples was stained with ethidium bromide, destained in DEPC treated dH_2O and photographed to confirm the quantity and integrity of the RNA. The other half of the gel was blotted as previously described for

Southern blotting, omitting the denaturation steps and using DEPC treated SSC transfer buffer. Once again, hybridization was performed using a ^{32}P-labeled 1.4 kb α1AT cDNA fragment which corresponds to the size of the transcript.

5.1.5 In situ Hybridization

The plasmid pα1AT was linearized and used to generate ^{35}S-UTP labeled sense and antisense riboprobes. Synthesis was carried out with either T3 or T7 polymerase and Stratagene RNA transcription kit according to the manufacturer's instructions. Lung sections were placed two to a slide to allow hybridization with sense and antisense riboprobes under identical conditions. The sections on the slides were deparaffinized, rehydrated, digested with 1 μg/ml proteinase K at 37°C for 30 minutes, rinsed in 0.1M triethylamine buffer for 10 minutes, washed twice with 2X SSC and dehydrated before prehybridization.

Sections were incubated with hybridization buffer consisting of 50% formamide for 1-3 hours at 58°C which was then replaced by hybridization buffer containing the riboprobes. Hybridization continued overnight (14-18 hours) at 58°C. Sections were then washed twice at room temperature for 10 minutes in 4X SSC and 10 mM DTT, at room temperature for 15 minutes in 0.5X SSC and 10 mM DTT, and at 60°C for 15 minutes in 0.1X SSC and 10 mM DTT. Next, slides were rinsed with 0.5 M NaCl, 10 mM Tris, 1 mM EDTA and incubated with 20 μg/ml RNase A at 37°C for 30 minutes and rinsed again with the same buffer. Once again, the sections were washed in 2X SSC at room temperature for 30 minutes, 0.1X SSC at 60°C for 15 minutes, 0.1X SSC at room temperature for 30 minutes. Finally the sections were dehydrated, dried, and coated with

50% Kodak NTB-2 emulsion in distilled water at 42°C. Slides were exposed for 2-4 weeks at 4°C and developed with Kodak D-19 developer at 14-16°C.

5.1.6 Light Microscopic Immunohistochemistry

Polyclonal anti-human α1AT antibody was purchased from Boehringer Mannheim (Mannheim, Germany) and used at a dilution of 1:200. Formalin-fixed lung sections were deparaffinized, pretreated with 0.1% protease (Sigma) in Tris buffer for 30 minutes, and then stained using an immunoperoxidase staining kit (Biostain Super ABC kit. Biomeda, Foster City, CA). Sections were incubated for 5 minutes with protein blocker solution and then with the primary antibody overnight at room temperature. A 30-60 minute incubation with the Detector Reagant followed a 30 minute incubation with biotinylated anti-rabbit IgG secondary antibody. Amino-ethylcarbozole (AEC) was used as the substrate chromogen reagent. Lung from a nontransgenic littermate was used as a negative control in addition to the standard control of sections incubated without primary antibody. Cirrhotic human liver was used as a positive control in some instances.

5.1.7 Electron Microscopic Immunohistochemistry

Lung samples from transgenic lines and CD-1 control animals were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde mixture for 2-3 hours, washed in 0.2M phosphate buffer, dehydrated and embedded in Lowicryl HM20 resin. Ultrathin sections were cut and placed onto mesh nickel grids. The sections were pretreated with 50mM ammonium chloride for 45 minutes to block unreacted aldehyde groups (Roth & Heitz, 1989) and further blocked with 0.1% ovalbumin for 15 minutes. The sections were

incubated overnight in a humid chamber on a drop of polyclonal primary anti-human α1AT antibody (DAKO) diluted 1:100 in Tris buffer pH 8.2 containing 0.1% Tween 20. This antibody was used because initial studies showed that it produced a considerably stronger and cleaner signal than the Boehringer antibody used for light microscopic immunochemistry. Detection of mouse α1AT was carried out in a similar fashion using a rabbit polyclonal anti-mouse α1AT primary antibody raised in our laboratory. The grids were washed six times with buffer (Tris pH 8.2) and incubated for 45 minutes on a drop of Protein A-gold conjugate (20nm: Pelco, Redding, CA) diluted 1:50 in Tris pH 8.2, 0.1% Tween 20. The sections were washed three times with buffer followed by three washes with distilled water. Buffer and water for the final washes was prewarmed at 37°C to minimize salt precipitation on the tissue sections. Finally, the sections were washed with a spray of distilled water, dried, and stained very lightly with lead citrate.

5.1.8 Enzyme Linked Immunosorbent Assay (ELISA)

5.1.8.1 Human ELISA

Purified human α1AT (Calbiochem) was used as a standard. The 96 well plates were coated with 100ng/well of human α1AT (in carbonate buffer, pH 9.6) and blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin at pH 7.4. A 100 µl sample (unconcentrated lavage or diluted serum) was added into the wells, followed by 100 µl of anti-human α1AT antibody (Boehringer Mannheim) diluted 1:2000 and depleted for mouse serum (the antibody was depleted for antibodies reacting against mouse α1AT by washing it through a CNBr-activated Sepharose 4B column (Pharmacia

Biotech) attached to mouse α1AT (Sigma)). After mixing the solution the plate was washed five times with PBS. 100 µl of 1:10000 diluted secondary antibody (goat anti-rabbit IgG-HRP, ICN Biochemicals) was measured into each well. After 1 hour incubation at room temperature the plate was washed five times with PBS. The color reaction was developed using TMB ELISA substrate (ICN Biochemicals). 100 µl substrate was added into each well and incubated until adequate color development occurred. The reaction was stopped with 100 µl of 2M HCl solution. Absorbance was measured at 450 nm.

5.1.8.2 Mouse ELISA

Purified mouse α1AT was used as the standard. The 96 well plates were coated with 100 µl purified mouse α1AT in carbonate buffer, pH 9.6. The assay was carried out exactly as described for human ELISAs except that 100 µl of 1:50 anti-mouse α1AT depleted for human serum was used.

Purification of Mouse α1AT (done by Ms. Katalin Zay and myself)

Mouse α1AT was isolated from whole mouse serum by a modification of the method of McGilligan and Thomas (1991). After ammonium sulphate precipitation, the supernatant was dialyzed against 30 mM phosphate buffer pH 7.4, and applied to a DEAE-Sephacel column (Pharmacia). Proteins were eluted from the column with a salt and pH gradient, and fractions containing α1AT were determined by inhibition of porcine pancreatic elastase (PPE). The fractions were concentrated and applied to a fast-flow

Cibacron Blue 3GA (Sigma) column to remove albumin (Travis et al., 1976). Purity of the eluted product was determined by SDS-PAGE and silver staining. The product was tested for elastase inhibitory capacity (see below). The purified product showed a band of 54 kilodaltons.

Mouse Antibody Purification (done by Ms. Katalin Zay and myself)

New Zealand white rabbits were immunized with biweekly injections of 400 μg of purified α1AT mixed with Freund's adjuvant. Blood samples (45ml) were taken from the ear vein four weeks after the second injection and antibody was purified using a standard immunoglobulin isolation protocol (Harboe & Ingild, 1988). Briefly, immunoglobulins were precipitated from serum with ammonium sulphate, centrifuged, and washed twice with 1.75M ammonium sulphate. The antibody-containing precipitate was dialyzed against distilled water and then against 0.05M sodium acetate, 0.02M acetic acid, pH 5.0 at 4°C. Next, the solution was transferred to a 25 ml DEAE-Sephadex column and the IgGs were the first fraction eluted from the column with acetate buffer, pH 5.0. Once again the IgG fraction was dialyzed against 0.1 M NaCl/15mM NaN3. Finally, the isolated antibody fraction was depleted for antibodies reacting against human α1AT by washing it through a CNBr-activated Sepharose 4B column (Pharmacia Biotech) attached to human α1AT (Sigma).

5.1.9 Purification of Protease

Protease® (Bayer Corporation) was reconstituted in 20 mM sodium phosphate buffer pH 7.6 containing 0.01% sodium azide (to prevent bacterial contamination during

the purification process). The resulting solution was filtered with an Ultrafiltration-15 100K centrifugal filter device (Millipore Inc., Beverly, MA) to eliminate all contaminating proteins of 100 kDa size or greater. The concentrate was washed several times with buffer and the resulting filtrates were collected and pooled. The pooled filtrate was run through a Cibacron blue 3GA column (Pharmacia) at 4°C to selectively eliminate albumin (the major contaminating protein; Travis et al., 1976). The human α1AT was washed out with sodium phosphate buffer pH 7.6. Finally, the solution was concentrated in an Amicon ultrafiltration cell using a 10K Diaflo membrane (Amicon Inc.). Purity of the resulting solution was checked on a 12.5% homogenous Phastgel (Pharmacia) with silver staining.

Measurement of Protein Concentrations

A BCA protein assay kit (Pierce) was used to measure the concentration of α1AT in the purified Protease® or for total protein concentration in mouse serums. Samples were diluted appropriately to fit within the range of the assay. Twenty-five µl of each sample and BSA standard (in duplicate) was incubated with 200 µl of *working reagant* (50 parts of Reagant A + 1 part Reagant B) in 96 well covered ELISA plate for 30 minutes at 37°C. After allowing the plate to cool to room temperature the absorbance was measured at 540 nm.

In some cases the BioRad Protein Assay kit was used. In this case, 100 µl of samples and 100 µl of albumin standards were incubated with the *Protein reagant* (40% BioRad Protein Reagant in dH2O) for 5 minutes at room temperature and the absorbance measured at 620 nm.

Acute Smoke and LPS Experiments

5.1.10 Bronchoalveolar Lavage (BAL) of Mouse Lungs

The lungs were removed from the chest cavity and an 18 gauge catheter inserted and tied into the trachea. The lungs were lavaged 6 times with 1 ml of ice-cold saline for cell counts or with distilled water for connective tissue degradation analysis (water is used because concentration of salts during sample preparation for the HPLC procedure interferes with the analysis of desmosine (Zay et al., 1999)).

5.1.11 Total cell counts and cell differential counts

The BAL fluid was centrifuged at 200g for 10 minutes and the supernatant was removed (stored at -80°C for later analysis or discarded). The cell pellet was resuspended in 200 µl of cold saline for cell counts using a hemacytometer. Ten µl of the resuspended cell solution was heat dried onto a slide and stained with hematoxylin and eosin for cell differential counts. Either 100 or 200 cells were counted in one or more fields of view as required. PMNs were identified as distinctly multi-lobulated cells.

5.1.12 Elastase Inhibitory Capacity (EIC)

Activity of the purified Protease® and antiprotease activity in lavage was determined with this assay, as well as the activity of purified mouse α1AT fractions. In a 96 well plate, 100 µl of 0.2M Tris buffer pH 8.0 was incubated for 10 minutes with 50 µl of sample and 50 µl of 1mM PPE. Immediately before measuring the absorbance, 50 µl

of substrate (300 µl of SAAAN in DMSO + 5 ml of 0.2M Tris buffer pH 8.0) was added. The residual PPE activity after inhibition by α1AT was measured.

5.1.13 High Pressure Liquid Chromatography (HPLC) Analyses
(performed by Mr. Chang-shi Xie)

For measurements of ECM breakdown products, BAL was done with dH_2O because high salt concentrations interfere with the analysis. The BAL fluid was lyophilized, then hydrolyzed in 2 ml of 6M hydrochloric acid for 48 hours at 110°C. Part of this hydrolysate was used for desmosine analysis and a separate portion used for hydroxyproline analysis as described in Li, et al. (1996).

Desmosine Analysis

Half a millilitre of hydrolysate was loaded onto a 50 mm cellulose column (Whatman cellulose CF11, Fisher Scientific). The column was then washed with 15 ml of a 4:1:1 *n*-butanol-acedic acid water solution, and desmosines were eluted with 5 ml of water. The eluate was lyophilized, and the residue was dissolved in 1 ml of distilled water. Analysis was performed on a Waters HPLC system with a model 486 ultraviolet detector at 275 nm. Separation was obtained with a Nova-Pak C18, 4 µm, 4.6 X 150 mm column, and an eluant of 0.1M phosphate buffer-acetonitrile (2.8:1 vol/vol). Twenty micromolar SDS was added as an ion-pairing agent. This procedure separates desmosine from isodesmosine. The latter is actually the major component in BAL fluid, but both are reported together as "desmosine".

Hydroxyproline Analysis

Fifty microlitres of the hydrochloric acid hydrolysate was dried with a Waters Pico-Tag Vacuum Station and redried with 50 µl of a mixture of 2:1:1 ethanol-water-triethylamine, and the dried sample was derivatized with 50 µl of a mixture of 7:1:1 ethanol-water-triethylamine-phenylisothiocyanate for 20 minutes at room temperature. The sample was then dried again and finally dissolved in 700 µl of phosphate buffer for analysis. Analysis was performed with a Waters model 486 detector at 254 nm. To achieve separation, a Whatman Partisil ODS-2 C18, 10 µm, 4.6 X 250 mm column was used. The mobile phase was programmed at a flow rate of 1.6 ml per minute, starting with 100% *solvent A* (60 ml of acetonitrile mixed with 940 ml of a 138 mM acetate buffer, pH 6.4, containing 0.05% triethylamine), followed by a linear gradient to 50% *solvent B* (60% acetonitrile in water) for 15 minutes.

5.1.14 BAL Fluid Elastase Activity

Elastase activity in BAL fluid was measured by colorimetric assay. BAL fluid samples were lyophilized and reconstituted in water to make a solution that was 5X more concentrated than the original sample. N-succinyl-Ala-Ala-Ala-p-nitroanalide (SAAAN; Sigma) was used as the substrate. The assay was carried out in 0.2M Tris-HCl, pH 8.0 with and without the addition of 10 mM EDTA as a metalloelastase inhibitor or 10 M N-methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (CMT) as a serine elastase inhibitor.

BAL samples were assayed in duplicate wells of 96 well flat bottom plates (VWR Canlab). Each well contained 100 µl of the appropriate assay buffer as described above,

50 µl of 0.5 mg/ml SAAAN substrate and 50 µl of sample. Negative control wells contained 150 µl of assay buffer and 50 µl of substrate. Background absorbance of each BAL sample was assessed by incubating 150 µl of assay buffer with 50 µl of BAL fluid (this value was substracted from the absorbance of the test wells). The absorbance of the wells was measured at 405 nm.

5.1.15 Western Blot Analysis of Human α1AT

Diluted serum samples or unconcentrated BAL fluid samples were separated on a 12% polyacrylamide gel with purified human α1AT (Sigma) as a positive control. After electrophoresis the protein bands were immobilized on a nitrocellulose membrane and the membrane was probed with 1:2000 dilution of anti-human α1AT primary antibody (Boehringer Manheim) and goat anti-rabbit/horseradish peroxidase-conjugated antibody (ICN Biochemicals) and developed by enhanced chemiluminescence (ECL system, Amersham Pharmacia Biotech).

5.1.16 Airspace Size Morphometry

The mean linear intercept (Lm) is the average distance between alveolar walls in the parenchyma. To measure the Lm, animals were killed by exsanguination and the lungs were inflated with 2.5% glutaraldehyde at 25 cmH_20 for 15 minutes. The trachea was then tied and the lungs immersed in 2.5 % glutaraldehyde. The lungs were sectioned in a sagittal plane for morphometric analysis using the methodology of Thurlbeck (1967). Ten random sections were examined in each lung, and the measurements were performed at 20X magnification using a grid with a line of 1.02 mm total length and 42 points.

5.1.17 Statistics

Comparisons were done by analysis of variance using Systat or Microsoft Excel. Because PMN counts were often zero (especially in control animals and the PMN depletion or α1AT treatment groups), comparisons were performed with the non-parametric Kruskal-Wallis test, although the graphs show means and standard deviations. For the chronic LPS experiments the one-tailed two sample t-test was used. Values of $p<0.05$ or less were considered significant.

5.2 Experimental Protocols

5.2.1 Serum Levels of Human α1AT

Initial experiments were performed to determine the increase in serum human α1AT in mice following injection of various amounts of purified product and to determine the time course of the serum elevation following i.p. injection. For this purpose, blood was drawn from the tail vein and analyzed by a human α1AT ELISA (described above). After examination of the preliminary data, a 20 mg/ml final solution was made up for subsequent injections. Serum protein electrophoresis on cellulose acetate gels was also done on the serum samples by a clinical chemistry laboratory to confirm that there was an increase in total α1AT levels in the mice. The protein electrophoresis data represented the percentage of total α1 proteins in the serum and this value was multiplied by the total protein concentration (as described above) of each serum sample to determine the absolute concentration of total α1AT (as α1AT is the major component of the serum α1 peak).

To determine the safety of repeated administration of Protease, a 20mg/ml solution of purified human α1AT was given i.p. to transgenic and normal CD-1 mice (n=6) twice a week, on Monday and Wednesday. Blood was taken from the tail vein immediately prior to Protease injection, and either 24 hours or 48 hours after the injection. Serum levels of human α1AT were determined with the human α1AT ELISA and, once again, confirmed by serum protein electrophoresis.

5.2.2 Acute Lung Injury Experiments

Smoke Exposure

Each experimental smoke group consisted of 5 C57-BL/6 mice between 6 and 8 weeks of age (Jackson Laboratories). Mice were exposed to the whole smoke from 1, 2, or 3 full Kentucky 2R1 cigarettes (obtained from the University of Kentucky) using a standard smoking apparatus previously described (Simani et al., 1974). Briefly, a calibrated amount of smoke ("one puff") was delivered to a chamber to which the noses of two mice (restrained in plexiglass tubes) were directed to two openings in each chamber. Mice were alternately exposed to smoke for one minute and air for one minute until the entire cigarette was used up. (One cigarette = 8 puffs). Two cigarettes was the maximum tolerated dose and a dose of 3 cigarettes approached lethality (some deaths occurred in these dose groups, but the animals were replaced if possible). Control mice were exposed to air.

Depletion of PMNs

PMNs were depleted by administration of an anti-mouse PMN antibody (APA, Accurate Scientific). Two i.p. injections of 0.2 ml of antibody were given 24 hours apart. Test lavages and blood counts 24 hours after intratracheal instillation of 50 μg of LPS given 24 hours after the second antibody dose showed that this procedure succeeded in depleting 100% of the PMNs from the peripheral blood and greater than 90% of the PMNs in the lavage. Mice were exposed to smoke or air 24 hours after the second antibody dose.

Administration of Purified α1AT (Protease)

To determine the ability of exogenous α1AT to protect against cigarette smoke induced connective tissue breakdown, purified human α1AT (20mg in 1 ml) was administered intraperitoneally (i.p.) 24 hours prior to cigarette smoke exposure.

5.2.3 Acute LPS Experiments

LPS Instillations

Each LPS experimental group also consisted of 5 C57-BL/6 mice (6 to 8 weeks old). Mice were anaesthetized with 0.02 ml/gram body weight of 2% avertin (Hogan et al. 1986) and vertically suspended by their upper front teeth against a wooden board. A fibre optic illuminator was focused at throat level to illuminate the inside of the throat area and visualize the tracheal opening. Fifty micrograms of LPS (Escherichia coli, serotype 0127:B8; Sigma) in 50 μl saline was injected into the trachea followed by 200 μl of air. The mouse was left vertically suspended for 30 seconds following the instillation

to allow movement of the instilled fluid into the lungs. Approximately 30 to 50 minutes were required for mice to awaken after the anaesthetic. Mice were sacrificed at various times following smoke exposure or LPS instillation by halothane overdose for removal of lungs to perform bronchoalveolar lavages.

Administration of Purified α1AT (Protease)

To determine the ability of exogenous α1AT to protect against LPS-induced connective tissue breakdown, purified human α1AT (20mg in 1 ml) was administered intraperitoneally (i.p.) 24 hours prior to LPS instillation.

5.2.3 Chronic LPS Injury Experiment

Groups of 6 normal CD-1 mice and SpAT3 transgenic mice (in CD-1 background) received weekly intratracheal instillations of 25 μg of LPS as described above (4.2.1) for either 8 or 10 weeks in two separate experiments. Groups of 5 CD-1 mice and 5 transgenic age-matched mice receiving no instillations served as controls. At the end of the experimental periods, lungs were removed for morphometry (measurement of Lm).

6. Pulmonary Expression of Human α1AT in Transgenic Mice[1]

6.1 Results

6.1.1 Identification of Positive Transgenic Offspring

Mice were screened for the presence of the transgene by Southern blot analysis of genomic DNA from their tails. For each transgene, DNA was digested with a restriction enzyme having a unique site within the transgene. A line carrying the Haptoglobin-α1AT transgene (HpAT1) was identified by digesting the DNA with *Hind*III and a 2.3 kb band was seen on a Southern blot (not shown). The two CC10-α1AT transgenic lines (CCAT1 and CCAT2) were identified by digesting the genomic DNA with *Eco*RV and the 3.7 kb signals corresponding to the complete length of the transgene are seen (Figure 6). This indicates that the founders contain multiple copies of the transgene integrated in a head-to-tail array. For the SPC-α1AT lines, genomic DNA was cut with *Pst*I (which also cuts only once within the transgene) and one founder mouse gives a signal of 5.1 kb which corresponds to the length of the transgene (SpAT2) indicating multiple copies in a head-to-tail array. The second founder animal (SpAT3) shows an intense signal at 5.6 kb and less intense signals at smaller molecular weights. This suggests rearrangement of the transgene, although subsequent studies (see below) indicate transcription and translation of appropriate molecular weight mRNA and protein indicating at least one intact and functional copy of the transgene.

[1] Results and Discussion in this chapter appear in J. Mol. Med. (1999) 77: 377-385. Dhami, R., et al.

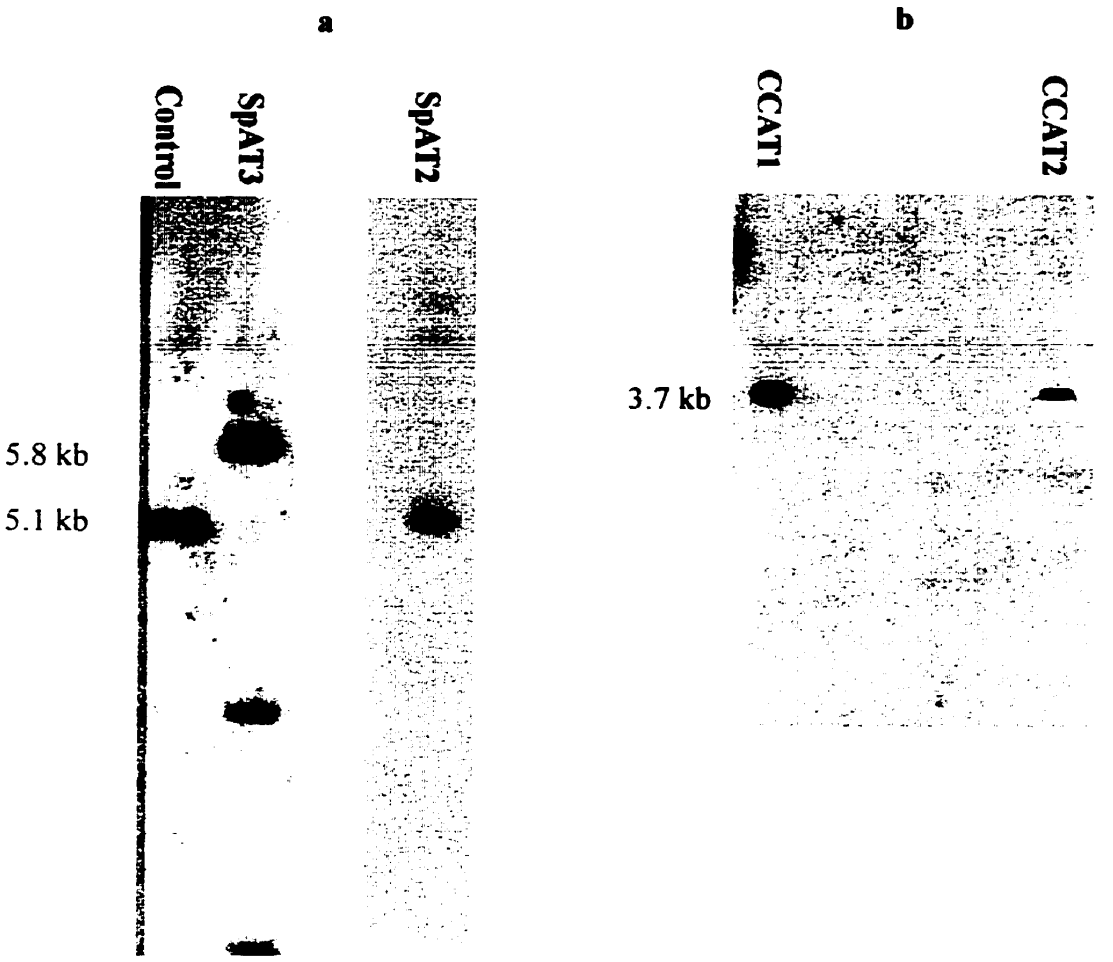


Figure 6. Southern analysis on genomic DNA from offspring of mice receiving eggs with injected transgene constructs. (a) Two lines carrying the SPC-AAT transgene were identified and named SPAT2 and SPAT3. (b) Two lines carrying the CC10-AAT transgene were identified and named CCAT1 and CCAT2. (The intervening lanes contain genomic DNA from non-transgenic littermates).

6.1.2 Northern Blot Analysis

Northern analysis reveals α1AT mRNA expression in the lungs of mice from the transgenic lines carrying the SPC and CC10 promoters (Figure 7). Lines CCAT1 and CCAT2 also show α1AT expression in the trachea. None of the lines show transgene expression in their livers. In an attempt to create a model in which there was direct interstitial production of α1AT, mice were created with a human α1AT gene driven by a 0.9 kb haptoglobin promoter, following the report by D'Armiento et al.(1992) that this promoter results in transgene expression in the pulmonary interstitium. However, there is no detectable mRNA in the lung, or liver in this line (not shown). As a result, these animals are not characterized further.

6.1.3 In situ Hybridization

In the two CC10 lines, CCAT1 and CCAT2, α1AT expression localizes to the alveolar parenchyma, small airways and also larger airways (Figure 8a; only CCAT1 shown). The distribution of staining in the alveolar parenchyma is consistent with localization in type II cells. Line SpAT2 shows a very similar distribution of gene expression (Figure 8b). In contrast, line SpAT3 demonstrates α1AT expression only in the alveolar parenchyma (Figure 8c), again, in a distribution consistent with expression in type II cells. Sections treated with the sense probe show minimal background grains (Figure 8d), and lung tissue from nontransgenic littermates are negative for human α1AT expression (not shown).

