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THE EFFECT OF PIRFENIDONE ON CHRYSOTILE ASBESTOS-INDUCED PULMONARY FIBROSIS IN THE HAMSTER

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THE EFFECT OF PIRFENIDONE ON CHRYSOTILE ASBESTOS-INDUCED PULMONARY FIBROSIS IN THE HAMSTER

by

Scott Wayne Grimm

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For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN TOXICOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

J. Wesley Clayton, Jr. July 21 1986

J. Wesley Clayton, Jr. Date

Professor of Pharmacology and Toxicology

DEDICATION

I would like to dedicate this thesis to Kathy, my wife, for her love, understanding, and support during our first years of marriage, and to Harold and B. Jean Grimm, my parents, for providing me with the environment to believe in myself and strive for the goals that I have undertaken to achieve.

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ABSTRACT

Male and female Golden Syrian hamsters were dosed intratracheally 0.5 mg chrysotile asbestos twice weekly for two weeks. At 60 days after instillation, groups 3,4, and 5 received daily oral doses of 25, 250, or 500 mg pirfenidone/kg/day, respectively, for 10 to 40 days, while group 6 received 1 mg cortisone/day. Group 1 was control while group 2 received asbestos only. Prolyl hydroxylase activity was increased in group 2 at 30 and 60 days and hydroxyproline content was increased at 30 days. Lactate dehydrogenase and alkaline phosphatase activities in lavage fluid of group 2 animals were increased at 30 days. Changes in inflammation scores and the numbers of collagen and reticulin fibers in pirfenidone-treated animals were variable. Decreases were observed in males but not in females. Improvement in the fibrotic state due to pirfenidone could not be definitively concluded.

INTRODUCTION

Toxicology of the Lung

The lung's primary function is to provide a means for the exchange of oxygen and carbon dioxide in the circulation. Its extensive surface area, conducting airways, and the minute separation between airspace and the circulation also make the lung an excellent organ for the absorption of toxicants. These toxicants can enter the lung as solids, liquids, or liquid aerosols and because of the vital nature of the lung, the actions of toxicants can be acutely or chronically important to health.

The deposition and retention of inhaled gases and aerosols are influenced by many anatomic features of the lung. The respiratory tract can be divided into three primary regions: the nasopharyngeal, the tracheobronchial, and the pulmonary regions. The nasopharynx consists of the anterior nares extending to the larynx. This region filters out large inhaled particles and also humidifies and moderates the temperature of the air that passes into the lung. The tracheobronchial region consists of the trachea, bronchi, and bronchioles, which serve to conduct the air into the pulmonary region. The pulmonary region consists of respiratory bronchioles, alveolar ducts, and alveoli.

Alveolar epithelium along with the basement membrane and the capillary epithelium make up the very thin air-blood barrier across which oxygen and carbon dioxide must diffuse.

On a cellular level, the lung can be divided into parenchymal (alveoli, alveolar ducts, and capillaries) and non-parenchymal tissue (conducting airways, conductive blood vessels, connective tissue structures, and the pleura)(Hollinger, 1985). Approximately 40 different cell types make up these structures along with connective tissue (collagen, elastin, and proteoglycans). Estimates based on animal studies suggest that at least 86% of the total lung cell population is parenchymal in nature. These include alveolar type I