6.1.4 Light Microscopic Immunohistochemistry

Light microscopic immunohistochemistry shows a staining pattern generally corresponding to the in situ hybridization results. However, there are differences in staining

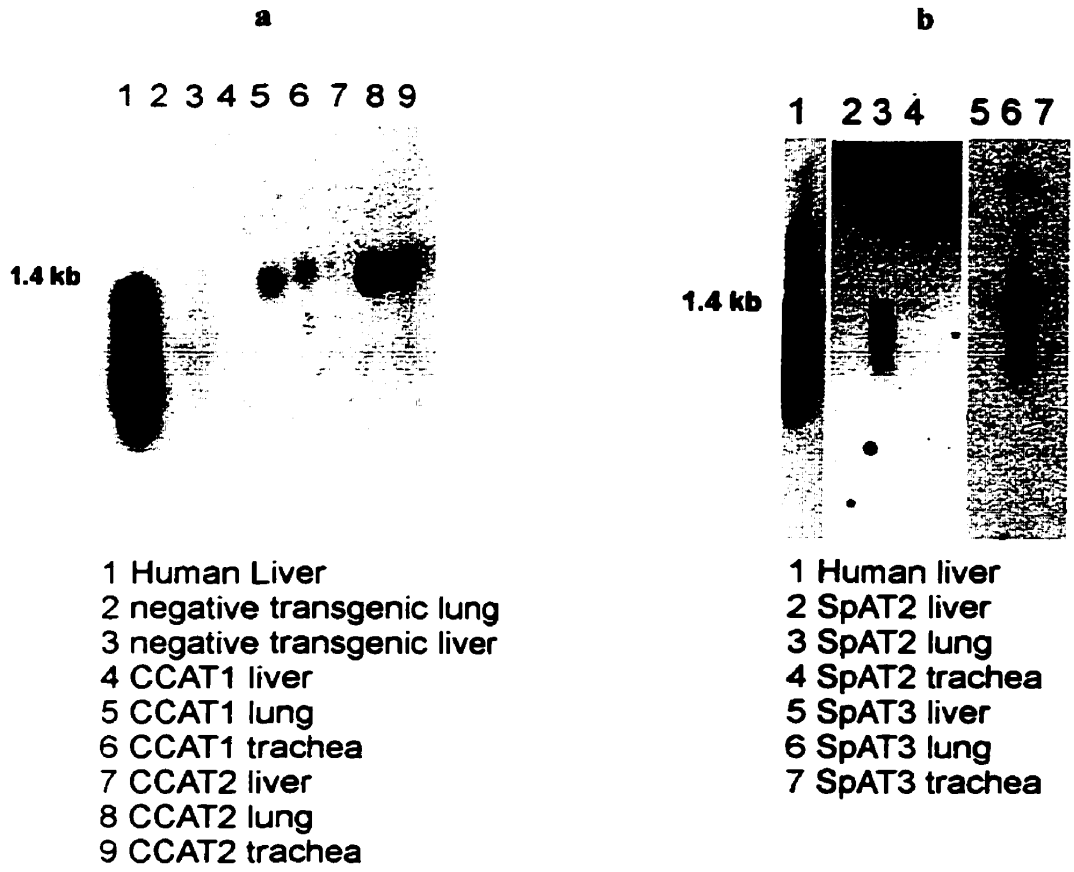


Figure 7. Northern blot analysis to determine the expression of the human AAT gene in the four transgenic lines. (a) CC10-driven lines. (b) SPC-driven lines. Total RNA was isolated from the liver, lung, and trachea of each animal. The transcript size corresponds to the full length cDNA (1.4 kb) which was also used as the probe. Human liver RNA (partially degraded) was used as a positive control.

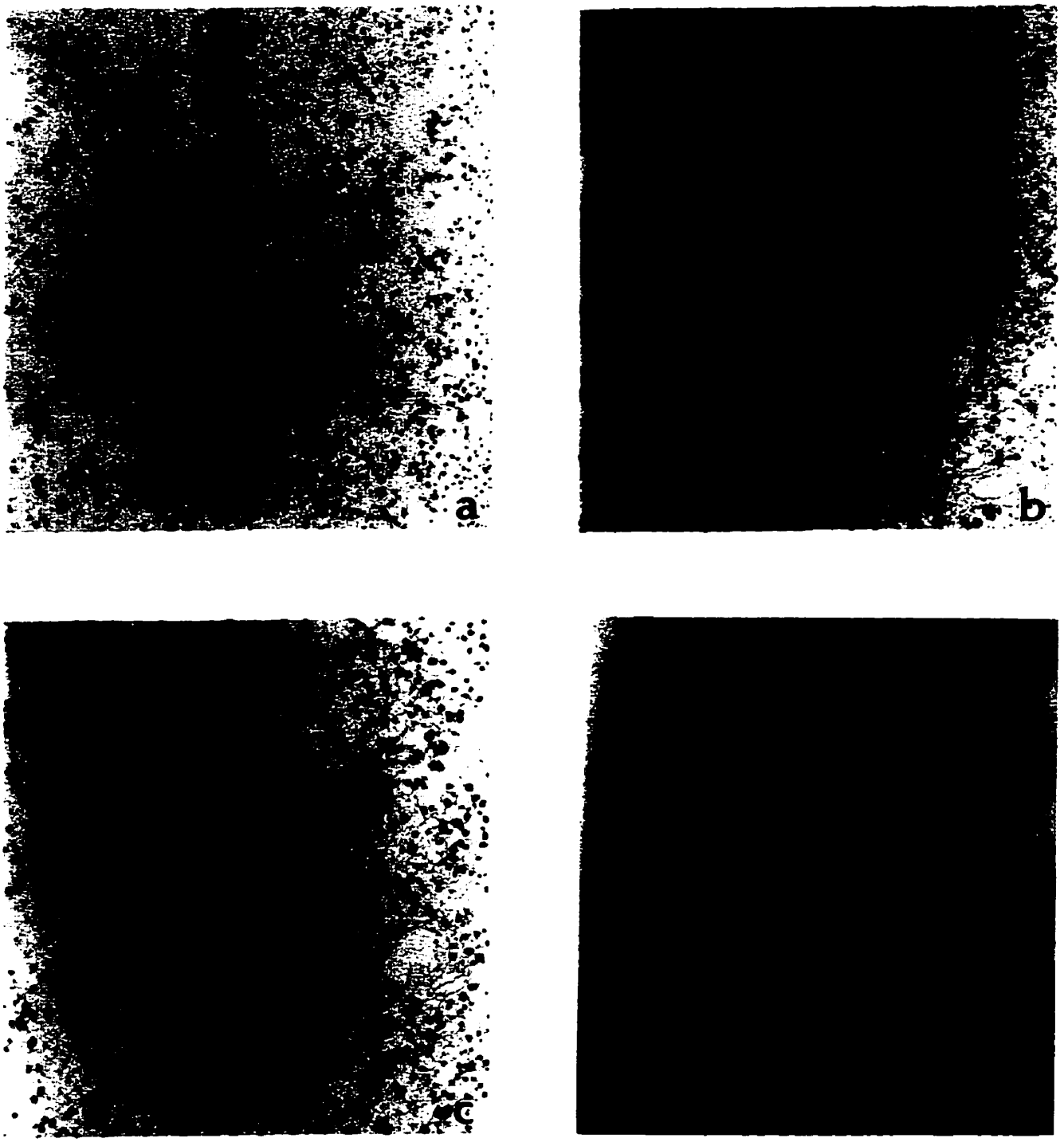


Figure 8. In situ hybridization to demonstrate localization of human α1AT mRNA within the lungs of CCAT and SpAT transgenic mice. **a** Line CCAT1, showing expression in both airway epithelium and type II cells. **b** Line SpAT2, showing a similar pattern of expression. **c** Line SpAT3, showing only type II cell expression. **d** Sense probe from line SpAT3 showing absence of signal. Sense probe corresponding to **a** and **b** were similarly negative (Magnification X150).

A

B

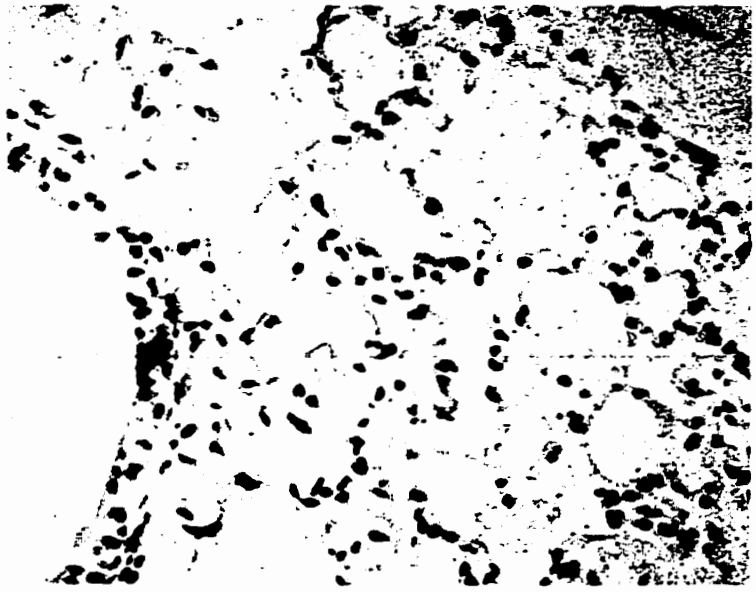


Figure 9. Light immunohistochemistry to demonstrate cellular protein production in line CCAT1 (A). Prominent staining is seen in the respiratory epithelium and much lighter staining is present in type II epithelial cells. There is no staining in non-transgenic mouse lungs (B). (Magnification, X500).

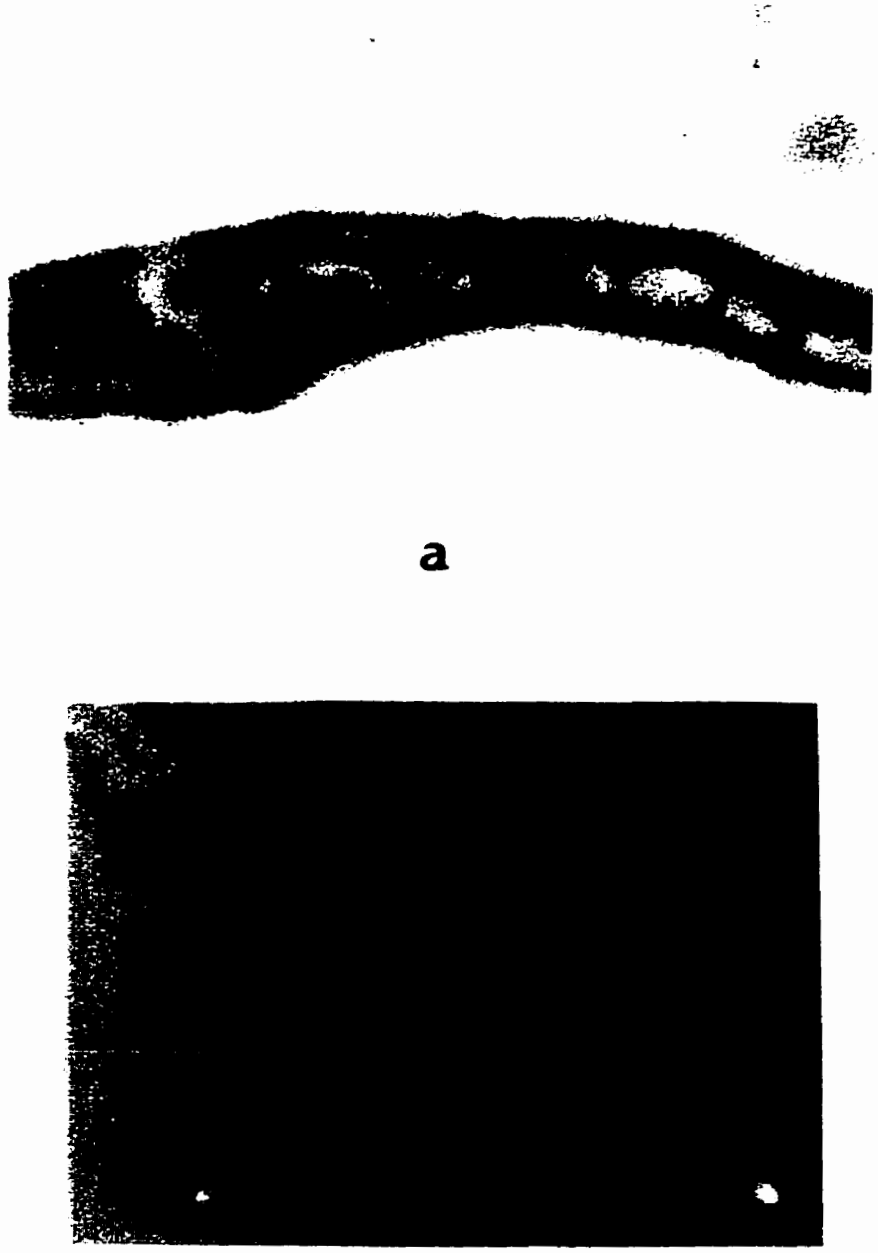


Figure 10. **a** Immunogold electron microscopic image showing the localization of human α1AT in the interstitium in line SpAT3. **b** Immunogold electron microscopic image showing the localization of mouse α1AT in the interstitium. (Magnification X 33,000).

intensity. The strongest staining is seen in the airway epithelial cells in the two CC10-driven lines (Figure 9). Airway epithelial cell staining in the SPC transgenic lines appears weaker. The parenchyma shows much less staining than the airway epithelium and is present in type II cells and pulmonary macrophages.

6.1.5 Electron Microscopic Immunohistochemistry

In transgenic animals immunogold labeling (indicating human α1AT) is seen in small amounts in type II alveolar cells, occasionally in type I cells, and consistently in the interstitial tissues (Figure 10a). A similar distribution is observed for endogenous (mouse) α1AT (Figure 10b). Omission of the primary antibody results in no staining, and use of lung tissue from nontransgenic control animals also shows negative results and revealed only small numbers of randomly scattered gold particles.

6.1.6 α1AT Protein Levels in Transgenic Mice

ELISA measurements show demonstrable levels of human α1AT in the whole lung tissue and also in the serum of animals in all lines (Table 3). No human protein is detected in the lavage fluid of any of the transgenic lines; dilution of the epithelial lining fluid on the surface of the epithelial cells is an inevitable consequence of lavage and may contribute to this negative result. A weak band is observed on western blot of lavage fluid using antibody against human α1AT (data not shown). In contrast to the low or undetectable levels of human α1AT in lavage fluid, mouse α1AT levels are approximately 200 μg/ml in the same samples.

Table 3: Whole lung and serum levels of human α1AT (ND= not detected)

Transgenic Line	Lung Protein (μg/g lung tissue)	*Serum (μg/ml)
Transgene -ve controls	ND	ND
SpAT2	0.59 ± 0.51	0.82 ± 0.20
SpAT3	2.9 ± 1.4	1.2 ± 0.18
CCAT1	0.37 ± 0.37	0.72 ± 0.41
CCAT2	1.3 ± 0.61	1.3 ± 1.2

* Normal serum α1AT levels: 2-5 mg/ml in humans, 4-6 mg/ml in mice

6.1.6 Purification of human α1AT from Protease®

Figure 11 shows the final purified human α1AT protein after completion of steps to remove the major contaminating protein, human albumin. In the original product purchased from Bayer (Protease®), approximately 50% of the lyophilized product is proteins other than α1AT, with albumin making up the majority of these contaminants. Virtually all of the albumin is removed after washing the original Protease solution through the Cibacron Blue 3GA column. Larger molecular weight contaminants are removed by washing through a Centricon 100 MW filter. The elastase inhibitory capacity assay shows that the final purified product inhibited 100% of PPE activity (data not shown). The purified human α1AT was stored at 4°C in azide buffer and, in most cases, used within a maximum of two weeks after purification.

6.1.7 Pharmacokinetics of intraperitoneal α1AT Administration

Figure 12 shows the serum concentrations of human α1AT in transgenic mice (n=4 SpAT3) over a period of 7 days after a single injection of 20mg α1AT (measured with a human α1AT ELISA). The highest concentration (12mg/ml) is measured 24 hours after the injection and this is decreased to 4mg/ml by 48 hours. There are still measurable levels of human protein 3 days after the injection. Cellulose acetate protein electrophoresis measurements of total serum α1 proteins confirm that a 2-3 fold increase in total α1AT levels is achieved at day 1 with persistent elevation for 2-3 days. Measurement of mouse α1AT levels in these same animals (with a mouse α1AT ELISA) shows that the increase in total serum α1AT levels is not due to an increase in endogenous mouse α1AT and is a result of the injected human α1AT protein.

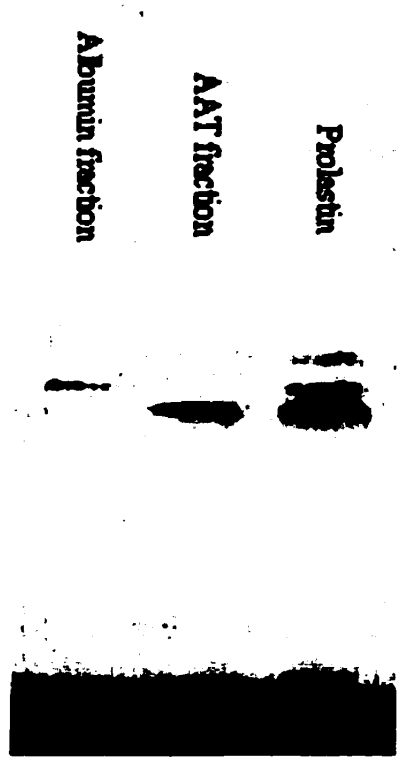


Figure 11. Purification of Prolastin (human AAT). The original product (Prolastin) contains human alpha-1-antitrypsin and albumin, as well as higher and lower molecular weight proteins. AAT fraction is the final purified product. The lane marked Albumin fraction shows the protein removed after Cibacron Blue 3GA column (see Methods). Relative concentrations cannot be determined from this gel as all the samples are not the same dilution. Samples are separated on a 12% polyacrylamide gel and silver stained.

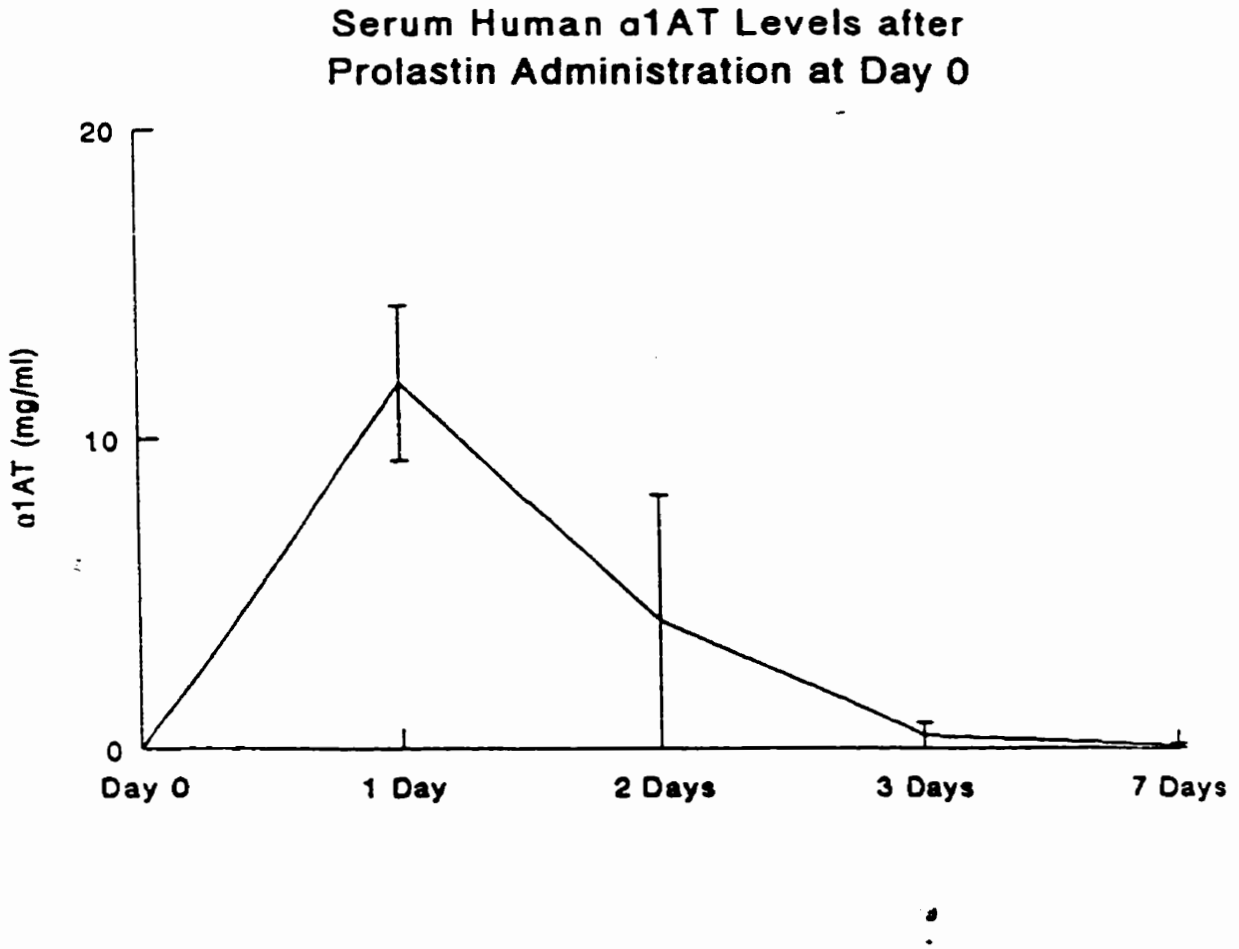


Figure 12. The time course of purified human α1AT (Prolastin) in mouse serum over a period of 7 days after a single i.p. injection of 20 mg Prolastin. N=4. Values are mean ± SD.

Figure 13 shows the concentration of human α1AT in mouse serum from three transgenic mice over a period of 48 hours. Mice receiving human α1AT injections for the first time (naïve) show peak levels at 6 hours after the injection, while mice receiving human α1AT for the fifth time (sensitized) show peak levels after 12 hours and higher levels are maintained for a longer period. Once again, there are measurable levels of the injected α1AT at 48 hours suggesting that injection of human α1AT every 48 hours is sufficient to maintain augmented levels of total α1AT and the time course of the injected human α1AT is not altered significantly after repeated injections. (Absolute concentrations of human α1AT determined in this experiment cannot be compared to those in other experiments because a different batch of human α1AT ELISA plates were used and inconsistencies between batches of plates were noted).

Table 4 compares the serum and bronchoalveolar lavage concentrations of human α1AT in the same animals after a single injection of 20 mg of Protease. On average 3.5% of the serum human α1AT passes into the pulmonary airspaces. The mouse α1AT concentration in the lavage (200μ/ml) is approximately 4% of that in the serum (4-5mg/ml).

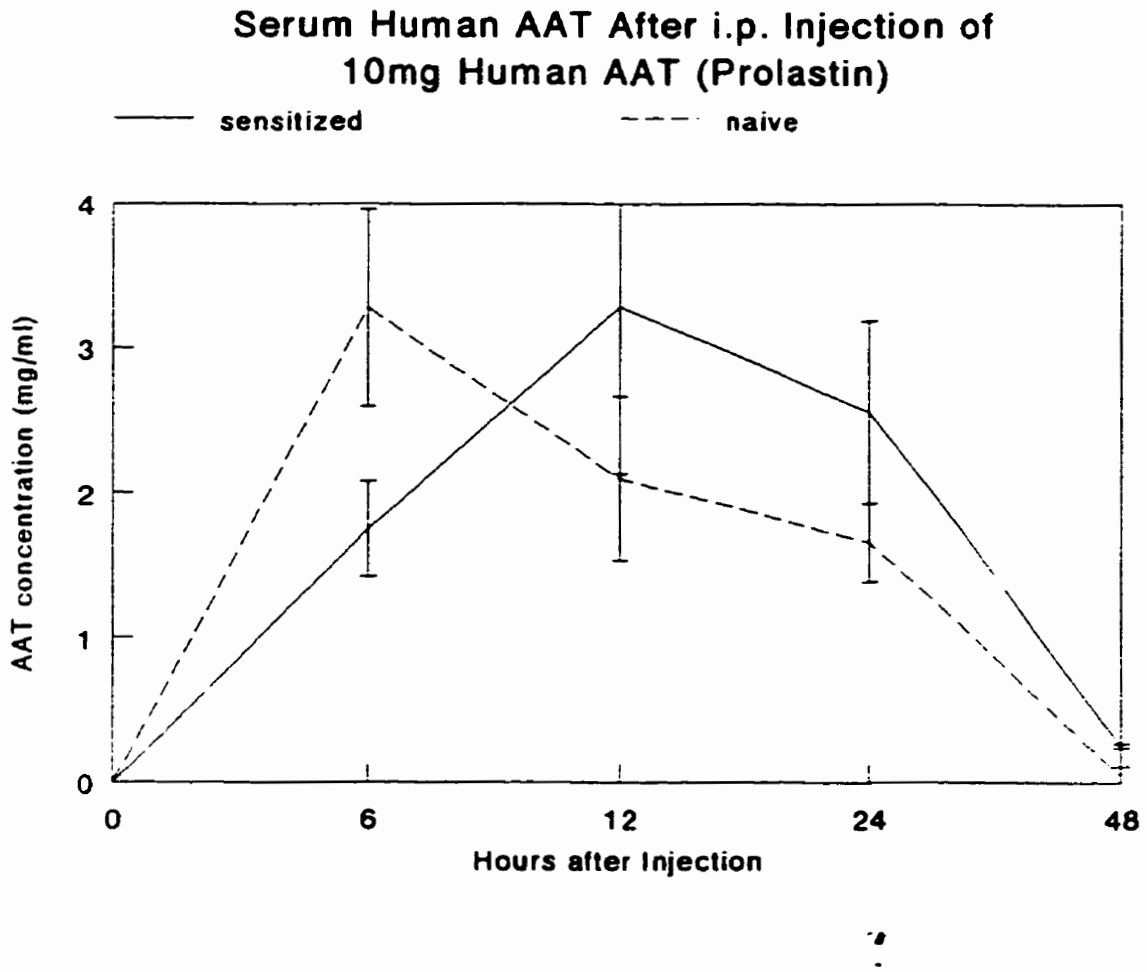


Figure 13. The time course of purified human α1AT over a period of 48 hours after a single i.p. injection of 10 mg Prolastin. Naïve = mice receiving human α1AT injections for the first time. Sensitized = mice that have received human α1AT injections before. N=3. Values are mean ± SD.

Table 4: Serum and Lavage Levels of Human α1AT in Mice 24 hours After a Single i.p. Injection of 20mg Protease (measured with the human α1AT ELISA).

Mouse	Serum concentration (mg/ml)	Lavage concentration (μg/ml)
1	8.07	324
2	10.65	570
3	12.69	324
4	11.61	354
5	11.79	330

6.1.8 Feasibility of Long-term α1AT Administration

In an experiment spanning 18 days, human α1AT transgenic and non-transgenic CD-1 mice received biweekly injections of 20 mg of purified human α1AT for a total of 6 injections. Each group consisted of 5 mice. One mouse from the non-transgenic group was found dead after the fourth injection. At the end of the experimental period the transgenic mice appeared healthy, however, the mice in the non-transgenic group had developed serum sickness as evidenced by the appearance of disheveled fur, lethargy, weight loss, and diarrhea.

Figure 14 shows the serum concentration of human α1AT at various time points during the experimental period as mice received biweekly injections of Protease®. A minimum concentration of approximately 4mg/ml is maintained throughout most of the 18 day period. Normal mouse serum α1AT levels are approximately 4-5mg/ml, therefore, a minimum of a two-fold increase in total (human and mouse) serum α1AT levels is maintained. Similar concentrations of human α1AT at various times are observed between transgenic (immunologically tolerant to human α1AT) and non-transgenic mice, however, there is a suggestion on day 18 that the non-transgenic mice are beginning to clear the human protein faster.

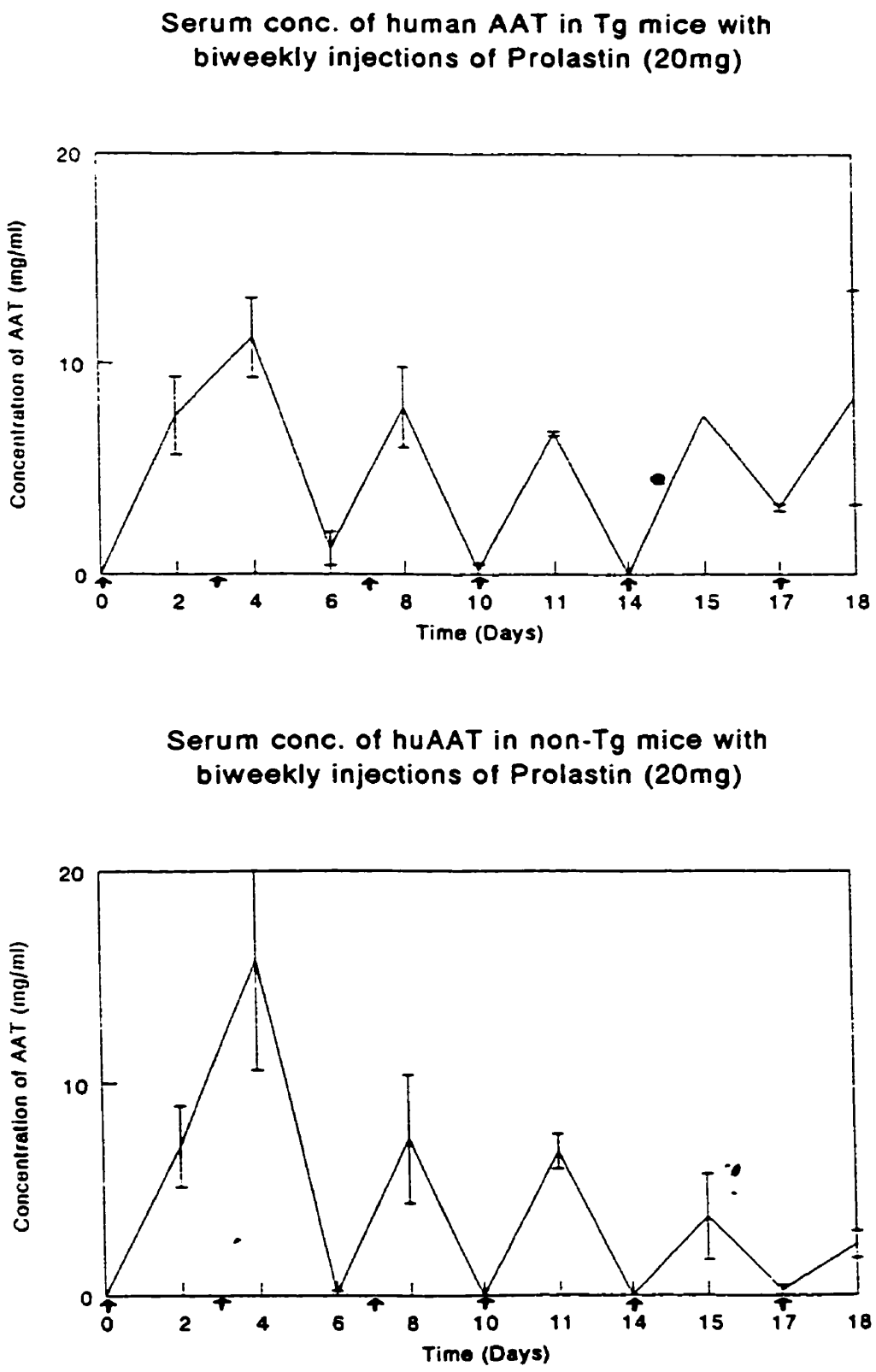


Figure 14. The serum concentrations of human α1AT in SpAT3 transgenic mice (top graph) and non-transgenic (bottom graph) CD-1 mice with biweekly i.p. injections of 20 mg purified human α1AT (Prolastin, a total six injections were given). Arrows indicate time of Prolastin injection. N=4. Values are mean ± SD.

6.2 Discussion

As discussed in the Introduction, attempts to boost pulmonary α1AT levels have attracted great interest and a wide variety of approaches, including numerous forms of gene therapy, have been employed. The accessibility of the respiratory epithelium to aerosolized vectors make it an obvious target for gene therapy and it has been shown that direct delivery of α1AT protein to the lungs as an aerosol is a more efficient means of increasing lung parenchymal levels of α1AT than intravenous delivery. Smith et al. (1989) demonstrated that, whereas only 2% of intravenously infused α1AT is present in the lungs of dogs at equilibrium, 32% of a lower dose of aerosolized α1AT administered to sheep was retained in the lungs. However, the results of attempts to increase α1AT levels to date are disappointing in that, with the exception of intravenous administration of partially purified human α1AT (Hutchison, et al., 1997, Pierce, 1997, Wencker, et al., 1998, Alpha-1-Antitrypsin Deficiency Registry Group, 1998), resulting protein levels have generally been very low. As well, expression has typically been transient, particularly when driven by viral vectors that elicit host antibody production (Rosenfeld, et al., 1991, Setoguchi, et al., 1994). A final consideration is that the relative efficacy of enhancing systemic rather than pulmonary expression of α1AT levels is unclear. In humans treated with purified α1AT, Barker et al. (1997) found a poor correlation of serum and lavage α1AT levels; moreover, lavage levels were disappointingly low.

In model systems in which pulmonary expression of α1AT has been introduced, there is little documentation that the protein actually reaches the interstitium. This is a particularly important issue, as most normal secretory products (for example, mucus or

surfactant) of pulmonary epithelial cells tend to be secreted into the alveolus or airway lumen, while, as noted previously, the presence of α1AT in the interstitium is probably required for protection against connective tissue breakdown, and this requires either basal secretion by epithelial cells or diffusion from the airspace back into the interstitium. Siegfried et al. (1995) showed that adenoviral vector transfection of human respiratory epithelial cells lead to both apical and basal secretion of α1AT in a ratio of 2:1 in monolayer culture systems, but DeGryse et al. (1996) could not demonstrate interstitial α1AT by EM immunochemistry in transgenic mice with the α1AT gene driven by the SPC promoter although they detected the protein in the plasma. In contrast to the low levels of human α1AT expression in mice with the α1AT gene under control of the SPC promoter, both Ruther et al. (1987) and Sifers et al. (1987) reported high levels of human α1AT in the serum of transgenic mice with the human α1AT gene under control of its own promoter, resulting in expression of the gene in hepatocytes. In neither of these studies were levels of α1AT in the lung assessed.

In this study we created transgenic mice using two lung epithelial specific promoters, CC10 and SPC, in the hope of producing permanent high level protein expression in a location very close to the crucial locus of action, that is, the pulmonary interstitium. The epithelial localization of α1AT expression seen in our animals with both the CC10 and SPC promoters is somewhat different from that found in studies using these promoters to drive expression of other proteins. In the normal animal SPC expression is confined to type II cells and CC10 expression to Clara cells in the airways. This latter distribution has been observed for most transgenic proteins expressed under the control of the CC10 promoter. For example, Stripp et al. (1992) showed that the

CC10 promoter caused expression of chloramphenicol acetyltransferase (CAT) in the epithelial cells lining the trachea, bronchi and bronchioles. They found only very low numbers of silver grains in the alveolar parenchyma, a result which they attributed to possible low level expression of the transgene. Our observation of extensive type II cell expression of human α1AT under the control of the CC10 promoter is thus unusual. On the other hand, Glasser et al. (1991) observed expression of CAT by in situ hybridization in both bronchiolar and alveolar cells in 4 different transgenic lines when the protein was under the control of the SPC promoter. Although there was marked variation in the relative abundance of expression, comparing airways to parenchyma in the four different lines, the overall distribution was similar to that which we found in our line SPAT2, with both type II cell and small airway epithelial cell expression. Again, our line SPAT3 differs in that only alveolar parenchymal expression was seen. Despite these variations, **our results suggest that either SPC or CC10 driven constructs can be used to produce diffuse pulmonary alveolar parenchymal production of α1AT.**

Our immunochemistry results are of interest in that the most intense staining was seen in airway epithelial cells, probably because the protein product is produced and stored in the airway cells for a period of time, rather than being immediately secreted. The type II cell signal was much weaker. These findings, along with the presence of signal in the interstitium by EM immunochemistry, and the ELISA/Western blot data showing measurable levels of protein in whole lung and serum but extremely low levels in lavage, suggest that most of the human α1AT produced in type II cells is not stored in the cells but is very rapidly exported through the basal surface of the cell, passing through the interstitium before reaching the circulation. This conclusion is consistent with the

observation that some other types of proteins produced by type II cells, for example, gelatinase, are predominantly secreted in a basal direction (d'Ortho, et al., 1997), and also with the observation that α1AT is produced in hepatocytes at very high levels, but very little or no immunochemically reactive protein can be demonstrated in hepatocytes because the protein is immediately secreted into the liver sinusoids. **This expression of α1AT in pulmonary epithelial cells may prove to be a potentially effective method of boosting interstitial α1AT levels *in vivo*.**

While this approach is promising, it suffers, like many of those discussed above, from low levels of protein production. To put this in perspective it is useful to note that in these same animals the levels of mouse α1AT are about 200μg/ml in lavage and 5mg/ml in serum. The normal range for serum α1AT in humans, by comparison, is 2-5 mg/ml and lavage levels are 10% of serum levels (McElvaney & Crystal, 1997b). Our findings, and the other reports in the literature in which gene therapy treatment models using α1AT have been described, thus raise an important question. Most attempts to use gene therapy, for example in the treatment of cystic fibrosis, are meant to provide an intracellular protein that is required in relatively small amounts. However, α1AT replacement therapy requires production of very large amounts of an extracellular protein not normally synthesized by any pulmonary cells, and it is unclear at this point whether adequate levels of protein expression in the lung can be achieved by gene therapy, or whether such massive levels of α1AT production, even if they could be achieved, would compromise the normal function of pulmonary epithelial cells. In contrast to the low levels of α1AT after gene therapy or in transgenic animals where expression is directed

to the pulmonary epithelium, both delivery of adenoviral vectors to hepatocytes and transgenic animals with expression in hepatocytes have shown high level α1AT expression with serum levels in the range considered therapeutic (Kay, et al., 1995, Ruther, et al., 1987, Sifers, et al., 1987). Similarly encouraging results have resulted from direct injection of skeletal muscle with adeno-associated virus vectors containing the α1AT gene (Song, et al., 1998). Although gene therapy vectors are more readily delivered to the respiratory epithelium these cells may not be able to produce therapeutic protein levels, while hepatocytes or some other cell type may ultimately prove capable of synthesizing sufficient protein to be protective.

Despite the low level expression of human α1AT, the animals described here will be useful experimentally, however, as unlike nontransgenic mice they tolerate repeated doses of human α1AT protein without developing serum sickness. A two-fold increase in total serum α1AT levels can be achieved with biweekly injections of 20 mg of purified human α1AT throughout most of a weekly period. In considering the expense involved with augmentation therapy, this is a realistic timetable and could be easily maintained for a long experimental period in mice. The main objective with augmentation therapy, however, is to increase lung levels of α1AT and the ability to achieve this has also been demonstrated in our transgenic mice.

Conclusion: The human α1AT transgenic mice can serve as an animal model for studying the protective effect of exogenous human α1AT protein in chronic experimental lung injuries, including chronic cigarette smoke exposure.

7. Acute Cigarette Smoke-induced Lung Injury[2]

Hypothesis: Acute cigarette smoke exposure in mice results in detectable pulmonary connective tissue breakdown caused by PMN-derived proteases and which can be prevented by intraperitoneal administration of human α1AT.

7.1 Results

7.1.1 Dose Response

A dose response in the degree of acute inflammation and connective tissue breakdown in the lung is observed in mice 24 hours after cigarette smoke exposure. The number of lavage PMNs increases approximately four-fold with exposure to smoke from 2 and 3 cigarettes, but the number of macrophages show no changes from control (0 cigarettes) levels (Figure 15). Figure 16 shows the levels of lavage desmosine and hydroxyproline 24 hours after exposure to cigarette smoke. Levels of both connective tissue breakdown markers (desmosine and hydroxyproline) are elevated in the BALF after smoke exposure. Desmosine levels increase approximately 3 to 4 fold with exposure to smoke from 2 and 3 cigarettes compared to control levels. There is no measurable desmosine in the serum of smoke-exposed mice so we are confident that the BALF desmosine is of pulmonary origin and is not leaked from the serum. Hydroxyproline levels increase approximately two-fold from basal (control) levels. The elevations in lavage connective tissue breakdown products correlates with the elevations in PMN numbers in the pulmonary airspaces. The increase in lavage PMNs, desmosine and hydroxyproline (markers of elastin and collagen breakdown, respectively) with exposure to 2 and 3 cigarettes is significantly different from control (0 cigarettes) levels.

[2] Results and Discussion in this chapter appear in part in Am. J. Resp. Cell Mol. Biol. (in press)

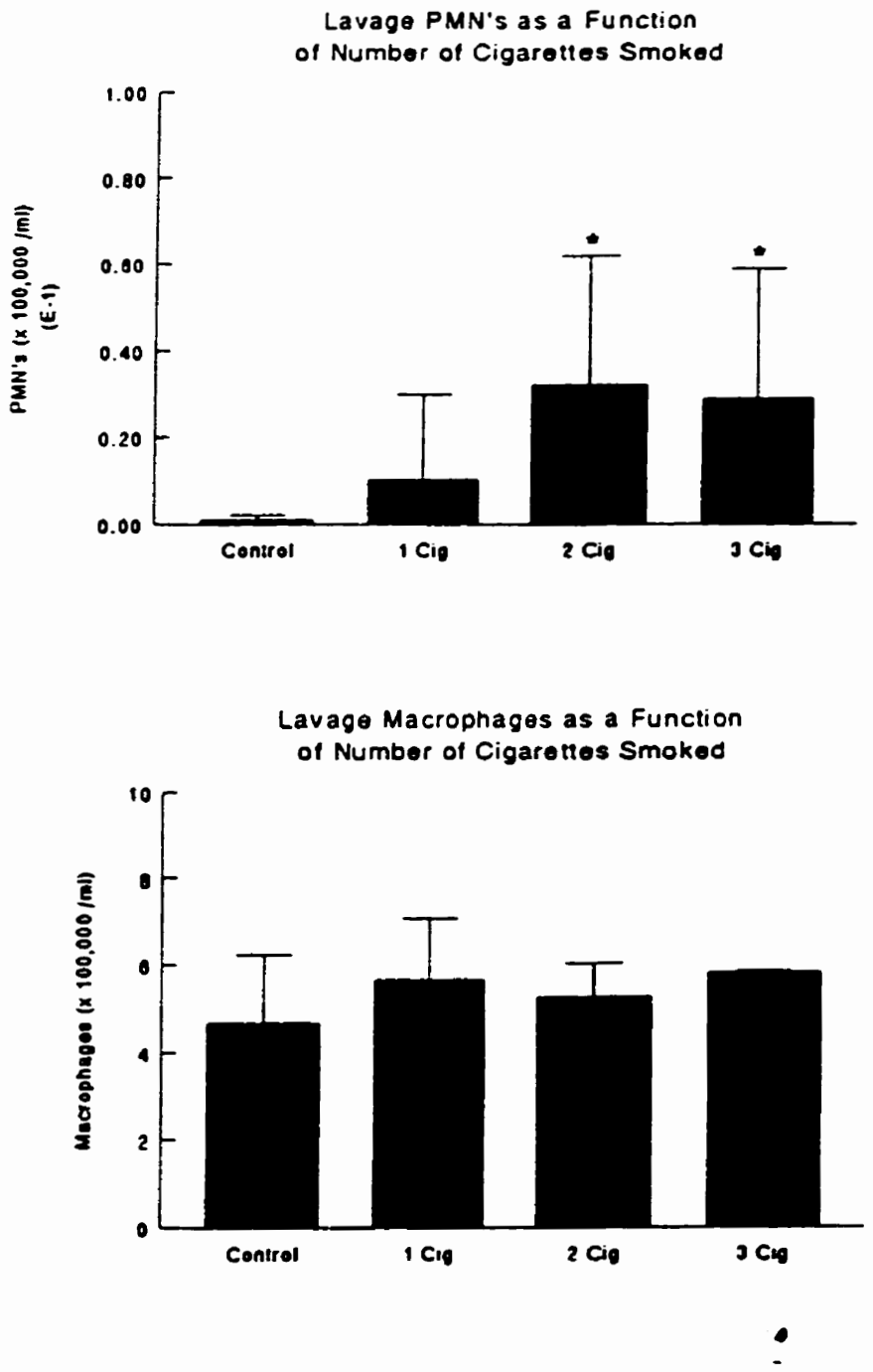


Figure 15. The number of PMNs and macrophages in the bronchoalveolar lavage fluid of C57-BL/6J 24 hours after exposure to various doses of cigarette smoke. The number of PMNs were increased significantly after exposure to 2 and 3 cigarettes compared to control (0 cigarettes) mice. The number of macrophages were unchanged compared to controls at all doses. N=5. Values are mean ± SD. * indicates significantly greater than control values.

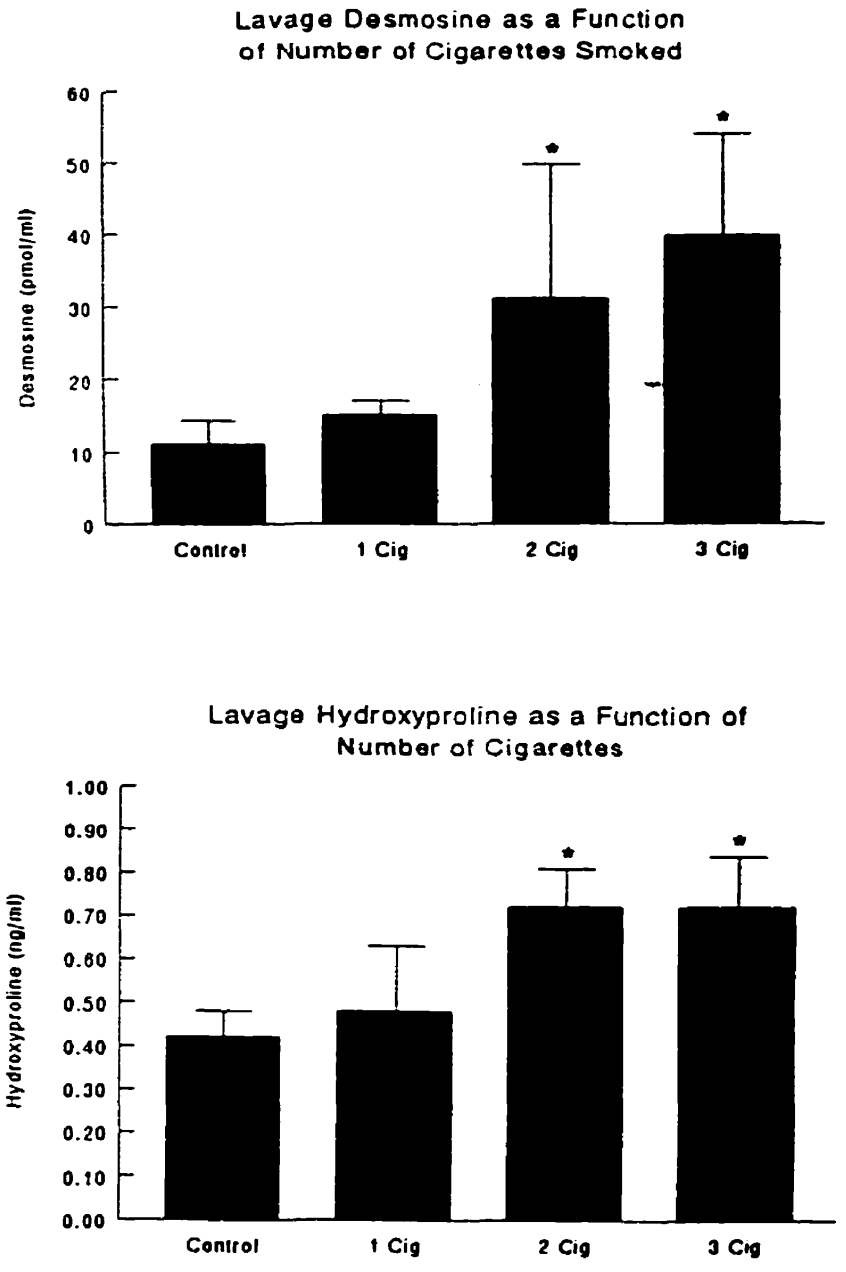


Figure 16. The levels of connective tissue breakdown products in the bronchoalveolar lavage fluid of C57-BL/6J 24 hours after exposure to various doses of cigarette smoke. Both desmosine and hydroxyproline levels are increased in the lavage after exposure to smoke from 2 and 3 cigarettes compared to control (0 cigarettes) mice. N=5. Values are mean ± SD. * = indicates significantly greater than control values.

7.1.2 Time Response

Figure 17 shows that PMN numbers in the lavage increase significantly by 6 hours and begin to return to control levels again by 48 hours while macrophage numbers in the airspaces are not affected by the cigarette smoke. Absolute numbers of PMNs are small with control lavages containing approximately 10,000 cells/ml; at 24 hours there are approximately 40,000 cells/ml. Absolute macrophage numbers in the lavage range from 400,000 to 500,000/ml. Figure 18 shows the accompanying changes in the connective tissue breakdown markers over a period of 48 hours. The desmosine and hydroxyproline levels in the lavage fluid show a significant increase from control values at both 6 hours and 24 hours. There is no significant difference in desmosine and hydroxyproline (markers of elastin and collagen breakdown, respectively) levels between control and smoke-exposed animals at 48 hours.

7.1.3 Response to Anti-PMN Antibody

Figure 19 shows the numbers of PMNs and macrophages in the lavage fluid of mice exposed to smoke from 2 cigarettes or air 24 hours after receiving or not receiving anti-PMN antibody administered i.p. Inflammatory cell counts 24 hours after smoke exposure indicate that the antibody reduces lavage PMN levels to 0 and does not affect the number of macrophages in the lavage. Figure 20 shows the corresponding changes in desmosine and hydroxyproline levels in the lavage fluid. The mice receiving the anti-PMN antibody prior to cigarette smoke exposure show no increase in desmosine or hydroxyproline compared to air-exposed mice while there is a significant increase in both desmosine and hydroxyproline (markers of elastin and collagen breakdown, respectively) lavage levels in smoke-exposed mice not receiving the anti-PMN antibody.

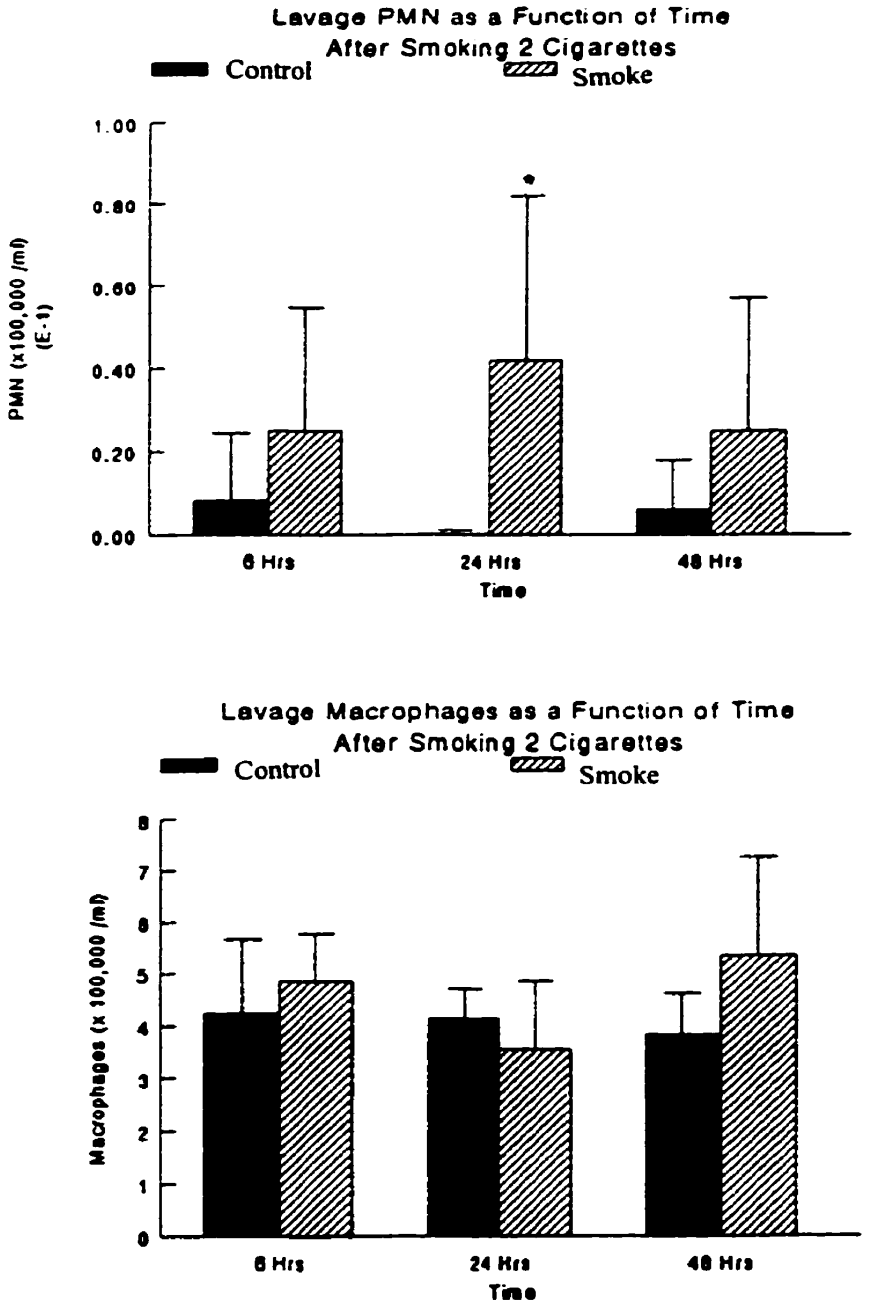


Figure 17. The number of PMNs and macrophages in the bronchoalveolar lavage fluid of C57-BL/6J mice at various time points after exposure to smoke from 2 cigarettes. The number of PMNs were increased significantly after 24 hours in smoke-exposed mice compared to control (0 cigarettes) mice. The number of macrophages were unchanged compared to controls at all time points. Solid bars represent control (air-exposed) mice and striped bars represent smoke-exposed mice. N=5. Values are mean ± SD. • indicates significantly greater than control values.

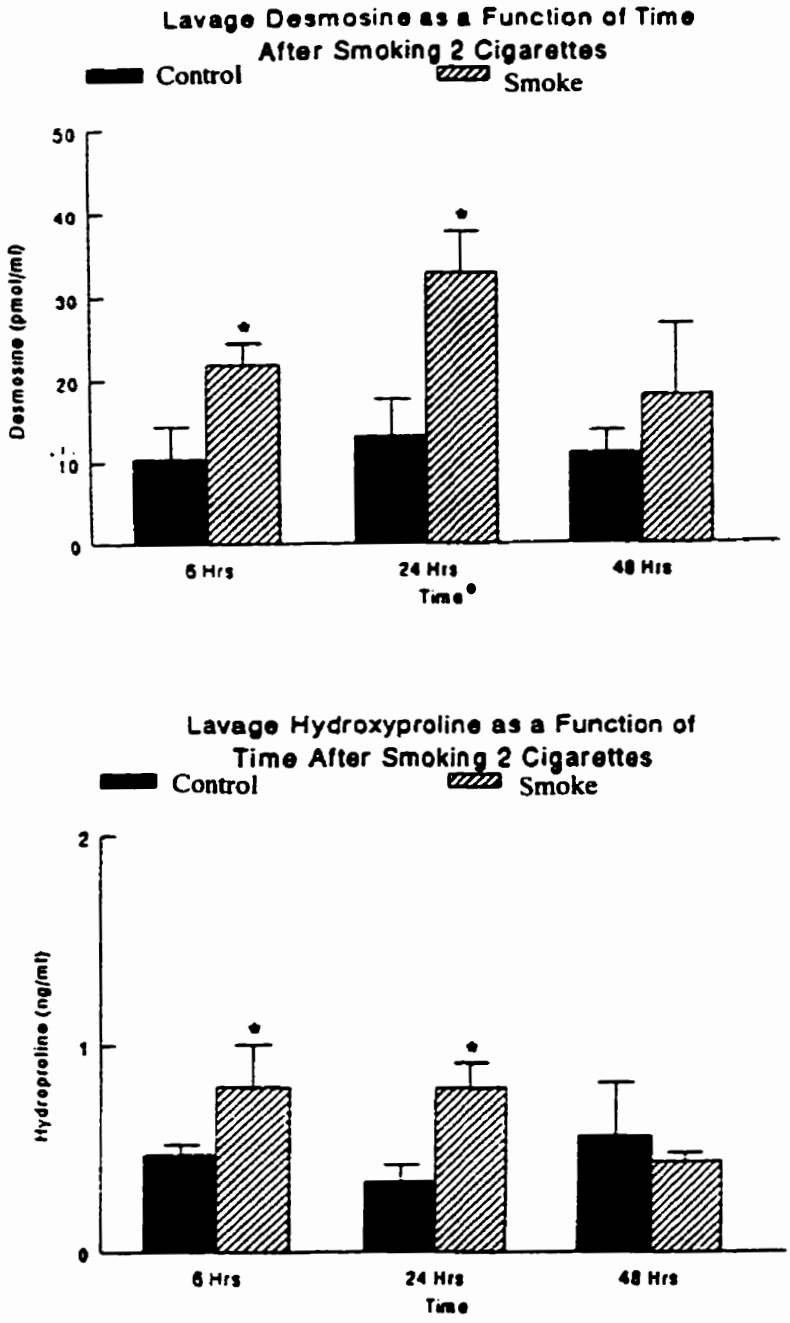


Figure 18. The levels of connective tissue breakdown products in the bronchoalveolar lavage fluid of C57-BL/6J mice at various time points after exposure to smoke from 2 cigarettes. Both desmosine and hydroxyproline levels are increased in the lavage after 6 and 24 hours compared to control (0 cigarettes) mice. Solid bars represent control (air-exposed) mice and striped bars represent smoke-exposed mice. N=5. Values are mean ± SD. * indicates significantly greater than control values.

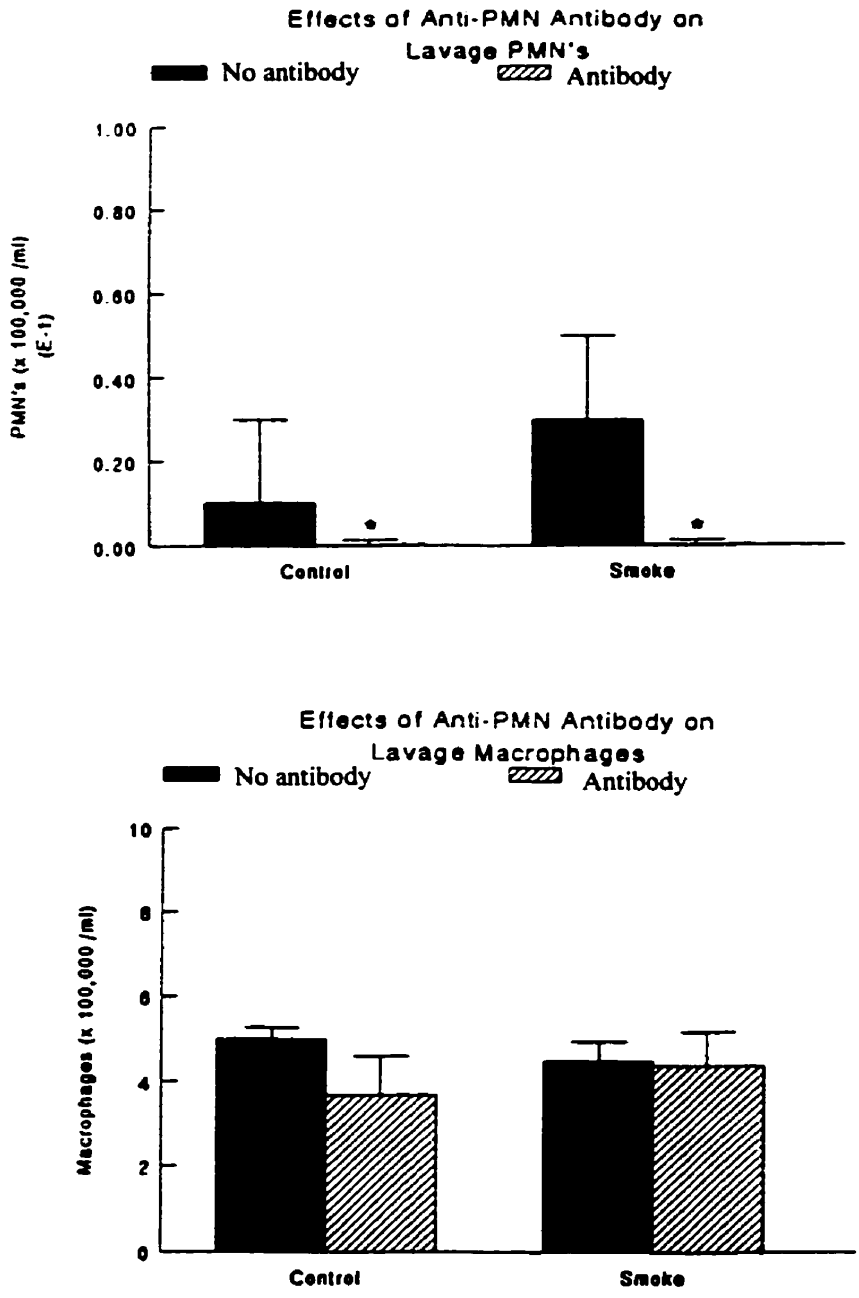
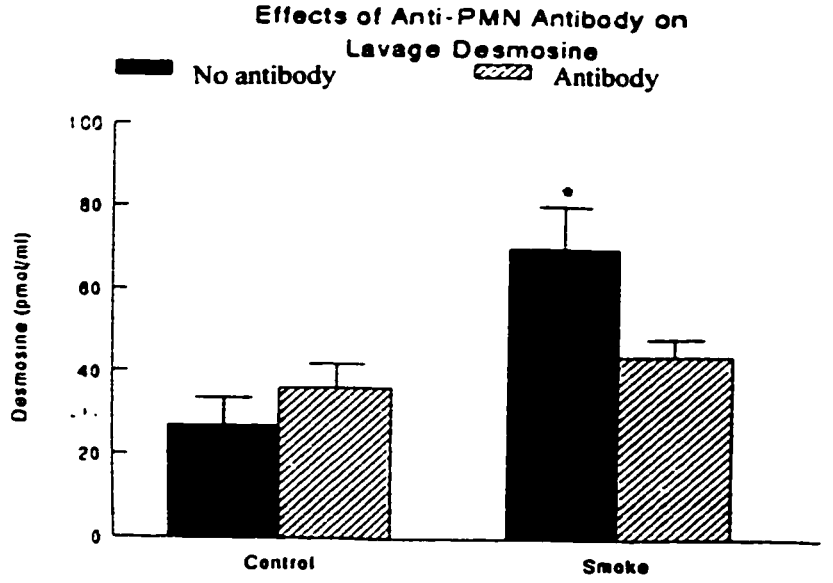


Figure 19. The effects of anti-PMN antibody on lavage cell numbers 24 hours after exposure to smoke from 2 cigarettes. Anti-PMN antibody reduces PMN counts to 0 (bar in graph is a place marker) in both control (air-exposed) and smoke-exposed mice. Lavage macrophages numbers are not changed with antibody administration. Solid bars represent control (air-exposed) mice and striped bars represent smoke-exposed mice. N=5. Values are mean ± SD. *indicates significantly greater than control values.



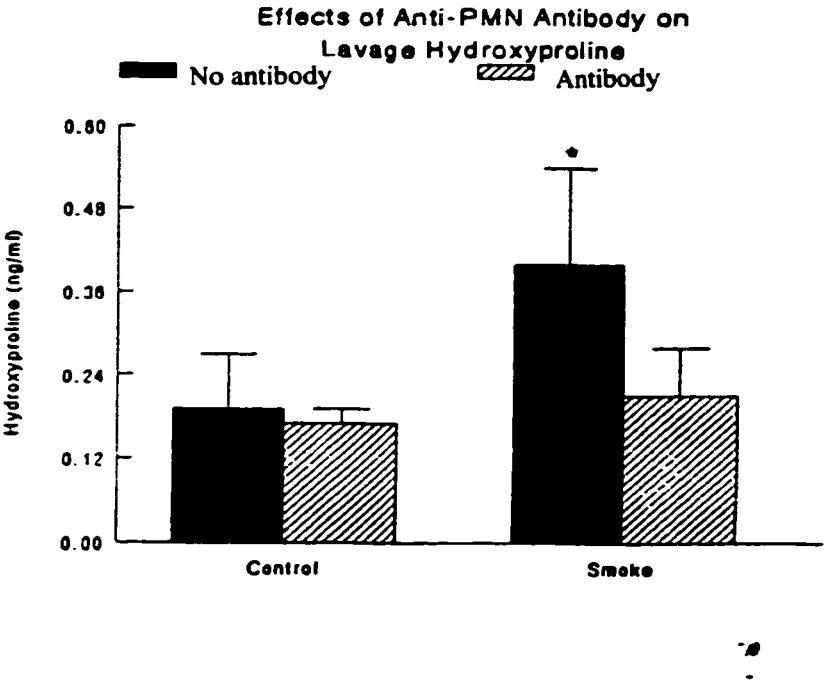


Figure 20. The effects of anti-PMN antibody on lavage connective tissue breakdown products 24 hours after exposure to smoke from 2 cigarettes. Anti-PMN antibody prevents the smoke-induced elevations of desmosine and hydroxyproline. Solid bars represent mice not receiving the antibody and striped bars represent mice receiving the anti-PMN antibody. N=5. Values are mean ± SD. * indicates significantly greater than control values.

7.1.4 Time Course of Exogenous Human α1AT in Mouse Serum

A single i.p. administration of 20 mg of purified α1AT in a volume of 1.0 ml was given to a group of 5 mice. The serum level of human α1AT was monitored over a period of 14 days using a human α1AT ELISA. Figure 12 shows that significant levels of the injected protein (approximately 2 mg/ml) could be detected in the serum for up to three days.

7.1.5 Western Blot of BALF After α1AT Administration

The western blots show that the human α1AT protein is present in the lavage fluid of both smoke (Figure 21B) and air exposed animals (Figure 21A) 48 hours after i.p. administration. Only a band of approximately 52 kilodaltons (the normal size of human α1AT) is visible on the blots suggesting that the protein is intact and not present in degraded forms.

7.1.6 Effects of Exogenous α1AT on Connective Tissue Breakdown

Figures 22 and 23 show the effects of exogenous α1AT administration on the inflammatory cell numbers and connective tissue breakdown products in the BAL fluid. For the acute smoke experiments, bronchoalveolar lavages were done a total of 48 hours after the Protease injection and 24 hours after cigarette smoke exposure. Protease given i.p. 24 hours prior to smoke exposure completely prevents the smoke-mediated increases in PMNs, but the macrophage numbers are not affected by smoke exposure or by exogenous α1AT treatment (Figure 22). There is a corresponding decrease in lavage desmosine (a marker of elastin breakdown) levels after smoke exposure in mice receiving prior α1AT administration, as well as, a decrease in lavage hydroxyproline (a marker of collagen breakdown) levels (Figure 23).

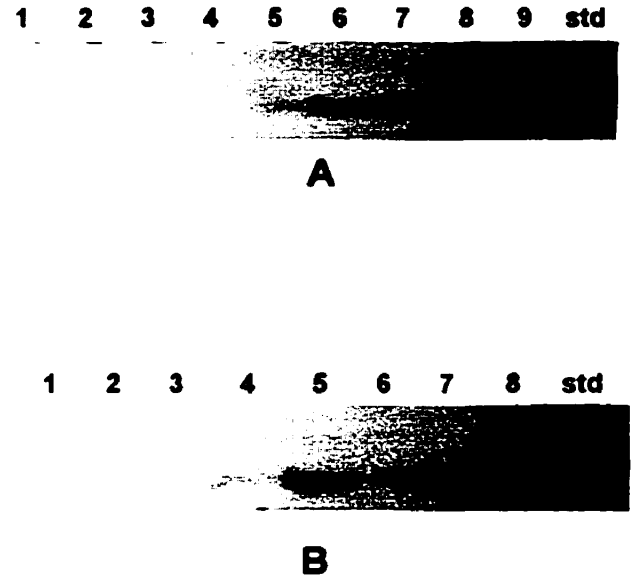


Figure 21. Western blot of lavage fluid at 48 hours after AAT administration (24 hours after smoke exposure) using antibody against human AAT (Sigma). A) lanes 1-5: control (air-exposed) mice with no human AAT, lanes 6-9: control mice receiving 20 mg human AAT i.p. std: pure human AAT. B) lanes 1-4: smoke-exposed mice with no human AAT, lanes 5-8: smoke-exposed mice receiving 20 mg human AAT i.p. std: pure human AAT.

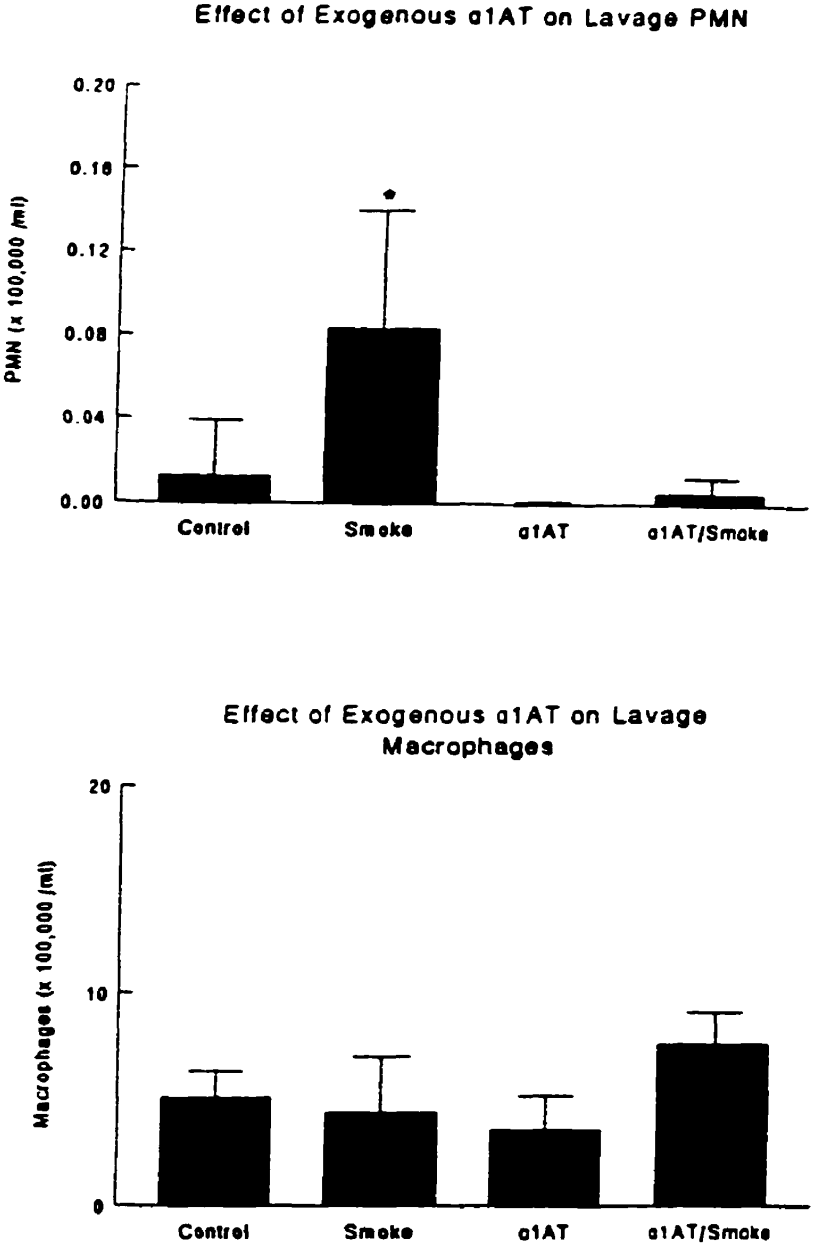


Figure 22. The effects of exogenous human α1AT (Prolastin), administered 24 hours prior to smoke exposure) on bronchoalveolar lavage cell counts 24 hours after exposure to smoke from 2 cigarettes. PMNs are increased after smoke exposure compared to control (air-exposed) values and this increase is not seen in mice receiving exogenous α1AT. The numbers of macrophages are unaffected by smoke exposure or α1AT treatment. N=5. Values are mean ± SD. * indicates significantly greater than control values.

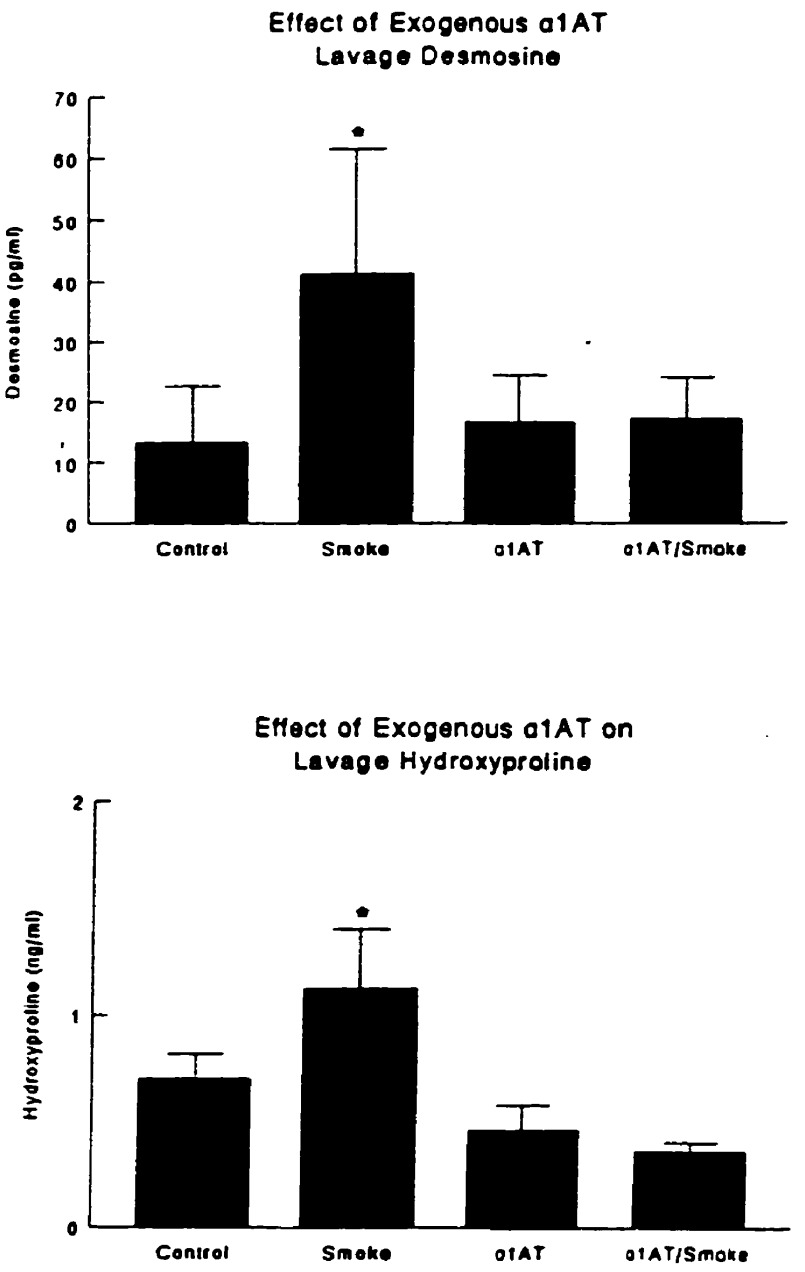


Figure 23. The effects of exogenous human α1AT (Prolastin, administered 24 hours prior smoke exposure) on bronchoalveolar lavage desmosine and hydroxyproline levels 24 hours after exposure to smoke from 2 cigarettes. Desmosine and hydroxyproline levels are increased after smoke exposure compared to control (air-exposed) values and these increases are not seen in mice receiving exogenous α1AT. N=5. Values are mean ± SD. * indicates significantly greater than control values.

7.1.7 Lavage Serine and Metalloelastase Activity

Figure 24 compares the serine and metalloelastase activities in the lavage fluid 24 hours after exposure to 2 cigarettes. Cigarette smoke exposure elevates both serine and metalloelastase activities (two- and three-fold respectively), compared to basal levels. Exogenous α1AT administration prior to the cigarette smoke exposure prevents the increase in serine but not the metalloelastase levels compared to control values. Activity is expressed in arbitrary units and differences indicated by * are statistically significant compared to controls. Sample groups consist of 5 animals each.

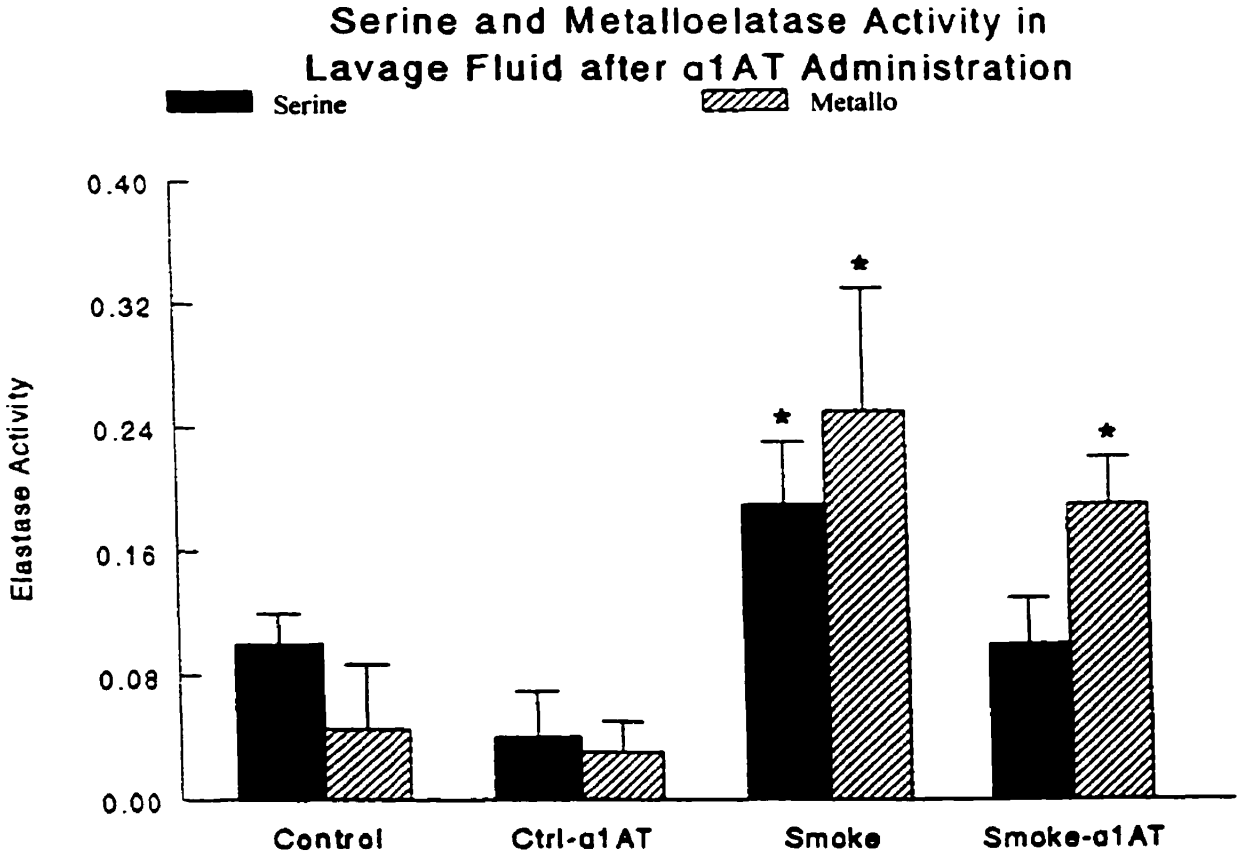


Figure 24. Elastase activities in the bronchoalveolar lavage fluid of mice 24 hours after exposure to smoke from 2 cigarettes or air exposure (control) with or without receiving an i.p. injection of 20 mg purified human α1AT 24 hours prior to treatment. Smoke exposure elevates both serine and metalloelastase activities. Administration of human α1AT reduces the lavage serine elastase activity to control values, but does not affect the metalloelastase activity. Solid bars represent control (air-exposed) mice and striped bars represent smoke-exposed mice. N=5. Values are mean ± SD. * indicates significantly greater than control values.

7.2 Discussion

In this study we have examined the acute effects of cigarette smoke exposure on pulmonary connective tissue breakdown and characterized the cells and proteases involved. The controversies regarding the effector cell(s) and protease(s) important in the pathogenesis of emphysema have been discussed in Chapter 2. Alpha-1-antitrypsin augmentation therapy has been shown to be a feasible and safe method of correcting the biochemical deficiency, and unrandomized comparisons of patients with and without replacement therapy suggest that it results in improvement in the decline in lung function, as measured by FEV_1 (Seersholm, et al. 1997, Wencker et al., 1998, α1AT Registry Study Group, 1998). The protease-antiprotease imbalance hypothesis has also been proposed to explain cigarette smoke-induced emphysema with the traditional view that the increase in proteolytic activity in smokers is derived from PMNs (Janoff, 1985, Tetley, 1995, Shapiro, 1995) and that cigarette smoke also inactivates the major neutrophil elastase inhibitor in the lung, α1AT. Thus elastin and collagen breakdown can occur in the lower respiratory tract in cigarette smokers as a result of increased proteolytic activity and/or decreased antiproteolytic activity (Evans & Pryor, 1994, Snider et al., 1994).

The quantitation of connective tissue breakdown in humans with COPD, however, has been problematic and reports using various methods are inconsistent. Early reports using radioimmunoassays conclude that there is no detectable difference in urine desmosine between control and α1AT deficient subjects (Pelham et al., 1985) and no correlation between urine desmosine and smoking status (Davies et al., 1983). Apart from problems of interfering substances, measurement of urinary or plasma desmosine is not completely specific as it includes the normal elastin turnover from all organs in the

body. Gottlieb et al. (1996) note that, in normal individuals, only 19% of urinary desmosine is derived from the lung. Measurements of lung lavage desmosine are potentially more informative, but few data are available. Recent studies suggest that plasma and also BAL fluid elastin-derived peptides are increased in smokers with signs of emphysema (Dillon et al., 1992 and Schriver et al., 1992) and Ofulue et al. (1998) reported that, using a radioimmunoassay, elevated levels of both desmosine and elastin split products could be detected in the lavage fluid of rats two months after initial smoke exposure, but not earlier. The present experiments demonstrate this phenomenon more at an earlier time. Levels of desmosine in the BAL fluid were elevated within 6 hours of smoke exposure, suggesting that connective tissue breakdown is an early event. The fact that hydroxyproline levels were also elevated at this time reinforces the idea that the proteolytic attack resulting from cigarette smoke exposure degrades a whole spectrum of matrix components, not just elastin. Studies have shown that collagen breakdown and abnormal resynthesis is associated with emphysema development in animal models of smoke-induced emphysema (Wright et al., 1995) and in humans who smoke (Cardosa et al., 1993). Also, transgenic mice expressing collagenase in the pulmonary interstitium were shown to develop emphysema-like lesions (D'Armiento et al., 1992) and increased collagenase activity was present in the lungs of smoke-exposed guinea pigs with emphysema (Selman et al., 1996).

Increased epithelial permeability occurs after exposure to cigarette smoke, so the possibility of smoke-induced leakage of serum desmosine and hydroxyproline into the lung was considered. However, there was no detectable desmosine in normal mouse serum with our HPLC technique, which has a detection limit of 3 pmol/ml, and since the

normal mouse serum volume is approximately 1.5 ml, the total contribution of desmosine from serum could have been at most 4.5 pmol/ml. The lavage desmosine in our control animals was 20-30 pmol/ml and the smoke-exposed animals had desmosine levels of up to 70 pmol/ml. Therefore we are confident that the increases in connective tissue breakdown products in the bronchoalveolar lavage fluid in the lungs of smoke-exposed mice are of lung origin.

Although at an intuitive level the pathogenesis of emphysema in hereditary α1AT deficiency is probably similar to that of cigarette smoke-induced emphysema, the role for Neutrophil elastase or α1AT in the lung destruction caused by cigarette smoke is controversial. In addition to Neutrophil elastase, a number of other enzymes, including macrophage metalloelastase, 72- and 92-kDa gelatinase, and matrilysin all have some elastase activity and may play an important role in increased proteolytic activity occurring in response to smoke (Gronski et al., 1997, Shapiro, 1994, Senior et al., 1991). In support of this possibility, Hautamaki et al. (1997) created mice lacking the gene for macrophage metalloelastase and demonstrated that these mice do not develop emphysema in response to long-term cigarette smoke exposure. Recently, Ofulue et al. (1998) have shown that the time course of cigarette smoke-induced emphysema in rats is closely related to the time course of increased macrophage-derived elastolytic activity and does not mirror changes in PMN numbers. These studies implicate a central role for macrophage elastase in cigarette smoke-induced emphysema. The significance of macrophages in the pathogenesis of emphysema is further implicated by reports of increased number of macrophages in the airspaces and respiratory bronchioles and parenchyma of smoker's lungs (Niewoehner et al., 1974).

Evidence from our experiments, however, is consistent with the traditional view that PMNs are the effector cells in emphysema, at least acutely, in the early stages after cigarette smoke exposure. PMNs constitute, on average, 1-3% of lavage cells in our smoke-exposed mice, values comparable to those seen in human smokers (Hunninghake & Crystal, 1983). This increase is potentially more significant than might be thought from such small numbers, since absolute numbers of PMNs were increased approximately 10 to 30 fold in the various experiments and lavage numbers reflect only 1 to 2% of PMNs present in the lung tissue (Downey et al, 1993). In studies using lung tissue from chronic smokers, it was demonstrated that the alveolar walls of smokers' contain a higher absolute number of PMNs than those of non-smokers, although the number of PMNs did not correlate with degree of lung destruction (Eidelman et al., 1990, Finkelstein et al., 1995). The importance of elevated PMN numbers is evident from the experiments using anti-PMN antibody. By reducing lung PMNs to essentially 0, connective tissue breakdown was abolished.

Similarly, the efficacy of exogenous α1AT in preventing connective tissue breakdown is strong evidence that a serine protease is involved, and the effect of α1AT in abolishing the elevated serine elastase activity in the smoke-exposed mice is evident in Figure 25. While our experiments do not prove that that protease is neutrophil elastase, this is the mostly likely culprit since it is the most potent serine elastase in the PMN armamentarium and accounts for most PMN-derived elastolytic activity. However, other PMN serine proteases such as cathepsin G and proteinase 3 cannot be ruled out.

An interesting observation from our studies is that α1AT administration not only results in decreased levels of desmosine and hydroxyproline in the lavage fluid, but it also

prevents PMN recruitment to the lungs (Figure 23). This suggests that α1AT is preventing connective tissue breakdown by decreasing PMN recruitment into the lungs. It has been suggested that inhibitors of cathepsin G and α1-chymotrypsin prevent PMN chemotaxis by binding to cell surface proteases (Lomas et al., 1995) and α1AT has also been shown to decrease PMN chemotaxis in vitro (Stockley et al., 1990). Another explanation for the reduction in PMN recruitment is the elimination of connective tissue breakdown products through inhibition of PMN-mediated ECM proteolysis. Both elastin and collagen-derived peptides have chemotactic activity for PMNs (Senior et al., 1980, Riley et al., 1988). Therefore, α1AT could prevent formation of chemotactic peptide fragments, blocking a positive feedback cycle. Our acute smoke experiments do not specifically address these possibilities, but when LPS is administered to mice, α1AT injection prevents elastin degradation, despite the fact that lavage PMN numbers remain significantly elevated (see Chapter 8). This suggests that direct inhibition of proteolytic activity is important in preventing connective tissue breakdown in our model of acute cigarette smoke exposure.

Decreased function of oxidized α1AT in smokers has been reported (Carp et al., 1982), as well as, a decreased association rate between α1AT and neutrophil elastase (Ogushi et al., 1991). Both of these factors would result in a reduction in the antiproteolytic screen of the lower respiratory tract, however, some studies have suggested that cigarette smoke does not impair the functional activity of α1AT and that there is no difference between the amount of inactive α1AT in BAL fluid and serum from smokers and nonsmokers (Boudier et al., 1983, Lellouch et al., 1985). Lack of

indisputable proof for the role of α1AT in cigarette smoke-induced emphysema generates wariness in recommending such a costly treatment.

In light of these reports, it is interesting that in our studies, C57-BL/6J mice which, in human terms, have normal levels of α1AT (Gardi et al., 1994) demonstrate acute pulmonary connective tissue breakdown in response to cigarette smoke exposure and strain 129/svJ mice which also have normal levels of α1AT develop emphysema after daily exposure to cigarette smoke for a period of six months (Hautamaki et al., 1997). This evidence indicates that the protease burden derived from acute cigarette smoke exposure is sufficient to overwhelm the protective effects of endogenous α1AT in C57-BL/6J mice. An additional note of interest is that a recent report associates cigarette smoke induced COPD with the MZ phenotype of α1AT, where serum concentrations of α1AT are only 50% of those in MM (normal) individuals, but not in MS subjects where the level of serum α1AT is approximately 75% of MM subjects (Sanford et al., 1999). This observation not only emphasizes the importance of α1AT (and by implication, its major substrate neutrophil elastase), but suggests that even fairly small variations in α1AT levels may lead to a situation in which smoke-induced proteolysis defeats the host defenses. Another report demonstrates that the F type α1AT which is expressed at normal levels in the serum inhibits Neutrophil elastase at a much slower rate than the M type enzyme (Cook et al., 1996). As a result FZ individuals have a high risk for the development of emphysema and future studies may reveal an increased risk for individuals who are homozygous for the F allele and other alleles that express a functionally impaired inhibitor that is present in normal amounts in the serum. Thus,

even individuals with normal levels of α1AT, albeit functionally impaired α1AT, may benefit from augmentation therapy.

We have used a model of acute cigarette smoke exposure to investigate aspects of connective tissue destruction and attempted to clarify the importance of PMNs and Neutrophil elastase in this process. We have shown that exposure of mice to cigarette smoke results in significant destruction of connective tissue components (elastin and collagen) after 24 hours and this damage correlates with an increased number of PMNs in the BAL and is prevented by prior injection of an anti-PMN antibody or injection of α1AT. Macrophage numbers remain unchanged under all experimental conditions. These data support the notion that PMNs evoked by cigarette smoke are responsible for the connective tissue breakdown and are also consistent with human data showing a correlation between elastin degradation products, NE-α1AT complexes, and NE-specific elastolytic activity (Betsuyaku et al.,1996).

As discussed, our results clearly differ from several recent reports in the literature which emphasize the role of macrophages and metalloproteases in smoke-mediated proteolysis. The fact that our study examines only an acute effect is an important consideration. We cannot speculate as to whether PMNs play the principal role in connective tissue breakdown during the progression of emphysema as our mice do not develop airspace enlargement. Ofulue et al. (1998) have suggested that macrophages rather than PMNs are temporally associated with the development of emphysema in a rat model of chronic smoke exposure, however, they did show that PMN accumulation in the lavage and pulmonary interstitium persists for at least one month from initiation of smoke exposure. During this early period the increased elastin breakdown may be attributed

primarily to Neutrophil elastase. It has also been postulated that alveolar macrophages serve as a reservoir for NE released by PMNs during their brief delay in the lungs and is later released by macrophages during disease progression (Betsuyaku et al., 1994). It is likely that the acute response we describe occurs repeatedly after each cigarette is smoked and, over a long term, PMNs and their proteases play a role in the pathogenesis of emphysema, as well as, macrophages. A relationship between amount smoked and number of BALF PMNs has previously been shown in humans (Bosken, et al, 1992). The metalloelastase activity we observed may be due to macrophage metalloproteases or PMN-derived proteases such as gelatinase B (MMP-9) although these enzymes do not contribute significantly to the elastin or collagen breakdown in the first 48 hours after smoke exposure as the serine protease inhibitor, α1AT, was able to completely prevent degradation of these connective tissue components.

Conclusion: We have demonstrated that acute exposure of mice to cigarette smoke results in lung connective tissue breakdown associated with increased lavage PMNs, but not with macrophages. The connective tissue breakdown was prevented by the neutrophil elastase inhibitor, α1AT, and also by depleting the PMNs from the lungs with an anti-PMN antibody. Our data suggests that neutrophil elastase, rather than macrophage-derived metalloelastase is important in the early events leading to the development of emphysema.

8. LPS-mediated Lung Injury

Hypothesis: 1) Acute LPS-mediated lung injury in mice is associated with a pulmonary inflammatory response causing connective tissue breakdown which is prevented by intraperitoneal administration of human α1AT. 2) Chronic LPS exposure in strain CD-1 mice (achieved by weekly intratracheal instillations of LPS) results in the development of emphysema.

8.1 Results

8.1.1 BALF Cell Numbers

Table 5 lists the inflammatory cell numbers in the lavage of mice 1 and 7 days after receiving a single intratracheal instillation of 50 μg of LPS. Mice receiving intratracheal instillations of saline were used as controls because LPS was delivered as a solution in saline. The inflammatory cell numbers indicate that saline causes a minor inflammatory response that is resolved by 7 days although it is not as dramatic as that observed in the LPS treatment groups. Compared to saline controls total cell numbers are increased three-fold compared to saline controls at 24 hours and five-fold at 7 days after the LPS instillation. At 24 hours after LPS instillation PMN numbers are elevated and make up 96% of the BALF cells. At 7 days PMN numbers remain elevated and they account for 51% of the cells in the BAL fluid of mice receiving LPS. Macrophage numbers drop after LPS instillation but are significantly increased at 7 days, compared to saline controls. Treatment of mice with exogenous α1AT 24 hours prior to the LPS instillation does not significantly affect cell numbers, compared to the LPS only group at 24 hours or 7 days. All groups consist of 5 mice each.

Table 5: Inflammatory cells in the BALF after intratracheal instillations of 50 μg LPS

	Total Cells (millions)	PMNs (millions)	Macrophages (millions)
24 hrs. saline control	1.17 ± 0.48	.50 ± .44	.71 ± .10
24 hours. LPS	3.38 ± 1.7	3.25 ± 1.67*	.12 ± .04*
24 hrs. LPS + α1AT	2.22 ± 0.94	2.1 ± .99*	.18 ± .07*
7 days. saline control	0.54 ± 0.02	0	.53 ± .02
7 days. LPS	2.6 ± 0.82	1.38 ± .79*	1.22 ± .50*
7 days. LPS + α1AT	2.75 ± 0.67	1.14 ± .84*	1.06 ± .60*

N= 5 for each experimental group

* indicates significantly different than corresponding saline control numbers

8.1.2 BALF Desmosine Levels

Figure 25 shows that 24 hours after the LPS instillation, lavage desmosine levels increase significantly (three-fold) and remain elevated for 7 days. Administration of α1AT 24 hours prior to the LPS instillation completely prevents the desmosine increase at both 24 hours and 7 days.

8.1.3 BALF Hydroxyproline Levels

Hydroxyproline levels in the BAL fluid of mice are unchanged 24 hours after LPS instillation compared to control levels, but are significantly increased after 7 days (Figure 26). Administration of α1AT 24 hours prior to the LPS instillation does not prevent the hydroxyproline increase at 7 days.

8.1.4 BALF Elastase Activities

Twenty-four hours after LPS instillation, the lavage serine elastase activity, but not metalloelastase activity is significantly increased (Figure 27). The serine elastase activity returns to control levels with prior α1AT (Protease) administration. There is no detectable difference in elastase activities (serine and metallo-) between control and LPS mice after 7 days (Figure 28). Elastase activities of the 7 day saline control animals are not included because both metallo- and serine elastase activities were several-fold higher than the other groups including the 24 hour saline controls, suggesting that these samples are contaminated. Unfortunately, samples of lung for histological examination were not

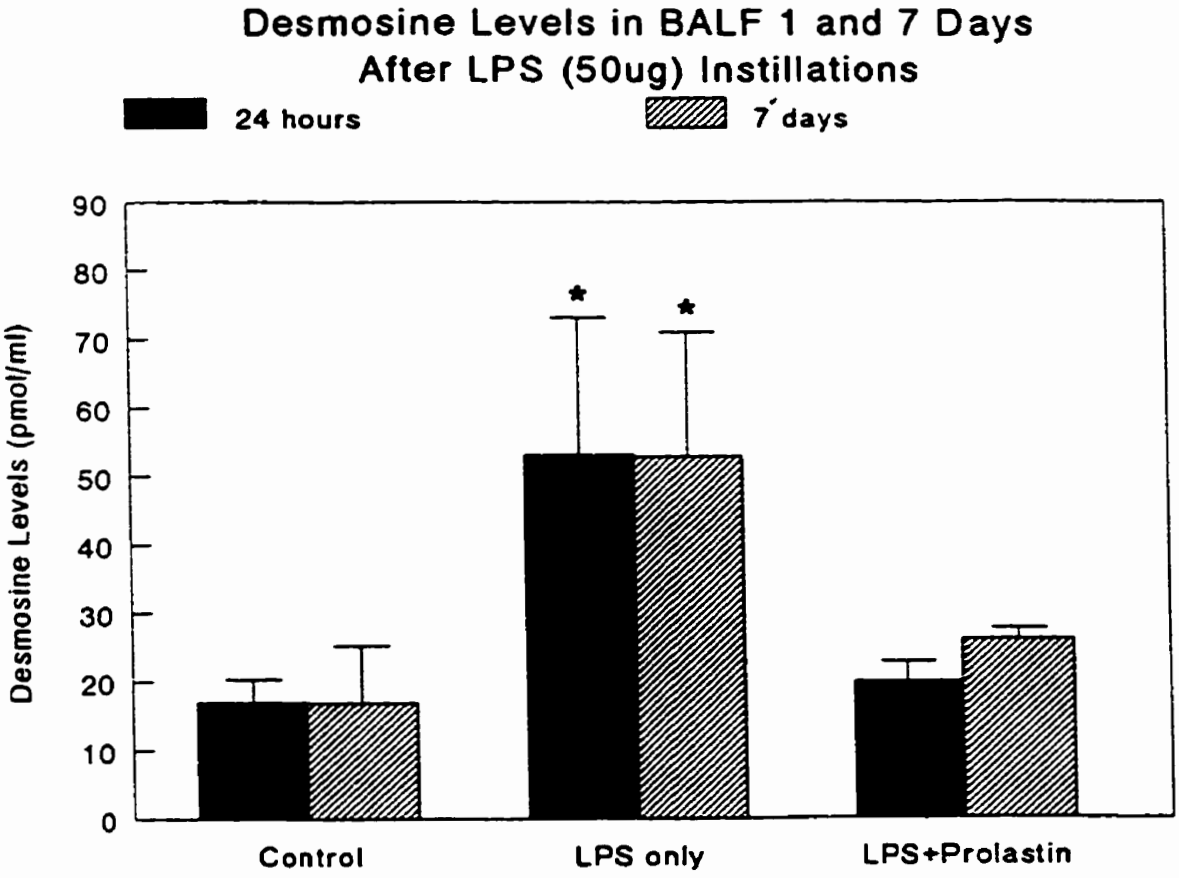


Figure 25. Lavage desmosine is increased 24 hours and 7 days after a single intratracheal instillation of 50 μg LPS compared to control mice receiving saline instillations. Administration of 20 mg Prolastin (purified human α1AT) 24 hours prior to the LPS instillation prevents the desmosine increase. Solid bars represent values at 24 hours and striped bars represent values at 7 days. N=5 for each group and values are expressed as mean ± SD. * indicates significantly greater than control.

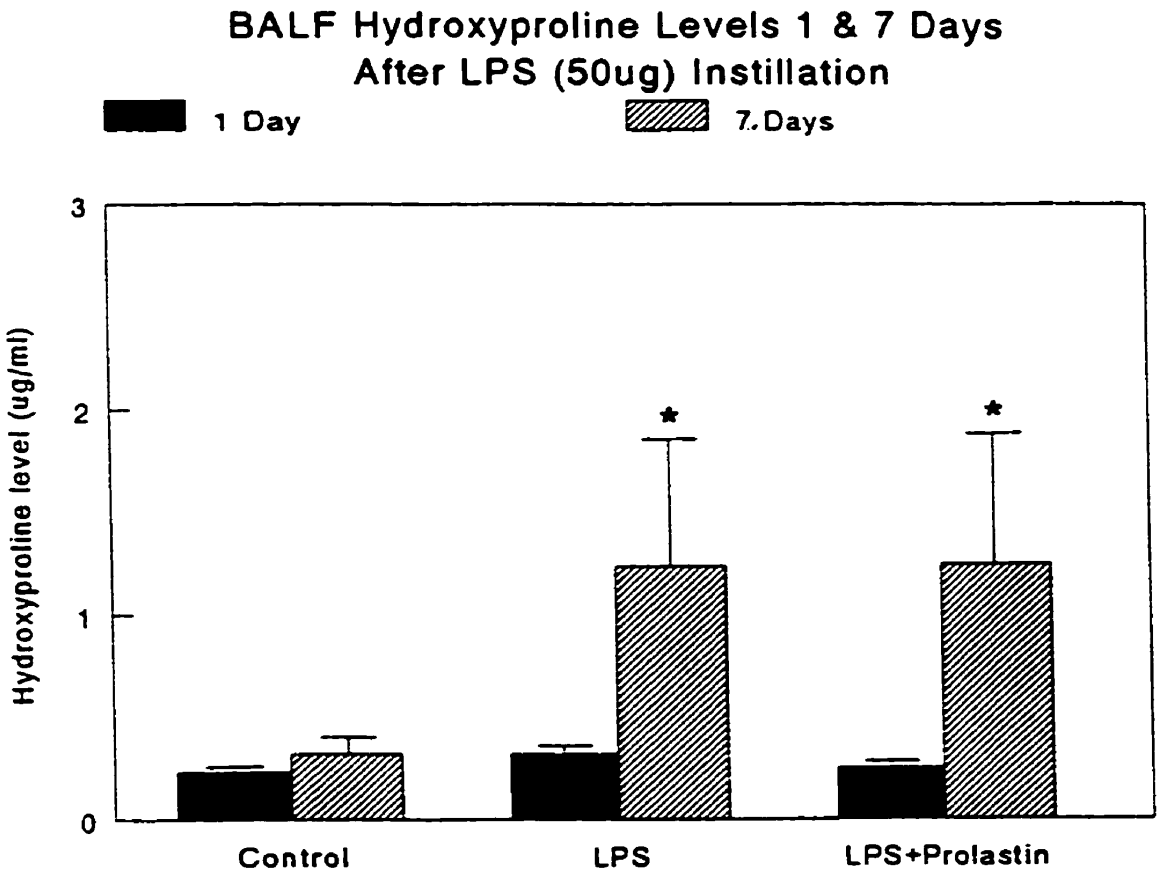


Figure 26. Lavage hydroxyproline is not increased 24 hours after a single intratracheal instillation of 50 μg LPS, but is significantly increased 7 days after the instillation compared to control mice receiving saline instillations. Administration of 20 mg Prolastin (purified human α1AT) 24 hours prior to the LPS instillation does not prevent the hydroxyproline increase at 7 days. Solid bars represent values at 24 hours and striped bars represent values at 7 days. N=5 for each group and values are expressed as mean ± SD. * indicates significantly greater than control.

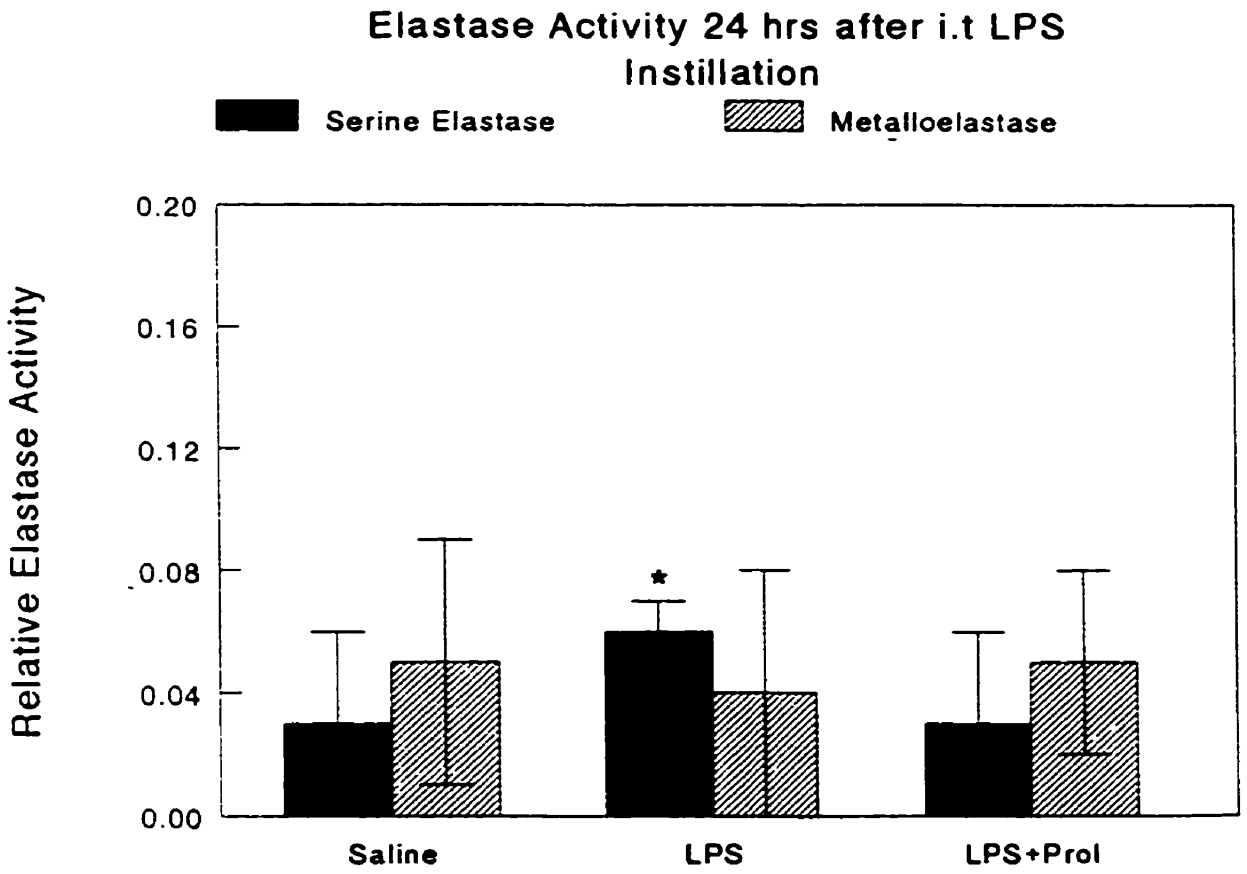


Figure 27. Elastase activities in the bronchoalveolar lavage fluid of mice 24 hours after receiving i.t. instillations of saline (control), LPS only, and LPS with prior Prolastin (purified human α1AT) administration. Solid bars represent serine elastase activity and striped bars represent metalloelastase activity. Activities are expressed in arbitrary units. N=5. Values are mean ± SD. * indicates significantly greater than control.

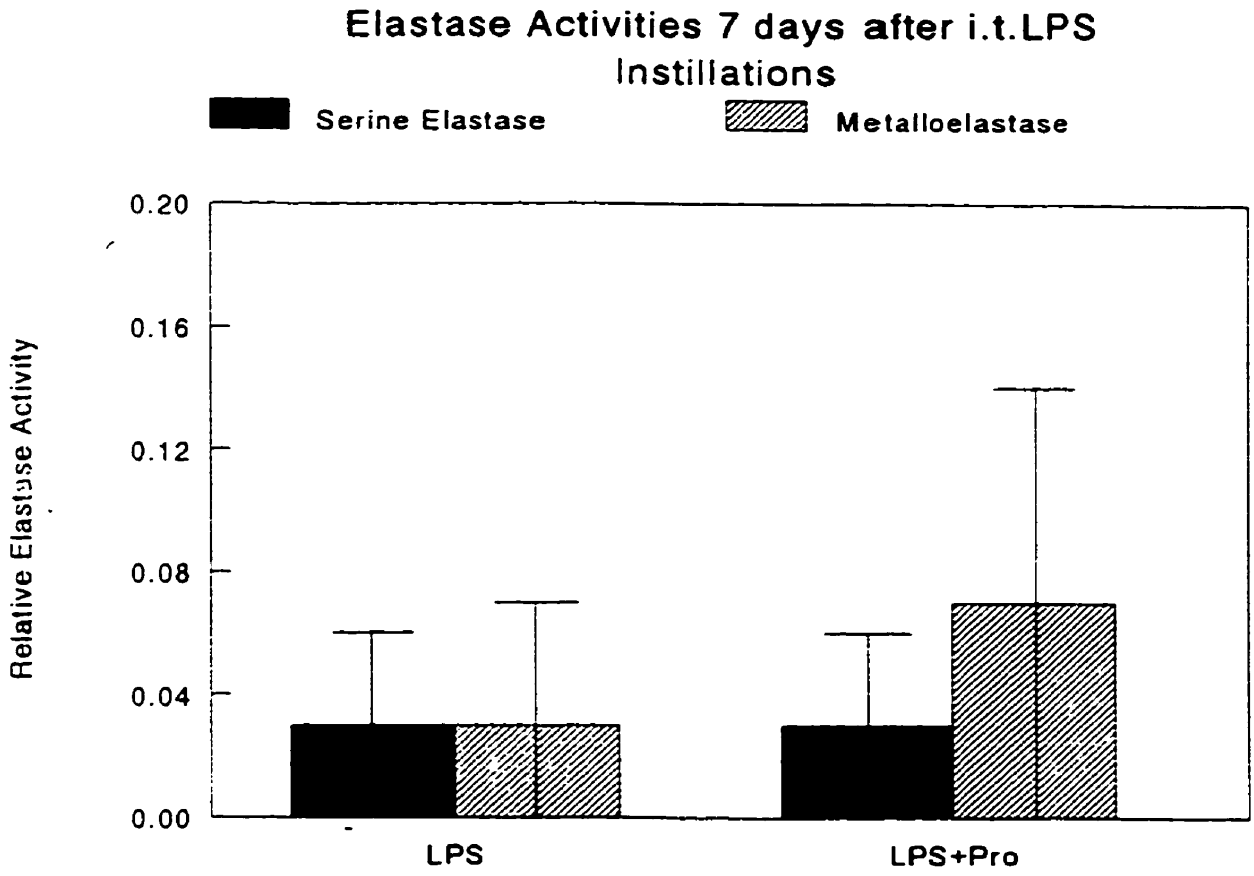


Figure 28. Elastase activities in the bronchoalveolar lavage fluid of mice 7 days after receiving i.t. instillations of LPS only, and LPS with prior Prolastin (purified human α1AT) administration. Control values are not plotted due to probable contamination of those samples. Solid bars represent serine elastase activity and striped bars represent metalloelastase activity. Activities are expressed in arbitrary units. N=5. Values are mean ± SD. **indicates significantly greater than control.

taken from these animals so assessment of possible pulmonary infection could not be done retrospectively.

8.1.5 Chronic LPS Experiment

The effects of 10 weeks of weekly LPS (25μg) instillations in transgenic and non-transgenic mice is shown and compared to age-matched control mice not receiving LPS in Table 6. There is a significant increase in mean linear intercept (Lm) in both transgenic and non-transgenic mice receiving LPS compared to the control transgenic group. Instillation of 25 μg of LPS causes a substantial increase in total lavage cell numbers (greater than 90% PMNs) after 24 hours, but this increase is less than that observed with instillation of the higher (50 μg) dose of LPS used for the acute experiments. Mice recovered from each instillation of LPS quickly and tolerated repeated instillations very well. Recovery time from the 50 μg dose was approximately 6-8 hours compared to 1-2 hours for the 25 μg dose of LPS. A total of 2 out of 5 mice from each of the SPAT3 and CD-1 treatment groups died at various time points throughout the experimental period. All deaths were a result of complications arising from the intratracheal instillations. Figure 30 shows light micrographs of transgenic CD-1 mice with and without (10 weeks) chronic i.t. LPS administration. In the mice receiving LPS there are large focal areas of obvious lung destruction which are not present in control CD-1 mice.

In order to determine whether the period required to induce pulmonary airspace enlargement in mice could be shortened, the above experiment was repeated with an experimental period of 8 weeks. A shorter experimental period would require fewer

injections of Protease in future experiments, thereby significantly reducing the associated expense. The effects of 8 weeks of weekly LPS instillations are shown in Table 7. There are significant differences between both the groups of transgenic mice and normal CD-1 mice. In many of the LPS treated mice in this experiment, however, there are some isolated areas of distinct airspace enlargement that are not present throughout the lung sections. This pattern is not observed in the control mice. At 8 weeks it appears that lung destruction has started to occur, but more impressive airspace enlargement is not evident until at least 10 weeks. These data underscore the progressive nature of the LPS-induced emphysema development.

Table 6. Morphometric Analysis of Transgenic (SPAT3) and Normal CD-1 Mouse Lungs After **10 weeks** of LPS Administration

	Transgenic Control (n=4)	Transgenic LPS (n=3)	CD-1 LPS (n=3)
Lm (μm)	58.5 ± 4.3	66.3 ± 5.8*	79.3 ± 8.8*

Lm = mean linear intercept
*differs significantly from control ($p < 0.05$)

Table 7. Morphometric Analysis of Transgenic (SPAT3) and Normal CD-1 Mouse Lungs After **8 weeks** of LPS

	Transgenic Control (n=5)	Transgenic LPS (n=5)	CD-1 Control (n=4)	CD-1 LPS (n=5)
Lm (μm)	59.3 ± 3.8	65.1 ± 1.7*	49.3 ± 4.7	55.6 ± 5.6*

Lm = mean linear intercept
*differs significantly from control ($p < 0.05$)

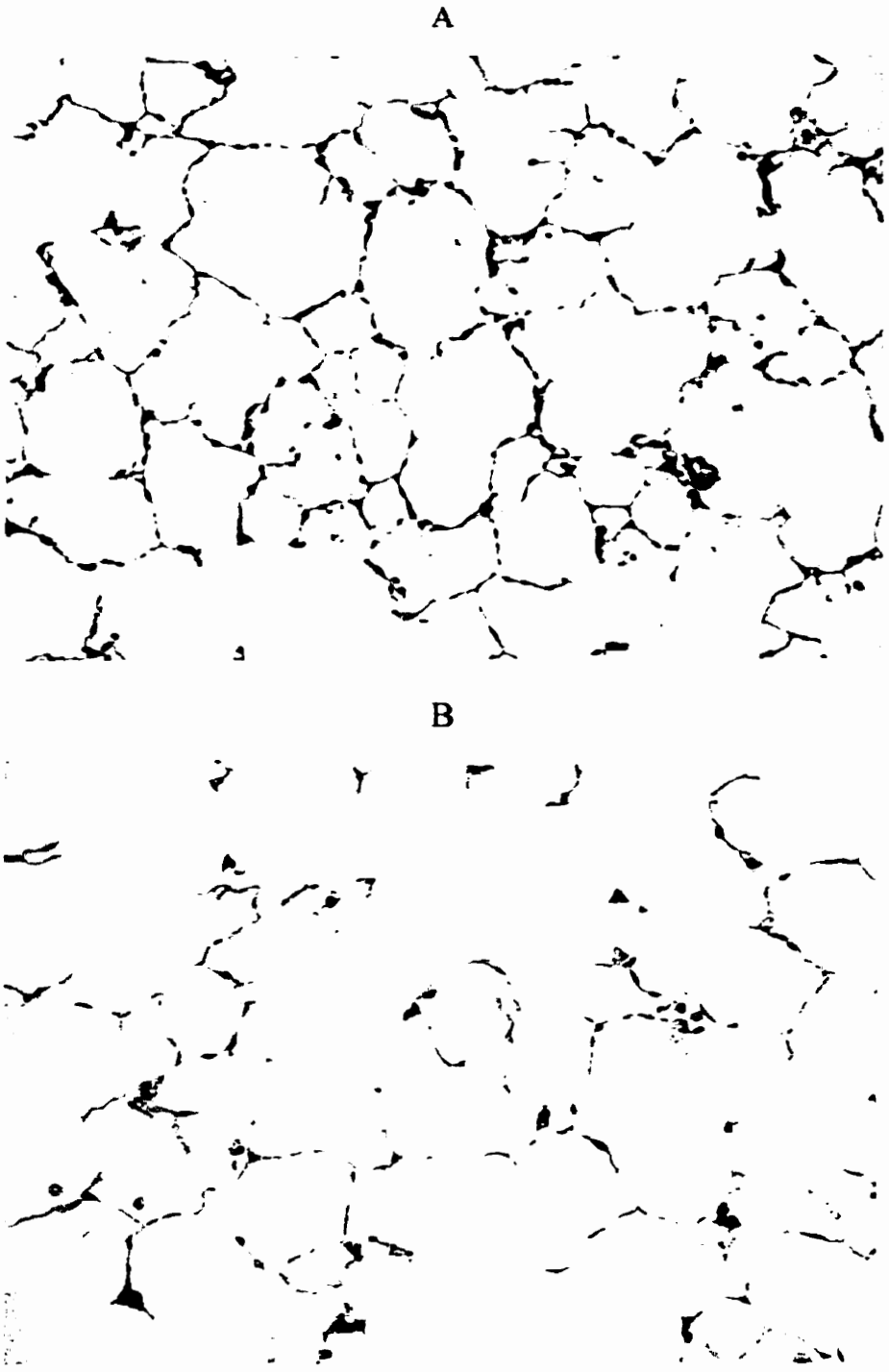


Figure 29. Photomigraphs of control CD-1 mice not receiving LPS (A) and CD-1 mice receiving weekly intratracheal instillations of LPS (B). Lungs are stained with hematoxylin and eosin. Both sections are photographed at the same magnification.

8.2 Discussion

8.2.1 Acute LPS Lung Injury

Lipopolysaccharide (LPS), or endotoxin, is a major component of the outer cell wall of gram-negative bacteria (Wicks et al., 1995). LPS has varied effects on different cell types and induces the release of inflammatory mediators from macrophages and T lymphocytes, stimulates T and B cell proliferation, stimulates B cell antibody production, and activates endothelial cells, natural killer cells, and PMNs (Wicks et al., 1995). Repeated intraperitoneal or intravascular administration of LPS in rats and dogs results in the sequestration of PMNs in the pulmonary microvasculature and alveolar wall destruction typical of emphysema (Blackwood et al, 1984, Guenter et al., 1981). Intratracheal administration of LPS into mice causes increased PMNs in the bronchoalveolar lavage fluid and the development of mild emphysema (Starcher & Williams, 1989). It has also been suggested that LPS-induced lung injury is a good model for adult respiratory distress syndrome (ARDS) because it leads to increased numbers of alveolar PMNs which are also typical of ARDS (Fowler et al., 1983). In this study, we have shown that acute intratracheal LPS administration in mice results in PMN influx and connective tissue breakdown which is prevented by α1AT.

Studies using intraperitoneal administration of LPS into mice show that although this leads to the sequestration of PMNs within the pulmonary vasculature, the cells do not migrate into the alveolar spaces (Hirano, 1996). This appears to be a species specific phenomenon as transinterstitial infiltration of PMNs in response to i.p. LPS does occur in rats and dogs (Blackwood et al., 1985, Guenter et al., 1981). Evidence of acute lung injury in mice as a result of intraperitoneally injected LPS has not been documented

(O'Malley et al., 1998). Intratracheal administration of LPS, on the other hand, results in recruitment of inflammatory cells, particularly PMNs, into the alveolar airspaces with subsequent lung injury. We have used this model of LPS-induced lung injury to demonstrate acute pulmonary connective tissue breakdown after a single LPS instillation.

Previous studies have demonstrated that intratracheal instillation of LPS into the lungs of mice results in an influx of PMNs into the lungs starting within 4 hours and remaining elevated for at least 48 hours (Starcher & Williams, 1989a). We have extended this observation to 7 days. Our studies show that BALF cell numbers are elevated after 24 hours, with PMNs making up greater than 95% of the cellular infiltrate (Table 5). The total cell numbers remain elevated at 7 days after the single LPS instillation, but at this time PMNs make up approximately 50% of the total cells and macrophages make up the remaining 50%. The connective tissue degradation data shows that elastin breakdown (indicated by desmosine levels) starts at 24 hours, coinciding with PMN increases in the airspaces and continues unabated for 7 days (Figure 25). This elastin destruction can be prevented with the prior administration of α1AT (Protease®). Collagen breakdown, however, (indicated by hydroxyproline levels) is only apparent after 7 days (Figure 26), correlating with increased numbers of BALF macrophages, and the degradation is unaffected by α1AT administration. This result is different from that observed with the acute smoke experiments (see Chapter 7) suggesting different proteolytic mechanisms in the two types of lung injuries. The LPS-induced inflammatory response consisting of first PMNs, and then macrophages has been previously reported in rats. Rinaldo et al. (1984) demonstrated that a single intraperitoneal injection of endotoxin in rats results in increased recovery of PMNs from the BALF starting at 24 hours and returning to control

levels by 6 days while macrophage numbers did not begin to increase until 6 days after the instillation. In our experiments, the dissociation between BALF hydroxyproline increases and PMN levels suggests that the collagen breakdown is not mediated by PMN proteases (such as 72-kDa gelatinase) and is more likely a result of macrophage metalloproteases such as MMP-1,-2,-3,-9,-10,-11 which all have collagenolytic activity (Owen & Campbell, 1999) or collagenases produced by connective tissue cells (Murphy & Docherty, 1992). Lung macrophages are also a source of potent cysteine proteases which are capable of degrading collagen, but are not inhibited by α1AT (Chapman et al., 1994, Burnett et al., 1983). Increased levels of the cysteine protease cathepsin B have been reported in the sputum from patients with acute respiratory infections (Burnett et al., 1983). The fact that hydroxyproline levels remained elevated at 7 days even in animals having received α1AT rules out the possibility of collagenolytic activity from a serine protease being responsible for the increase. Increased collagenase activity, mostly from metalloproteases, was measured in lavage recovered from patients with ARDS and persisted for greater than 3 weeks in some of the patients (Christner et al., 1985).

Since α1AT is a potent serine protease inhibitor and cannot inhibit metalloproteases, the elastin degradation is most likely caused by PMN serine elastases such as neutrophil elastase, cathepsin G, and proteinase 3. Measurements of elastase activity support this view. Lavage serine elastase activity is increased in LPS-treated animals at 24 hours and is returned to control levels in mice receiving Protease (Figure 27). There are no significant changes in metalloelastase activity in mice receiving LPS compared to mice receiving both Protease and LPS or to control mice. This is similar to a study with hamsters receiving intratracheal LPS instillations followed by FMLP in which

there was increased lavage serine elastase-like activity, but not metalloelastase activity after 24 hours (Mitsuhashi et al., 1997). Also in this study, administration of recombinant human SLPI or human α1AT attenuated the acute lung injury, as assessed by reduction of pulmonary hemorrhage, compared to control mice, with α1AT showing the more impressive results.

Increased urinary desmosine excretion has been associated with acute pulmonary injury in humans (Starcher et al. 1995). Individuals with ARDS were shown to have ten-fold higher urinary desmosine levels than control patient groups (Tenholder et al., 1991) and cystic fibrosis patients had 2 to 3 fold higher urinary markers for elastin and collagen breakdown products than control subjects (Stone et al., 1995b). Both conditions are associated with increased alveolar PMNs and/or Neutrophil elastase levels in many reported studies (Allen, 1996, Canonico et al., 1996, Khan et al., 1995, Donnelly et al., 1995). Other investigations, however, have not confirmed these results (Petty et al., 1991, Gadek & Pacht, 1996). It has been postulated that in both ARDS and CF the lung damage is a result of increased elastase activity released from pulmonary PMNs.

In CF a contribution to the increased proteolytic activity may also occur from elastases released from *Pseudomonas aeruginosa* (Starcher et al., 1995). The increased elastase levels in CF patients may arise as a result of insufficient elastase inhibitor function or overwhelming of inhibitors by the amount of elastase released from PMNs. The issue of whether α1AT in CF lungs is inactivated is controversial, but recent studies suggest that the increased elastase activity can be explained by elevated levels of NE present (O'Connor et al., 1993, Ying et al., 1996, reviewed in Allen, 1996, Britigan et al., 1999). In an effort to restore the protease-antiprotease balance in CF lungs, natural and

synthetic inhibitors of NE, including α1AT, are being tested in patients and show promising results (McElvaney et al., 1991, Stone et al., 1995b, Birrer, 1995).

In vitro and in vivo work shows that LPS directly activates PMNs to release elastase and produce an enhanced respiratory burst. The recruitment of PMNs to the lungs is mediated by macrophages, which rapidly produce TNF and IL-1 in response to LPS. TNF also directly stimulates elastase release by PMNs, and TNF and IL-1 induce the expression of adhesion receptors on the surface of the endothelium to facilitate PMN binding. The cytokines leukotriene B4, platelet-activating factor, and complement fraction C5a have all been associated with endotoxemia and may be involved in the induction of endothelial receptors on PMNs (reviewed in Welbourn & Young, 1992).

Prospective treatment of ARDS may be aimed at arresting the host's inflammatory response which could involve inhibiting cytokines and inflammatory mediators thereby limiting recruitment of inflammatory cells into the lungs. An alternative approach would be prevention of PMN-mediated injury (Welbourn & Young, 1992). PMN-derived mediators of lung injury may be oxygen free radicals and/or proteolytic enzymes. If proteolytic enzymes are mediators of lung injury in ARDS, then once again treatment with protease inhibitors may be a feasible form of therapy (Campbell & Cone, 1991). Rats with experimental α1-antitrypsin deficiency developed greater lung damage after systemic chronic endotoxin exposure than rats without α1-antitrypsin deficiency (Blackwood et al., 1984) suggesting that the release of neutrophil elastases during the acute inflammatory response is responsible for the pulmonary connective tissue degradation.

Conclusion: Our results support the postulate that serine elastases, inhibitable by α1AT, are responsible, at least in part, for endotoxin-induced acute lung injury, but collagenolytic metalloproteases, which are not inhibited by α1AT also contribute to the overall lung destruction.

8.2.2 A model of LPS-mediated emphysema

The importance of creating an animal model of emphysema has been discussed in Chapter 3. In order to test the efficacy of α1AT augmentation therapy in preventing or halting disease development, an animal model in which emphysema develops progressively is required. As mentioned already, a model of cigarette smoke-mediated emphysema would be the ideal situation, and an attempt was made in our laboratory to develop such a model. Strain CD-1 (background of transgenic mice) and C57-BL/6J mice were exposed to 2 cigarettes daily (5 days a week) in our laboratory for 8 months. Mild airspace enlargement was observed in both strains compared to control groups (JL Wright, personal communication). There was a suggestion from the data that the smoke-exposed C57-BL/6J mice had very slightly increased mean linear intercepts (although not significant) compared to the CD-1 mice. This is interesting from the point of view that measurements in our laboratory and others show that C57-BL/6J mice have lower serum levels of α1AT compared to CD-1 mice (approximately 20-30% lower). This suggests that variations in endogenous α1AT levels may affect the susceptibility of different mouse strains to cigarette smoke-induced lung injury. Our results, as well as, the previous demonstration of cigarette smoke-induced airspace enlargement in 129/svJ mice, which have plasma α1AT levels similar to that of C57-BL/6J mice, (Hautamaki et al.,

1997) indicates that a mouse model of cigarette smoke-induced emphysema is possible. Experience in our laboratory suggests that subtle variations in the smoking protocol involving delivery of smoke and flow in the fume hood affect the outcome of the long-term smoking experiments suggesting that we are at the threshold for producing detectable emphysematous lesions in mice exposed to cigarette smoke. Success in achieving a model of chronic cigarette smoke-induced emphysema may simply be a matter of determining the optimal conditions for smoke exposure.

As an alternative to cigarette smoke-induced emphysema, we have developed a model of chronic lung injury mediated by intratracheal LPS instillation using the human α1AT transgenic mice. Ten and 8 weeks of LPS administration resulted in histological changes in the lungs with areas of lung destruction and increased cellular infiltrate. The latter infiltrate mostly resolves within a week of the last instillation, in both transgenic and normal CD-1 mice receiving LPS. Repeated intraperitoneal or intravenous administration of LPS has been reported to cause airspace enlargement in rats (Blackwood, 1984), dogs (Guenter et al., 1981) and rhesus monkeys (Klut et al., 1996). Intravascular sequestration of PMNs was present in all of these models and our acute LPS experiments discussed above demonstrated increased elastolytic and collagenolytic activity after LPS instillation. Chronic endotoxemia, however, was not associated with emphysematous lung lesions in rabbits receiving 2-3 intravenous injections of LPS weekly over a period of 15 to 28 weeks (Klut et al., 1996). This may be a reflection of interspecies differences in either responses to LPS or protease-antiprotease balance in the lungs, making it essential that the development of airspace enlargement with chronic endotoxemia be demonstrated in our transgenic mice strain (CD-1 background), even

though LPS instillation has already been shown to be a feasible method of causing emphysematous lesions in C57-BL/6J mice (Starcher & Williams, 1989b). The route of administration may also be an important factor in the outcome of the LPS-mediated injury. Direct instillation of LPS into the lungs is a more direct method of administration compared to i.p. injection and may limit the effects of LPS to the lungs.

The disparity among the individual animals instilled with LPS (reflected in the large standard deviations of groups) and the patchiness of the lung destruction we observed is most likely a reflection of the variability of delivery and retention of the LPS. The method of intratracheal instillation does not ensure even dispersal of the LPS in the lungs. The inflammatory response was the same after each instillation of LPS, however, with lavage PMN numbers beginning to increase by 4 hours and remaining elevated for 4 to 5 days (Starcher & Williams, 1989 and personal observations).

It has been assumed in the LPS models described above that the lung lesions are a direct result of the accumulation of PMNs in the lungs, thus, obviating the role of macrophages in this process (Guenter et al., 1981, Starcher & Williams, 1989, Klut et al., 1996). Studies have shown that depletion of PMNs in animal models prevents the LPS-associated lung injury (Welbourn & Young, 1992). As discussed in this thesis, there is compelling evidence that macrophages are involved in the pathogenesis of cigarette smoke-mediated emphysema. Macrophages are also increased in the lungs after LPS instillation, although not to the same extent as PMNs (see above) and there is collagenolytic activity present 7 days after LPS instillation which is probably due to macrophage protease(s). Thus, the tissue damage secondary to chronic LPS exposure is more complex than simple NE-induced injury, with both PMNs and macrophages

probably contributing to connective tissue breakdown, and in this regard is similar to cigarette smoke-induced emphysema.

The chronic LPS animal model of emphysema described here is not ideal, but has several desirable qualities. First, significant airspace enlargement (increased Lm) is present at 8 and 10 weeks compared to the mouse cigarette smoke model which requires 8 months. This not only shortens the experimental period, but dramatically decreases the cost associated with the purified human α1AT (Protease) augmentation therapy. Second, unlike studies that involve instilling specific elastases into the lungs of animals, the premise of which is that increased elastolytic capacity in itself is directly responsible for the airspace enlargement, the LPS instillation model does not require this assumption. LPS injury may be caused by any of the PMN or macrophage proteases or by production of oxidizing agents or both. The fact that collagen breakdown, not inhibited by α1AT, is also occurring (discussed in acute LPS experiments section) is interesting and provides the opportunity to test the efficacy of α1AT augmentation in preventing the formation of emphysematous lesions in a model in which the mechanism of pathogenesis is complex, a situation more similar to that of cigarette smoke-mediated emphysema. Also, with the LPS models, physiological concentrations of the effector agents (that is, PMNs and macrophages) are present, whereas, with protease instillation models artificially large quantities of elastases were instilled into animals, a characteristic which is not relevant to human disease.

The LPS model has a limited ability to answer questions about pulmonary emphysema, but it is also relevant for other pulmonary diseases associated with lung injury including ARDS and cystic fibrosis. The chronic LPS-mediated lung injury model

described here can be used to address specific questions related to aspects of the disease considered to be associated with lung PMN accumulation. It is also appropriate for testing the efficacy of α1AT augmentation treatment in preventing or ameliorating pulmonary airspace enlargement secondary to serine proteases, in a model where they contribute to the lung injury, but are not necessarily the only mediators of lung damage. This is important because if α1AT can be shown to be effective in halting disease progression despite the complexity of the pathogenesis of LPS-induced disease, it will support testing the therapeutic benefits of α1AT in other settings in which several cell types and cellular products are present, as in cigarette smoke-induced emphysema. The general belief is that the primary role of a specific inflammatory cell and/or protease must be irrefutably established (Eidelman et al., 1990, Finkelstein et al., 1996, Hautamaki et al., 1997) before a treatment for cigarette smoke-induced emphysema can be considered, but **this may not be necessary if the benefit of a naturally occurring or synthetic antiprotease can be demonstrated in an animal model of emphysema.**

9. Summary and Conclusions

In this thesis we have hypothesized that α1AT can protect against the development or progression of cigarette smoke mediated emphysema, a major cause of death in the world today. α1AT is the major inhibitor of the serine protease neutrophil elastase released from PMNs that are a significant component of the pulmonary inflammatory response which can be evoked in mice by cigarette smoke exposure and intratracheal LPS instillation. We have used two approaches to determine the efficacy of α1-antitrypsin augmentation therapy in protecting against cigarette smoke and LPS-mediated lung injury. The first technique was to investigate the potential for gene therapy to increase local production of α1AT in the lungs and protect against cigarette smoke-induced emphysema. To advance this effort, we created and characterized four transgenic mouse lines with expression of the human α1AT gene directed by lung-specific promoters to determine if local production of the protein is effective in preventing pulmonary connective tissue destruction progressing to the development of emphysema. In all four transgenic lines, expression of α1AT was limited to the lung and present in both parenchymal and airway cells. Unfortunately, the protein was produced in unacceptably low concentrations, although it was detectable in the pulmonary interstitium, the site believed to be most susceptible to destruction by proteolytic enzymes released from inflammatory cells. **These transgenic mice provide evidence that α1AT gene therapy directed to the lung alveolar type II or respiratory epithelial cells will result in the production of protein and its movement into the pulmonary interstitium where its anti-proteolytic activity may be most beneficial.** Although,

these mice could not be used directly to test the hypothesis that pulmonary over-expression of α1AT protects against the development of cigarette smoke-induced emphysema, they are useful for experiments involving the chronic administration of commercially available human α1AT because they are immunologically tolerant to this human protein.

The second approach used to examine the potential effectiveness of increased α1AT levels in preventing emphysema, was to look at its role in acute lung injury associated with cigarette smoke exposure and intratracheal LPS instillation. First, it was established that acute cigarette smoke exposure and a single LPS instillation cause significant pulmonary connective tissue degradation. Further investigation revealed that elastin and collagen breakdown (in the cigarette smoke model) and elastin breakdown (in the LPS model) was associated with increased numbers of alveolar airspace PMNs and serine elastase activity. Intraperitoneal administration of purified human α1AT prior to cigarette smoke exposure completely prevented the connective tissue breakdown and ameliorated the LPS-mediated connective tissue degradation. These experiments support the concept of the protease-antiprotease imbalance being responsible for lung injury associated with acute pulmonary disorders and the early events in cigarette smoke-mediated emphysema development. The acute smoke experiments implicate NE as the primary protease responsible for connective tissue breakdown in cigarette smoke-induced acute lung injury, however, the acute LPS experiments suggest that additional proteases, not inhibitable by α1AT, are involved in this type of lung injury.

In summary, the above experiments establish that increasing α1AT levels in mouse models of acute lung injury protects against pulmonary connective tissue

degradation and that both of the lung cell-specific promoters SP-C ond CC10 can be used to deliver the human α1AT gene to the pulmonary interstitium. The transgenic mice are immunologically tolerant to human α1AT and, therefore, provide a model for chronic α1AT augmentation treatment. Furthermore, weekly intratracheal instillations of LPS into the transgenic mice were shown to cause regions of airspace enlargement characteristic of emphysema. Together, these studies provide a basis for long-term investigations to determine the efficacy of α1AT augmentation in the development of cigarette smoke or LPS-mediated emphysema in a mouse model.

10. References

Abboud, R., Fera, T., Richter, A., Tabona, M., Johal, S. 1985. Acute effect of smoking on the functional activity of α1-protease inhibitor in bronchoalveolar lavage fluid. Am. Rev. Respir. Dis. 131: 79-85.

Abrams, W., Cohen, A., Damiano, V., Eliraz, A. Kimbel, P., Meranze, D. 1981. J. Clin. Invest. 61: 1132-1139.

Afford, S., Burnett, D., Campbell, E., Cury, J., Stockley, R. 1988. The assessment of α1-proteinase inhibitor form and function in lung lavage fluid from healthy subjects. Clin. Chem. Acta. 369: 1065-1073.

Aksamit, T.R., Hunninghake, G.W. 1993. Interleukin-1. In: Cytokines of the Lung. Volume 61. Ed. J. Kelley. Marcel Dekker, Inc. New York.

Alino, S. 1997. Long-term expression of the human α-1-antitrypsin gene in mice employing anionic and cationic liposome vector. Biochem. Pharmacol. 54(1): 9-13.

Allen, E. 1996. Opportunities for the use of aerosolized α1-antitrypsin for the treatment of cystic fibrosis. Chest. 110: 256S-260S.

The Alpha-1-Antitrypsin Deficiency Registry Study Group. 1998. Survival and FEV1 decline in individuals with severe deficiency of α1-antitrypsin. Am. J. Respir. Crit. Care Med.158: 49-59.

Ausubel, F., Brent, R., Kingston, Moore, D., Seidman, J., Smith, J., Struhl, K.(eds). 1997. Current Protocols in molecular biology. John Wiley & sons, Inc.

Barker, A., Iwata-Morgan, I., Oveson, L., Roussel, R. 1997. Pharmacokinetic study of α1-antitrypsin infusion in α1-antitrypsin deficiency. Chest. 112: 607-613.

Bensi, G., Raugei, Klefenz, H., Cortese, R. 1985. Structure and expression of the human haptoglobin locus. EMBO J. 4(1): 119-126.

Betsuyaku, T., Yoshioka, A., Nishimura, M., Miyamoto, K., Kondo, T., Kawakami, Y. 1994.Neutrophil elastase associated with alveolar macrohages from older volunteers. Am. J. Respir. Crit. Care Med. 151: 436-432.

Betsuyaku, T., Nishimura, M., Yoshioka, A., Takeyabu, K., Miyamoto, K., Kawakami, Y. 1996. Elastin-derived peptides and Neutrophil elastase in bronchoalveolar lavage fluid.
. Am. J. Resp. Crit. Care Med. 154: 720-724.

Bieth, J. Elastase. In: Regulation of matrix accumulation. 1986. ed. Mecham, R.P. Academic Press. Orlando.

Bingle, L., Tetley, T., 1996. Secretory leukoprotease inhibitor: partnering α1-proteinase inhibitor to combat pulmonary inflammation. Thorax. 51: 1273-1274.

Birrer, P., McElvaney, N., Gillissen, A., Hoyt, R., Bloedown, D., Hubbard, R., Crystal, R. 1992. Intravenous recombinant secretory leukoprotease inhibitor augments antiNeutrophil elastase defense. J. Appl. Physiol. 73(1): 317-323.

Blackwood, R.A., Moret, J. Mandl, I., Turino, G.M. 1984. Emphysema induced by intravenously administered endotoxin in an alpha1-antitrypsin-deficient rat model. Am. Rev. Respir. Dis. 130: 231-236.

Blau, H., Khavari, P. 1997. Gene Therapy: progress, problems, prospects. Nat. Med. 3(6): 612-613.

Bless, N., Smith, D., Charlton, J., Czermak, B., Schmal, H., Friedl, H., Ward, P. 1997. Protective effects of an aptamer inhibitor of Neutrophil elastase in lung inflammatory injury. Curr. Biol. 7(11): 877-880.

Blue, M., Janoff, A. 1978. Possible mechanisms of emphysema in smokers: release of elastase from human polymorphonuclear cells by cigarette smoke condensate in vitro. Am. Rev. Respir. Dis. 117: 317-325.

Bosken, C., Hards, J., Gatter, K., Hogg, J. 1992. Characterization of the inflammatory reaction in the peripheral airways of cigarette smokers using immunocytochemistry. Am. Rev. Respir. Dis. 145: 922-917.

Boudier, C., Pelletier, A., Pauli, G., Bieth, J. 1983. The functional activity of α1-proteinase inhibitor in bronchoalveolar lavage fluids from healthy human smokers and non-smokers Clin. Chim. Acta. 132: 309-315.

Brandt, E.J., Swank, R.T., Nowak, E.K. 1981. The murine Chediak-Higashi mutation and other murine pigmentation mutations. In: Immunological defects in laboratory animals. Eds. Gershwin, M.E., Merchant, B. New York: Plenum Press. Pp. 99-117.

Brantly, M., Nukiwa, T., Crystal, R. 1988. Molecular basis of alpha-1-antitrypsin. Am. J. Med. 84(suppl 6A): 13-31.

Briet, S., Wakefield, D., Robinson, J., Luckhurst, E., Clark, P., Penny, R. 1985. The role of α1-antitrypsin deficiency in the pathogenesis of immune disorders. Clin. Immun. Immunopath. 35: 363-380.

Britigan, B., Railsback, M., Cox, C. 1999. The Pseudomonas aeruginosa secretory product pyocyanin inactivates alpha(1) protease inhibitor: implications for the pathogenesis of cystic fibrosis lung disease. Infect. Imm. 67(3): 1207-1212.

Brumwell, M., MacNee, W., Doerschuk, C., Wiggs, B., Hogg, J. 1991, in Pulmonary Emphysema: The Rationale for Therapeutic Intervention. Eds. Weinbaum, G., Giles, R., Krell, R., New York Academy of Sciences.

Buhl, R., Meyer, A., Vogelmeier, C. 1996. Oxidant-protease interaction in the lung. Chest. 110:267S-272S.

Burnett, D., Crocker, J., Stockley, R.A. 1983. Cathepsin B-like cysteine proteinase activity in sputum and immunohistologic identification of cathepsin B in alveolar macrophages. Am. Rev. Respir. Dis. 128: 915-919.

Burnett, D., Afford, S., Campbell, E., Rios, Mollineda, R., Buttle, D., Stockley, R. 1988. Evidence for lipid-associated serine proteases and metalloproteases in human bronchoalveolar lavage fluid. Clin. Sci. 75: 601-607.

Campbell, G.S. & Cone, J.B. 1991. Adult respiratory distress syndrome. Am. J. Surg. 161: 239-243.

The Ad Hoc Committee on Alpha-1-antitrypsin Replacement Therapy of the Standards Committee, Canadian Thoracic Society. Current status of α1AT replacement therapy: recommendations for the management of patients with severe hereditary deficiency. 1992. CMA Journal. 146: 841-844.

Canonico, A., Conary, J., Meyrick, B., Brigham, K. 1994. Aerosol and intravenous transfection of human α1-antitrypsin gene to lungs of rabbits. Am. J. Respir. Cell Molec. Biol. 10: 24-29.

Canonico, A., Brigham, K., Carmichael, L., Plitman, J., King, G., Blackwell, T., Christman, J. 1996. Plasmid-liposome transfer of the α1-antitrypsin gene to cystic fibrosis bronchial epithelial cells prevents elastase-induced cell detachment and cytokine release. Am. J. Resp. Cell Mol. Biol. 14 (4): 348-355.

Canonico, A., Brigham, K. 1997. Biology of acute lung injury. In: The lung, second edition. Eds Crystal, R. et al. Lippincott-Raven, Philadelphia. pp 2475-2498.

Cardoso, W.V., Sekhon, H.S., Hyde, D.M., Thurlbeck, W.M. 1993. Collagen and elastin in human pulmonary emphysema. Am. Rev. Respir. Dis. 147: 975-981.

Carlson, J., Rogers, B., Sifers, R., hawkins, H., Finegold, M., Woo, S. 1988. Multiple tissues express alpha1-antitrypsin in transgenic mice and man. J. Clin. Invest. 82: 26-36.

Carolan, E., Casale, T. 1996. PMN transepithelial migration is dependent upon epithelial characteristics. Am. J. Respir. Cell Mol. Biol. 15: 224-231.

Carolan, E., Mower, D., Casale, T. 1997. Cytokine-induced PMN transepithelial migration is dependent upon epithelial orientation. Am. J. Respir. Cell Mol. Biol. 17: 727-732.

Carp, H., Miller, F., Hoidal, J. et al. 1982. Alpha-1-antiproteinase inhibitor purified from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity. Proc. Nat. Acad. Sci. USA. 79: 2041-2045.

Cavarra, E., Martorana, P., Gambelli, F., de Santi, M., van Even, P., Lungarella, G., 1996. PMN recruitment into the lungs is associated with increased lung elastase burden, decreased lung elastin, and emphysema in α1 proteinase inhibitor-deficient mice. Lab. Invest. 74(2): 273-280.

Cavarra, E., Martorana, P., de Santi, M., Bartalesi, B., Cortese, S., Gambelli, F., Lungarella, G. 1999. PMN influx into the lungs of beige mice is followed by elastolytic damage and emphysema. Am. J. Respir. Cell Mol. Biol. 20: 264-269.

Cendon, S.P., Battlehner, C., Lorenzi Filho, g., Dohlnikoff, M., Pereira, P.M., Conceicao, G. M., Beppu, O.S., Saldiva, P.H. 1997. Pulmonary emphysema induced by passive smoking: an experimental study in rats. Braz. J. Med. Biol. Res. 30: 1241-1271.

Chang, J., Lesser, M. 1984. Quantitation of leukocytes in bronchoalveolar lavage sample from rats after intravascular injection of endotoxin. Am. Rev. Respir. Dis. 129: 72-75.

Chapman, H.A., Stone, O.L. 1984. Comparison of live human PMN and alveolar macrophage elastolytic activity in vitro. J. Clin. Invest. 74: 1693-1700.

Chapman, H. A., Munger, J.S., Shi, G-P. 1994. The role of thiol proteases in tissue injury and remodeling. Am. J. Resp. Crit. Med. 150(6): S155-S159.

Christner, P., Fein, A., Goldberg, S., Lippmann, M., Abrams, W., Weinbaum, G. 1985. Collagenase in the lower respiratory tract of patients with adult respiratory distress syndrome. Am. Rev. Respir. Dis. 131: 690-695.

Churg, A., Hobson, J., Wright, J. 1989. Functional and morphologic comparison of silica- and elastase-induced airflow obstruction. Exp. Lung Res. 15: 813-822.

Cocharane, C. 1988. Alpha-1-proteinase inhibitor in inflammatory states of humans and laboratory animals. Am. J. Med. 84(suppl 6A): 75-79.

Cohen, A., Girard, W., McLarty, J., Starcher, B., Davis, D., Stevens, M., Rosenbloom, J., Kucich, U. 1991. Reduction of Neutrophil elastase load in the lungs of patients with emphysema by reducing PMN enzyme secretion or chemotaxis. Ann. N.Y. Aca. Sci. 624: 244-256.

Colau, B., Chuchana, P., Bollen, A. 1984. Revised sequence of full-length complementary DNA coding for human α1-antitrypsin. DNA. 3 (4): 327-330.

Cook, L., Burdon, J.G., Brenton, S., Knight, K.R., Janus, D.D. 1996. Kinetic characterisation of α-1-antitrypsin F as an inhibitor of human Neutrophil elastase. Pathology. 28: 242-247.

Crespo, J., Blaya, C., Crespo, A., Alino, S. 1996. Long-term expression of the human α1-antitrypsin gene in mice employing anionic and cationic liposome vectors. Biochem. Pharmacol. 51(10): 1309-1314.

Crystal, R. 1990. α1-antitrypsin deficiency, emphysema, and liver disease. J. Clin. Invest. 85: 1343-1352.

Crystal, R. 1992. Gene therapy strategies for pulmonary disease. Am. J. Med. 92 (suppl 6A): 44S-51S.

Culver, K.W. Gene Therapy: A Handbook for Physicians. 1994 by Mary Ann Liebert Inc. New York.

Curiel, D. 1997. Gene-based therapies for inherited and acquired disorders of the lung. Chest. 111(6): 149S-152S.

Damiano, V., Tsang, A., Kucich, U., Abrams, W., Rosenbloom, J., Kimbel, P., Fallahnejad, M., Weinbaum, G. 1986. Immunolocalization of elastase in human emphysematous lungs. J. Clin. Invest. 78: 482-493.

D'Armiento, J., Dalal, S., Okada, Y., Berg, R., Chada, K. 1992. Collagenase expression in the lung of transgenic mice causes pulmonary emphysema. Cell. 71: 955-961.

Davies, S., Offord, K., Brown, M., Campe, H., Niewoehner, D. 1983. Urine desmosine is unrelated to cigarette smoking or to spirometric function. Am. Rev.Respir. Dis. 1983: 473-475.

Degryse, E., de Santi, M., Dietrich, M., Ali Hadji, D., Spetz, J., Villeval, D., Lungarella, G. 1996. A human SP-C promoter fragment targets α1-protienase inhibitor gene expression to lung alveolar type II cells in trangenic mice. Transgenic Res. 5: 139-143.

D'Ortho, M., Clerici, C., Yao, P., Delcourt, C., Delcaux, C., Fronco-Montoya, M., Harf, A., Lafuma, C. 1997. Alveolar epithelial cells in vitro produce gelatinase and tissue inhibitor of matrix metalloproteinase-2. Am. J. Physiol. 273: L663-L675.

de Santi, M., Martorana, P., Cavarra, E., Lungarella, G. 1995. Pallid mice with genetic emphysema. Lab. Invest. 73(1): 40-47.

Dillon, T., Walsh, R., Scicchitano, R., Eckert, B., Cleary, E., Mclennan, G. 1992. Plasma elastin-derived peptide levels in normal adults, children, emphysematous subjects. Am. Rev. Respir. Dis. 146: 1143-1148.

DiMatteo, M., Antonini, J., Van Dyke, K., Reasor, M. 1996. Characteristics of the acute-phase pulmonary response to silica in rats. J. Tox. Env. Health. 47: 93-108.

Donnelly, S., MacGregor, I., Zamani, A., Gordon, M., Robertson, C., Steedman, D., Little, K., Haslett, C. 1995. Plasma elastase levels and the development of the adult respiratory distress syndrome. Am. J. Resp. Crit. Care Med. 151: 1428-1433.

Downey, G., Worthen, S., Henson, P., Hyde, D. 1993. PMN sequestration and migration in localized pulmonary inflammation. Am. Rev. Respir. Dis. 147: 168-176.

Driscoll, K. 1994. Macrophage inflammatory proteins: biology and role in pulmonary inflammation. Exp. Lung Res. 20(6): 473-490.

Eidelman, D., Saetta, M., Ghezzo, H., Wang, N., Hoidal, J., King, M., Cosio, M. 1990. Cellularity of the alveolar walls in smokers and its relation to alveolar destruction. Am. Rev. Respir. Dis. 141: 1547-1552.

Eriksson, S. 1991. Emphysema before and after 1963: historic perspectives. Ann. N.Y. Acad. Sci. 624: 1-6.

Eriksson, S. 1996. a 30-year perspective on α1-antitrypsin deficiency. Chest. 110: 237S-242S.

Erle, D.J. 1996. Leukocyte recruitment to the lung and airways. In: Leukocyte recruitment in inflammatory disease. Ed. Peltz, G. R.G. Landes Company. New York.

Evans, M., Pryor, W. 1994. Cigarette smoking, emphysema, and damage to α1-proteinase inhibitor. Am. J. Phys. L593-L611.

Ferkol, T., Mularo, F., Hilliard, J., Lodish, S., perales, J., Ziady, A., Konstan, M. 1998. Transfer of the human alpha-1-antitrypsin gene into pulmonary macrophages in vivo. Am. J. Respir. Cell Mol. Biol. 18: 591-601.

Finkelstein, R., Fraser, R., Ghezzo, H., Cosio, M. 1996. Alveolar inflammation and its relation to emphysema in smokers. Am. J. Respir. Crit. Care Med. 152: 1666-1672.

Finlay, G., O'Driscoll, L., Russell, K., D'Arcy, E., Masterson, J., Fitzgerald, M., O'Connor. 1997. Matrix metalloproteinase expression and production by alveolar macrophages. Am. J. Respir. Crit. Care. Med. 156: 240-247.

Fujita, H., Nelson, N., Daughton, D. et al. 1990. Evaluation of elastase and antielastase balance in patients with chronic bronchitis and pulmonary emphysema. Am. Rev. Respir. Dis.142: 57-62.

Gadek, J., Fells, G., Crystal, R. 1979. Cigarette smoking induces functional antiprotease deficiency in the lower respiratory tract of humans. Science. 206: 1315-1316.

Gadek, J., 1992. Adverse effects of PMNs on the lung. Am. J. Med. 92 (suppl 6A): 27S-31S.

Gadek, J., Pacht, E. 1996a. Pathogenesis of hereditary emphysema and replacement therapy for α1-antitrypsin deficiency. Chest. 110:248S-250S.

Gadek, J., Pacht, E. 1996b. The interdepedence of lung antioxidants and antiprotease defense in ARDS. Chest. 110:273S-277S.

Gardi, C., Cavarra, E., Calzoni, P., Marcolongo, P., de Santi, M., et al., 1994. PMN lysosomal dysfunctions in mutant C57-Bl/6J mice: interstrain variations in content of lysosomal elastase, cathepsin G and their inhibitors. Biochem. J. 299: 237-245.

Gaskin, M-P., Porchet, H.C., Cotonnec, J., Ythier, A., Wallach, D., Piguet, P-F., Grau, G. 1992. Pharmacokinetics and tissue distribution of human urinary tumor necrosis factor binding protein in mice. Drug Metab. Dispos. 20 (4): 592-595.

Gipson, A., Bless, N., Shanley, T., Crouch, L., Bleavins, M., Younkin, E., Sarma, V., Gibbs, D., et al. Endogenous protease inhibitors in acute lung inflammatory injury. 1999. J. Immun. 162: 3653-3662.

Glasser, S., Korfhagen, T., Wert, S., Bruno, M., McWilliams, K., Varkroker, D., Whitsett, J. 1991. Genetic element from human surfactant protein (SP-C) gene confers bronchiolar-alveolar cell specificity in transgenic mice. Am. J. Phys. 261: L349-L356.

Glasser, S., Korfhagen, T., Wert, S., Whitsett, J. 1994. Transgenic models for study of pulmonary development and disease. Am. J. Physiol. 267: L489-L497.

Gottlieb, D., Stone, P., Sparrow, D., Gale, M., Weiss, S., Snider, G., O'Connor, S. 1996. Urinary desmosine excretion in smokers with and without rapid decline of lung function. Am. J. Respir. Care Med. 154: 1290-1295.

Gross, P., Pfitzer, E., A., Tolker, E., Babjak, M.A., Kaschak, M.. 1965. Experimental emphysema. Its production with papain in normal and silicotic rats. Arch. Environ. Health. 11: 50-58.

Guenter, C.A., Coalson, J., Jacques, J. 1981. Emphysema associated with intravascular leukocyte sequestration. Am. Rev. Respir. Dis. 123: 79-84.

Harboe, N. Ingild, A. 1988. In: Antibodies: A Laboratory Manual. Eds. E. Harlowe & D. Lane. Cold Spring Harbor Laboratory. Cold Spring Harbor.

Hautamaki, R., Kobayashi, D., Senior, R., Shapiro, S. 1997. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. Science. 277(26); 2002-2004.

Hay, J., Danel, C., Chu, C., Crystal, R. 1995. Human CC10 gene expression in airway epithelium and subchromosomal locus suggest linkage to airway disease. Am. J. Physiol. 268: L565-L575.

Hiemstra, P., van Wetering, S., Stolk, J. 1998. PMN serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium. Eur. Respir. J. 12: 1200-1208.

Higgins, M. 1991. Risk factors associated with chronic obstructive lung disease. Ann. N.Y. Aca. Sci. 624: 7-17.

Hirano, S. 1996. Migratory responses of PMN after intraperitoneal and intratracheal administration of lipopolysaccharide. Am. J. Physiol. 270: L836-L845.

Hogg, J.C. 1987. PMN kinetics and lung injury. Phys. Rev. 67, No. 4: 1249-1289.

Hoidal, J., Niewoehner, D. 1983. Cigarette smoke inhalation potentiates elastase-induced emphysema in hamsters. Am. Rev. Respir. Dis. 127: 478-481.

Huang, Paulauskis, J., Kobzik, L. 1992. Expression of macrophage inflammatory protein-2 and KC mRNA in pulmonary inflammation. Am. J. Path. 141(4): 981-987.

Huang, Y., Surichamorn, W., Cao, G., Meng, M., Pou, S., et al. 1998. Effect of triflouromethyl ketone-based elastase inhibitors on PMN function in vitro. J. Leukoc. Biol. 64: 322-330.

Hubbard, R., Crystal, R. 1988. Alpha-1-antitrypsin augmentation therapy for alpha-1-antitrypsin deficiency. Am. J. Med. 84(suppl 6A): 52-62.

Hubbard, R., Fells, G., Gadek, J., Pacholok, S., Humes, J., Crystal, R. 1991. PMN accumulation in the lung in α1-antitrypsin deficiency. J. Clin. Invest. 88: 891-897.

Huet-Duvillier, G., Balduyck, M., Watrigant, Y., Sesboue, R., Thiebaut, C., Lafitte, J., Degand, P. 1995. Relationship between a mild a α1-proteinase inhibitor deficiency and respiratory symptoms in a family. Ann. Clin. Biochem. 32: 545-549.

Hunninghake, G., Crystal, R. 1983. Cigarette smoking and lung destruction. Am. Rev.Respir. Dis. 128: 833-838.

Hutchison, D. Hughes, M. 1997. Alpha-1-antitrypsin replacement therapy: will its efficacy ever be proved? Eur. Respir. J. 10: 2191-2193.

Janoff, A. 1983a. Biochemical links between cigarette smoking and pulmonary emphysema. Am. J. Phys. 285-291.

Janoff, A., Raju, L., Dearing, R. 1983b. Levels of elastase activity in bronchoalveolar lavage fluids of healthy smokers and nonsmokers. Am. Rev. Respir. Dis. 127: 540-544.

Janoff, A. 1985. Elastases and Emphysema: current assessment of the protease-antiprotease hypothesis. Am. Rev. Respir. Dis. 132: 417-433.

Jenne, D. 1994. Structure of the azurocidin, proteinase 3, and Neutrophil elastase genes: implications for inflammation and autoimmune vasculitis. Am. J. Resp. Crit. Care Med. 150(6): S147-S154.

Jochum, A., Pelletier, A., Boudier, C., Pauli, G., Bieth, J. 1985. The concentration of leukocyte elastase-α1-proteinase inhibitor complex in bronchoalveolar lavage fluids from healthy human subjects. Am. Rev. Respir. Dis. 132: 913-914.

Johnson, D., Travis, J. 1978. The oxidative inactivation of human α1-proteinase inhibitor. J. Biol. Chem. 253: 7142-7144.

Kay, M., Graham, F., Leland, F., Woo, S. 1995. Therapeutic serum concentrations of human alpha-1-antitrypsin after adenoviral-mediated gene transfer into mouse hepatocytes. Hepat. 21: 815-819.

Keil, M., Lungarella, G., Cavarra, E., van Even, P., Martorana, P. 1996. A scanning electron microscopic investigation of genetic emphysema in tight-skin, pallid, and beige mice, three different C57-Bl/6J mutants. Lab. Invest. 74: 353-362.

Kelso, A. 1998. Cytokines: principles and prospects. Imm. Cell Biol. 76: 300-317.

Khan, T., Wagener, J., Bost, T., Martinez, J., Accurso, F., Riches, D. 1995. Early pulmonary inflammation in infants with cystic fibrosis. Am. J. Respir. Crit. Care Med. 151: 1075-1082.

Kleinerman, J., Rynbrandt, D., 1974. Fed. Proc. 33: A635.

Klut, M., van Eeden, S., Whalen, B., Verburgt, L., English, D., Hogg, J. 1996. PMN activation and lung injury associated with chronic endotoxemia in rabbits. Exp. Lung Res. 22: 449-465.

Konstan, M.W., Hilliard, K.A., Norvell, T.M., Berger, M. 1994. Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. Am. J. Respir. Crit. Care Med. 150(2): 448-454.

Korsgren, C., Cohen, C.M. 1994. CDNA sequence, gene sequence, and properties of murine pallidin (band 4.2), the protein implicated in the murine pallid mutation. Genomics. 21(3): 478-85.

Kramps, J., te Boerkhorst, A., Franson, J. Ginsel, L. Dijkman, J. 1989. Antileukoprotease is associated with elastin fibers in the extracellular matrix of the human lung. Am. Rev. Respir. Dis. 140: 471.

Kramps, J., et al., 1991, in Pulmonary Emphysema: The Rationale for Therapeutic Intervention. Eds. Weinbaum, G., Giles, R., Krell, R., New York Academy of Sciences.

Kueppers, F. 1986. Animal models of emphysema. In: Pulmonary emphysema and proteolysis. Academic Press.

Kunkel, S., Standiford, T., Kasahar, K., Strieter, R. 1991. Interleukin-8: the major PMN chemotactic factor in the lung. Exp. Lung Res. 17: 17-23.

Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., Ohyama, Y., Kurabayashi, M., kaname, T., Kume, E., Iwasaki, H., Iida, A., Shiraki-lida, T., Nishikawa, S., Nagai, R., Nabeshima, Y. 1997. Mutation of the mouse *klotho* gene leads to a syndrome resembling ageing. Nature. 390 (6655): 45-51.

Laskin, D.L., Kimura, T., Sakakibara, S., Riley, D.J., Berg, R.A. 1986. Chemotactic activity of collagen-like polypeptides for human peripheral blood PMNs. J. Leuk. Biol. 39: 255-266.

Laurell, C.B., Eriksson, S. The electrophoretic alpha1-globulin pattern of serum in alpha-1--antitrypsin deficiency. Scand. J. Clin. Lab. Invest. 15: 132-140.

Lellouch, J., Claude, J., Martin, J., Orssaud, G., Zaoui, D., Bieth, J. 1985. Smoking does not reduce the functional activity of serum α1-proteinase inhibitor. Am. Rev. Respir. Dis.132: 818-820.

Li, K., Keeling, B., Churg, A. 1996. Mineral dusts cause elastin and collagen brakdown in the rat lung: a potential mechanism of dust-induced emphysema. Am. J. Respir. Crit. Care Med. 153: 644-649.

Lomas, D.A., Stone, S.R., Llewellyn-Jones, C., Keogan, M-T., Wang, Z., Rubin, H., Carrell, R.W., Stockley, R.A. 1995. The control of PMN chemotaxis by inhibitors of cathepsin G and chymotrypsin. J. Biol. Chem. 270 (40): 23437-23443.

Ludwig, P., Schwartz, B., Hoidal, J., Niewoehner. 1985. Cigarette smoking causes accumulation of polymorphonuclear leukocytes in alveolar septum. Am. Rev. Respir. Dis. 131: 828-830.

Luft, F.C. 1999. Alpha-1-antitrypsin and its relevance to human disease. J. Mol. Med. 359-360.

Lukacs, N., Ward, P. 1998. Proinflammatory and antiinflammatory cytokines in the inflammatory response. In: Proinflammatory and antiinflammatory peptides. Ed. Said, S.I. Marcel Dekker, Inc. New York.

Luisetti, M., Piccioni, P., Donnetta, A. Bulgheroni, A., Peona, V. 1992. Protease-antiprotease imbalance local evaluation with bronchoalveolar lavage. Respiration. 59(suppl): 24-27.

Luisetti, M., Sturani, C., Sella, D., Madonini, E., Galavotti, V., Bruno, G., et al. 1996. MR889, a Neutrophil elastase inhibitor, in patient with chronic obstructive pulmonary disease: a double-blind, randomized, placebo-controlled clinical trial. Eur. Respir. J. 9: 1482-1486.

MacDonald, J., Johnson, C. 1995. Pathophysiology and treatment of α1-antitrypsin deficiency. Am. J. Health-Syst. Pharm. 52: 481-489.

MacNee, W., Wiggs, B., Belzberg, A., Hogg, J. 1989. Effect of cigarette smoking on PMN kinetics in human lung. N. Engl. J. Med. 321: 924-928.

Mahadeva, R., Stewart, S., Bilton, D., Lomas, D. 1998. Alpha-1-antitrypsin deficiency alleles and severe cystic fibrosis lung disease. Thorax. 53: 1022-1024.

Martin, T., Raugi, G., Merritt, T., Henderson, W. 1987. Relative contribution of leukotriene B4 to the PMN chemotactic activity produced by the resident human alveolar macrophage. J. Clin. Invest. 80: 1114-1124.

Martorana, P., Brand, T., Gardi, C., van Even, P., de Santi, M., Calzoni, P., Marcolongo, P., Lungarella, G. 1993. The pallid mouse: a model of genetic α1-antitrypsin deficienty. Lab. Invest. 68(2): 233-241.

McDonald, J., Cary, J. 1995. Pathophysiology and treatment of α1-antitrypsin deficiency. Am. J. Health-Syst Pharm. 52: 481-489.

McElvaney, N.G., Hubbard, R.C., Birrer, P., Chernik, M.S., Caplan, D.B., Frank, M.M., Crystal, R.G. 1991. Aerosol alpha-1-antitrypsin treatment for cystic fibrosis. Lancet. 337(8738): 392-394.

McElvaney, N., Crystal, R. 1997a. Antiproteases and lung defense. In: The Lung: Scientific Foundations. Volume 2. Lippincott-Raven. New York.

McElvaney, N., Crystal, R. 1997b. Inherited susceptibility of the lung to proteolytic injury. In: The Lung: Scientific Foundations. Volume 2. Lippincott-Raven. New York.

McGilligan, K., Thomas, D.W. 1991. Evaluation of assays for detecting α-1-protease inhibitor during purification from rat serum. Analyt. Biochem. 193: 260-265.

Mecham, R., Prosser, I., Fukuda, Y. 1991. Elastic fibers. In: The Lung: Scientific Foundations. pp 381-388. Lippincott-Raven. New York.

Miller, N., Vile, R. 1995. Targeted vectors for gene therapy. FASEB J. 9: 190-199.

Mio, T., Romberger, D.J., Thompson, A.B., Robbins, R.A., Heires, A., Rennard, S.I. 1997. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. Am. J. Respir. Crit. Care Med. 155(5): 1770-1776.

Mitsuhashi, H., Asano, S., Nonaka, T., Masuda, K., Kiyoki, M. 1997. Potency of truncated secretory leukoprotease inhibitor assessed in acute lung injury models in hamsters. J. Pharm. Exp. Ther. 282(2): 1005-1010.

Mitsuhashi, H., Nonaka, T., Hamamura, I., Kishimoto, T., Muratani, E., Fujii, K. 1999. Pharmacological activities of TEI-8362, a novel inhibitor of human Neutrophil elastase. Brit. J. Pharm. 120: 1147-1152.

Morgan, R., Anderson, W.F. 1993. Human gene therapy. Ann. Rev. Biochem. 62: 191-217.

Murphy, G. & J.P. Docherty. 1992. The matrix metalloproteinases and their inhibitors. Am. J. Respir. Cell Mol. Biol. 7: 120-125.

Murray, C., Lopez, A. 1997. Mortality by cause for eight regions of the world: global burden of disease study. The Lancet. 349: 1269-1276.

Nadziejko, C., Finkelstein, I., Balmes, J. 1996. Contribution of secretory leukocyte proteinase inhibitor to the antiprotease defense system of the peripheral lung: effect of ozone-induced acute inflammation. Am. J. Resp. Crit. Care Med.

Nagai, A., Aoshiba, K., Ishihara, Y., Inano, H., Sakamoto, K., Yamaguchi, H., Kagawa, J., Takizawa, T. 1992. Administration of α1-proteinase inhibitor ameliorates bleomycin-induced pulmonary fibrosis in hamsters. Am. Re. Respir. Dis. 145: 651-656.

Niewoehner, D., Kleinerman, J., Rice, D., Div, M. 1974. Pathologic changes in the peripheral airways of young cigarette smokers. N. Engl. J. Med. 291: 755-758.

Niewoehner, D. 1988. Cigarette smoking, lung inflammation, and the development of emphysema. J. Lab. Clin. Med. 111(1): 15-24.

Nowak, E.D., Hui, S-W, Swank, R.T. 1984. Platelet storage pool deficiency in mouse pigment mutations associated with seven distinct genetic loci. Blood. 63: 536-544.

O'Connor, C.M., Gaffney, K., Keane, J., Southey, A., Byrne, N., O'Mahoney, S., Fitzgerlad, M.X. 1993. Alpha-1-proteinase inhibitor, elastase activity, and lung disease severity in cystic fibrosis. Am. Rev. Respir. Dis. 148(6 Pt1): 1665-1670.

Ofulue, A., Ko, M., Abboud, R. 1998. Time course of PMN and macrophage elastinolytic activities in cigarette smoke-induced emphysema. Am. J. Phys. L1134-L1144.

Ogushi, R., Hubbard, R., Vogelmeier, C., Fells, G., Crystal, R. 1991. Risk factors for emphysema: cigarette smoking is associated with a reduction in the association rate constant of lung α1-antitrypsin for Neutrophil elastase. J. Clin. Invest. 87: 1060-65.

O'Hagan, J. 1996. Prevention of chronic obstructive pulmonary disease: a challenge for health professions. New Zeal. Med. J. 109(1014): 1-3.

Ohnishi, K., Takagi, M., Kurokawa, Y., Satomi, S., Konttinen, Y. 1998. Matrix metallo-proteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. Lab. Invest. 78(9): 1077-1086.

Owen, C., Campbell, M., Boukedes, S., Campbell, E. 1997. Cytokines regulate membrane-bound leukocyte elastase on PMNs: a novel mechanism for effector activity. Am. J. Phys. 272(16): L385-L393.

Owen, C., Campbell, E. 1999. The cell biology of leukocyte-mediated proteolysis. J. Leukoc. Biol. 65: 137-150.

Oxman, A.D., Muir, D., Shannon, H.S., Stock, S., Hnizdo, E., Lane, H. 1993. Occupational dust exposure and chronic obstructive pulmonary disease: a systematic overview of the evidence. Am. Rev. Respir. Dis. 148: 38-48.

Paakko, P., Kirby, M., du Bois, R., Gillissen, A., Ferrans, V., Cystal, R. 1996. Activated PMNs secrete stored α1-antitrypsin. Am. J. Respir. Crit. Care Med. 154: 1829-1833.

Parkos, C. 1997. Molecular events in PMN transepithelial migration. Bioessays. 19(10): 865-873.

Pelham, F., Wewers, M., Crystal, R., Buist, A., Janoff, A. 1985. Urinary excretion of desmosine in subjects with PiZZ alpha-1-antitrypsin deficiency, a phenotype associated with hereditary predisposition to pulmonary emphysema. Am. Rev. Respir. Dis. 132: 821-823.

Pesci, A., Balbi, B., Majori, M., Cacciani, G., Bertacco, S., Alciato, P., Donner, C. 1998. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. Eur. Respir. J. 12(2): 380-386.

Petty, T. 1988. Medical management of COPD. In: Chronic obstructive pulmonary d isease: a behavioral perspective. eds. McSweeny, A., Grant, I. by Marcel Dekker, Inc. New York.

Petty, T. 1991. Protease mechanisms in the pathogenesis of acute lung injury. Ann. N.Y. Aca. Sci. 624: 267-277.

Pierce, J. A. 1997. α1-antitrypsin augmentation therapy. Chest. 112(4): 872-873.

Pratt, S., Finley, T., Smith, M., Ladman, A. 1970. A comparison of alveolar macrophages and pulmonary surfactant obtained from the lungs of human smokers and nonsmokers by endobronchial lavage. Anat. Rec. 163: 497-508.

Pratt, P.C. Emphysema and chronic airways disease. In: Pulmonary pathology. Eds. D.H. Dail & S.P. Hammar. ©1994 by Springer-Verlag, New York. Pp.847-863.

Reilly, J.R., Chen, P., Sailor, L.Z., Wilcox, D., Mason, R.W., Chapman, H.A. 1991. Cigarette smoking induces an elastolytic cysteine proteinase in macrophages distinct from cathepsin L. Am. J. Phys. 261: L41-L48.

Riley, D.J., Berg, R.A., Soltys, R.A., Kerr, D.S., et al. 1988. PMN response following intratracheal instillation of collagen peptides into rat lungs. Exp. Lung Res. 14: 549-563.

Rinaldo, J.E., Dauber, J.H., Christman, J., Rogers, R.M. 1984. PMNs alveolitis following endotoxemia. Am. Rev. Respir. Dis. 130: 1065-1071.

Rosenfeld, M.A., Siefried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L, Paakko, P., Gilardi, P., Stratford-Perricaudet, L., Perricaudet, M., Jallat, S., Pavirani, A., Lecocq, J., Crystal, R.G. 1991. Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium in vivo. Science. 252: 431-434.

Rosenfeld, M.A., Yosimura, K., Trapnell, B., Yoneyama, K., Rosenthal, E., Dalemans, W. et al. 1992. In vivo tranfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell. 68: 143-155.

Rosenfeld, M.A., Collins, F. 1996. Gene therapy for cystic fibrosis. Chest. 109: 241-252.

Rossi, G., Hunninghake, G., Gadek, J., Szapiel, S., Kawakami, O., Ferrans, V., Crystal, R. 1984. Hereditary emphysema in the tight-skin mouse. Am. Rev. Respir. Dis. 129: 850-855.

Roth, J., Heitz, P. 1989. Immunolabelling with the protein A-gold technique: an overview. Ultrastruct. Pathol. 13: 476-484.

Rudolphus, A., Kramps, J., Mauve, I., Dijkman, J. 1994. Intratracheally-instilled antileukoprotease and α1-proteinase inhibitor: effect on human Neutrophil elastase-induced experimental emphysema and pulmonary localization. Histochem. J. 26: 817-824.

Ruther, U., Tripodi, M., Cortese, R., Wagner, E. 1987. The human alpha-1-antitrypsin gene is efficiently expressed from two tissue-specific promoters in transgenic mice. Nuc. Acids. Res. 15(18); 7519-7529.

Sallenave, J., Si-Ta har, M., Cox, G., Chignard, M., Gauldie, J. 1997. Secretory leukocyte proteinase inhibitor is a major leukocyte elastase inhibitor in human PMNs. J. Leukoc. Biol. 61: 695-702.

Sambrook, J., Fritsch, E., Maniatis, T. (eds). 1989. Molecular cloning: a laboratory manuel, 2nd edition. Cold Spring Harbor Laboratory Press. New York.

Sandford, A., Weir, T., Pare. 1997. Genetic risk factors for chronic obstructive pulmonary disease. Eur. Respir. J. 10: 1380-1391.

Sandford, A.J., Weir, T., Spinelli, J.J., Pare, P.D. 1999. Z and S mutations of the α-1-antitrypsin gene and the risk of chronic obstructive pulmnary disease. Am. J. Respir. Cell Molec. Biol. 20: 287-291.

Sansores, R., Abboud, R., et al. 1997. Effect of exposure of guinea pigs to cigarette smoke on elastolytic activity of pulmonary macrophages. Chest. 112: 214-219.

Saylors, R., Wall, D. 1998. Expression of human alpha-1-antitrypsin in murine hematopoietic cells in vivo after retrovirus-mediated gene transfer. Mol. Genet. Metab. 63(3): 198-204.

Schmidt, E., Rasche, B., Ulmer, W. 1988. Replacement therapy for alpha-1-protease inhibitor deficiency in PiZ subjects with chronic obstructive lung disease. Am. J. Med. 84(suppl 6A): 63-69.

Schriver, E., Davidson, J., Sutcliffe, M., Swindell, B., Bernard, G. 1992. Comparison of elastin peptide concentrations in body fluids from healthy volunteers, smokers, and patients with COPD. Am. Rev. Respir. Dis. 245: 762-766.

Schwaiblmair, M., Vogelmeier, C. 1998. α1-antitrypsin: hope on the horizon for emphysema sufferers? Drugs & Aging. 12(6): 429-440.

Seersholm, N., Wencker, M., Banik, N., et al. 1997. Does alpha-1-antitrypsin augmentation therapy slow the annual decline in FEV1 in patients with severe hereditary alpha-1-antitrypsin deficiency? Eur. Respir. J. 10: 2260-2263.

Seigfried, W., Rosenfeld, M., Stier, L., Stratford-Perricaudet, L., Perricaudet, M., Pavirani, A., Lecocq, J., Crystal, R. 1995. Polarity of secretion of alpha-1-antitrypsin by human respiratory epithelial cells after adenoviral transfer of a human alpha-1-antitrypsin cDNA. Am. J. Resp. Cell Mol. Biol. 12: 379-384.

Selby, C., Drost, E., Lannan, S., Wraith, K., MacNee, W. 1991. PMN retention in the lungs of patients with chronic obstructive pulmonary disease. Am. Rev. Respir.Dis.143: 1359-1364.

Selby, C., MacNee, W. 1993. Factors affecting PMN transit during acute pulmonary inflammation: minireview. Exp. Lung Res. 19: 407-428.

Selman, M., Montano, M., Ramos, C., Vanda, B., Becerril,C., Delgado,J., Sanores, R., Barrios,R., Pardo, A. 1996. Tobacco smoke-induced lung emphysema in guinea pigs is associated with increased interstitial collagenase. Am. J. Phys. L734-L739.

Senior, R.M., Griffin, G.L., Mecham, R.P. 1980. Chemotactic activity of elastin-derived peptides. J. Clin. Invest. 66: 859-862.

Senior, R., Connolly, N., Cury, J., Welgus, H., Campbell, E. 1989. Elastin degradation by human alveolar macrophages. Am. Rev.Respir. Dis. 139: 1251-1256.

Senior, R., Anthonisen, N. 1998. Chronic obstructive pulmonary disease (COPD). Am. J. Respir. Crit. Care Med. 157: S139-S147.

Setoguchi, Y., Jaffe, A., Chu, C., Crystal, R. 1994. Intraperitoneal *in vivo* gene therapy to deliver α1-antitrypsin to the systemic circulation. 10: 369-377.

Shapiro, S. 1991. Elastin degradation by mononuclear phagocytes. Ann. N.Y. Aca. Sci. 624: 69-80.

Shapiro, S. 1994. Elastolytic metalloproteinases produced by human mononuclear phagocytes: potential roles in destructive lung disease. Am. J. Resp. Crit. Care Med. 150(6): S160-S164.

Shapiro, S. 1995. The pathogenesis of emphysema: the elastase: antielastase hypothesis 30 years later. Proc. Ass. Am. Phys. 107(3): 346-352.

Shinguh, Y., Yamazaki, A., Inamura, N., Fujie, K., Okamoto, M., Nakahara, K., Notsu, Y., Okuhara, M., Ono, T. 1998. Biochemical and pharmacological characterization of FR134043, a novel elastase inhibitor. Eur. J. Pharm. 345: 299-308.

Sifers, R., Ledley, F., Reed-Fourquet, L., Ledbetter, D., Ledbetter, S., Woo, S. 1990. Complete cDNA sequence and chromosomal localization of mouse α1-antitrypsin. 6: 100-104.

Sifers, R., Carlson, J., Clift, S., DeMayo, F., Bullock, D., Woo, S. 1987. Tissue specific expression of the human alpha-1-antitrypsin gene in transgenic mice. 15(4): 1459-1475.

Simani, A. S., Inoue, S., Hogg, J.C. 1974. Penetration of respiratory epithelium of guinea pigs following exposure to cigarette smoke. Lab. Invest. 31: 75-87.

Smith, R., Traber, L., Traber, D., Spragg, R. 1989. Pulmonary deposition and clearance of aerosolized alpha-1-proteinase inhibitor administered to dogs and to sheep. J. Clin. Invest. 84: 1145-1154.

Snider, G.L., Kleinerman, J., Thurlbeck, W., Bengali, Z. 1985. The definition of emphysema. Am. Rev. Respir. Dis. 132: 182-185.

Snider, G.L., Lucey, E., Stone, P. 1986. Animal models of emphysema. Am. Rev. Resp. Dis. 133: 149-169.

Snider, G.L., Ciccolellea, D. Morris, S., Stone, P., Lucey, E. 1991. Putative role of Neutrophil elastase in the pathogenesis of emphysema. Ann. N.Y. Acad. Sci. 624: 45-59.

Snider, G.L., Lucey, E., Stone, P. 1994. Pitfalls in antiprotease therapy of emphysema. Am. J. Resp. Crit. Care. Med. 150(6): S131-S137.

Song, S., Morgan, M., Ellis, T., Poirier, A., Chesnut, K., Wang, J., Brantly, M., Muzyczka, N. et al. 1998. Sustained secretion of human alpha-1-antitrypsin from murine muscle transduced with adeno-associated virus vectors. Proc. Natl. Acad. Sci. USA. 95(24): 14384-14388.

Standiford, T.J., Kunkel, S.L., Lukacs, N., Greenberger, M.J., Danforth, J.M., Kunkel, R.G., Strieter, R.M. 1995. Macrophage inflammatory protein-1α mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. J. Immun. 155: 1515-1524.

Starcher, B., Williams, I. (a) 1989. A method for intratracheal instillation of endotoxin into the lungs of mice. Lab. Anim. 23: 234-240.

Starcher, B., Williams, I. 1(b) 1989. The beige mouse: role of Neutrophil elastase in the development of pulmonary emphysema. Exp. Lung Res. 15: 785-800.

Starcher, B., Green, M., Scott, M. 1995. Measurement of urinary desmosine as an indicator of acute pulmonary disease. Respiration. 62: 252-257.

Stockley, R.A., Shaw, J., Afford, S.C., Morrison, J.M., Burnett, D. 1990. Effect of α1-proteinase inhibitor on PMN chemotaxis. Am. J. Respir. Cell. Molec. Biol. 2: 163-170.

Stoller, J. 1998. Augmentation therapy for severe α1-antitrypsin deficiency: is the jury still out on a trial? Thorax. 53: 1007-1009.

Stolk, J., Rudolphus, A., Kramps, J. A. 1991. Lipopolysaccharide-induced alveolar wall destruction in the hamster is inhibited by intratracheal treatment with r-secretory leukocyte protease inhibitor. Ann. N. Y. Acad. Sci. 624: 350-352.

Stone, P. Bryan-Rhadfi, J., Lucey, D., Ciccolella, D., Crombie, G., Faris, B., Snider, G., Franzblau, C. 1991. Measurement of urinary desmosine by isotope dilution and high performance liquid chromatography. Am. Rev. Respir. Dis. 144: 284-290.

Stone, P., Gottlieb, D., O'Connor, G., et al. 1995. Elastin and collagen degradation products in urine of smokers with and without chronic obstructive pulmonary disease. Am. J.Respir. Crit. Care Med. 151: 952-59.

Stone, P.J., Konstan, M., Berger, M., Dorkin, H., Franzblau, C., Snider, G.L. 1995b. Elastin and collagen degradation products in urine of patients with cystic fibrosis. Am. J. Respir. Crit. Care Med. 152(1): 157-162.

Stone, P., Morris,T., Franzblau,C., Snider,G. 1995. Preliminary evidence that augmentation therapy diminishes degradation of cross-linked elastin in alpha-1-antitrypsin deficient humans. Respiration. 62: 76-79.

Streiter, R.M., Standiford, T.J., Rolfe, M.W., Kunkel, S.L. 1993. Interleukin-8. In: Cytokines of the Lung. Volume 61. Ed. J. Kelley. Marcel Dekker, Inc. New York.

Streiter, R.M., Kunkel, S.L. 1994. Acute lung injury: the role of cytokines in the elicitation of PMNs. J. Investig. Med. 42(4): 640-651.

Stribling, R., Brunette, E., Liggitt, D., Gaensler, K., Debs, R. 1992. Aerosol gene delivery in vivo. Proc. Natl. Acad. Sci. USA. 89: 11277-11281.

Stripp, B., Saway, P., Luse, D., Wikenheiser, K., Wert, S., Huffman, J., Lattier, D., Singh, G., Tatyal, S., Whitsett, J. 1992. Cis-acting elements that confer lung epithelial cell expression of the CC10 gene. J. Biol. Chem. 267(21): 14703-14712.

Sun, G., Stacey, M., Vittori, E., Marini, M., Bellini, A., Kleimberg, J., Mattoli, S. 1998. Cellular and molecular characteristics of inflammation in chronic bronchitis. Eur. J. Clin. Invest. 28(5): 364-372.

Takahashi, H., Ishidoh, K., Muno, D., Ohwada, A., Nukiwa, T., Kominami, E., Kira, S. 1993. Cathepsin L activity is increased in alveolar macrophages and bronchoalveolar lavage fluid of smokers. Am. Rev. Respir. Dis. 147: 1562-1568.

Tenholder, M.F., Rajagopal, K.R., Phillips, Y.Y, Dillard, T.A., Mundie, T.G. 1991. Urinary desmosine excretion as a marker of lung injury in the adult respiratory distress syndrome. Chest. 100: 1385-1390.

Tetley, T.D. 1993. Proteinase imbalance: its role in lung disease. Thorax. 48(5): 560-165.

Thomas, R., Vigerstad, T. 1989. Use of laboratory animal models in investigating emphysema and cigarette smoking in humans. Reg. Tox. Pharm. 10: 264-271.

Thornton, W., Adelstein, E., Edes, T. 1989. Leukotriene B4 is measurable in serum of smokers and nonsmokers. Clin. Chem. 35(3): 459-460.

Thurlbeck, W. M. 1967. Internal surface area and other measurements in emphysema. Thorax. 22: 483-496.

Thurlbeck, W.M. 1988. Chronic Airflow Obstruction. In: Pathology of the lung. pp 510-576. ed. WB Thurlbeck. Thieme Medical Publishers, New York.

Travis, J., Bowen, J., Tewksbury, D., Johnson, D., Pannell, R. 1976. Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. Biochem. J. 157(2): 301-306.

Travis, J. 1988. Structure, function, and control of PMN proteinases. Am. J. Med. 84(suppl 6A): 37-42.

Travis, J., Johnson, D. 1998. Human α1-proteinase inhibitor. In: Methods in Enzymology. pp 754-765.

Turino, G. 1991. Natural history and clinical management of emphysema in patients with and without alpha1-antitrypsin inhibitor deficiency. Ann. N.Y. Aca. Sci. 624: 7-17.

Valentine, R., Rucker, R.B., Chrisp, C.E., Fisher, G.L. 1983. Morphological and biochemical features of elastase-induced emphysema in strain A/J Mice. Tox. Appl. Pharmacol. 68: 451-461.

Vogelmeier, C., Gillissen, A., Buhl, R. 1996. Use of secretory leukoprotease inhibitor to augment lung antiNeutrophil elastase activity. Chest. 110: 261S-266s.

Vogelmeier, C., Bidermann, T., Maier, K., Mazur, G., Behr, J., Krombach, F., Buhl, R. 1997. Comparative loss of activity of recombinant secretory leukoprotease inhibitor and α1-protease inhibitor caued by different forms of oxidative stress. Eur. Respir. J. 10: 2114-2119.

Vogelmeir, C., Kirlath, I., Warrington, S., Banik, N., Ulbrich, E., Dubois, R. 1997. The intrapulmonary half-life and safety of aerosolized α1-protease inhibitor in normal volunteers. Am. J. Respir. Crit. Care Med. 155: 536-541.

Weibel, E., Crystal, R. 1991. Structural organization of the pulmonary interstitium. In: The Lung: Scientific Foundation. pp. 369-380. Lippincott-Raven. New York.

Weiss, S. 1989. Tissue destruction by PMNs. NEJM. 320(6): 365-376.

Weitz, J., Crowley, K., Landman, S., Lipman, B., Yu, J. 1987. Increased Neutrophil elastase activity in cigarette smokers. Ann. Int. Med. 107: 680-682.

Welbourn, C.R.B. & Young, Y. 1992. Endotoxin, septic shock and acute lung injury: PMNs, macrophages and inflammatory mediators. Br. J. Surg. 79: 998-1003.

Wencker, M., Banik, N., Buhl, R., Seidel, R., Konietzko, N. 1998. Long-term treatment of α1-antitrypsin deficiency-related pulmonary emphysema with human α1-antitrypsin. Eur. Resp. J. 11: 428-433.

West, J.B. Respiratory Physiology-the essentials. 4th edition. © 1990 by Williams & Wilkins. Baltimore.

West, J.B. Pulmonary Pathophysiology-the essentials. 4th edition. © 1992 by Williams & Wilkins. Baltimore.

Wewers, M., Casolaro, A., Sellers, S., Swayze, S., McPhaul, K., Wittes, J., Crystal, R. 1987. Replacement therapy for alpha1-antitrypsin deficiency associated with emphysema. N. Engl. J. Med. 316: 1055-1062.

White, R.A., Peters, L.L., Adkison, L.R., Korsgren, C., Cohen, C.M., Lux, S.E. 1992. The murine pallid mutation is a platelet storage pool disease associated with the protein 4.2 (pallidin) gene. Nat. Genet. 2(1): 80-82.

Wicks, I., Howell, M., Hancock, T., Kohsaka, H., Olee, T., Carson, D. 1995. Bacterial lipopolysaccharide copurifies with plasmid DNA: implications for animal models and human gene therpy. Hum. Gene Ther. 6: 317-323.

World Health Organization. 1995. Definitions, epidemiology, pathophysiology, diagnosis, and staging. Am. J. Resp. Crit. Care Med. 152: S78-S83.

Wright, J.L., Churg, A. 1990. Cigarette smoke causes physiologic and morphologic changes of emphysema in the guinea pig. Am. Rev. Respir. Dis. 142: 1422-1426.

Wright, J.L., Sun J.P. 1994. Effect of smoking cessation on pulmonary and cardiovascular function and structure: analysis of guinea pig model. J. Appl. Physiol. 76(5): 2163-2168.

Wright, J.L., Churg, A. 1995. Smoke-induced emphysema in the guinea pig is associated with morphometric evidence of collagen breakdown and repair. Am. J. Phys. 268: L17-L20.

Ying, Q., Kemme, M., Simon, S.1996. Alginate, the slime exopolysaccharide of Psuedomonas aeruginosa, binds human leukocyte elastase, retards inhibition by alpha-1-proteinase inhibitor, and accelerates inhibition by secretory leukoprotease inhibitor. Am. J. Respir. Cell Mol. Biol. 15(2): 283-291.

Yoshioka, A., Betsuyaku, T., Nishimura, M., Miyamoto,K., et al. 1995. Excessive PMN

elastase in bronchoalveolar lavage fluid in subclinical emphysema. Am. J. Respir. Crit. Care Med. 152: 2127-2132.

Zay, K., Devine, D., Churg, A. 1995. Quartz inactivates α1-antiproteinase: possible role in mineral dust-induced emphysema. J. Appl. Physiol. 78(1): 53-58.

Zay, K., Loo, S., Xie, C., Devine, D., Wright, J., Churg, A. 1999. Role of PMNs and alpha-1-antitrypsin in coal and silica-induced connective tissue breakdown. Am. J. Phys. 276(2 Pt 1): L269-279.

Zenati, M., Keenan, R.J., Courcoulas, A.P., Griffith, B.P. 1998. Lung volume reduction or lung transplantation for end-stage pulmonary emphysema? Europ. J. Cardio-Thor. Surg. 14: 27-32.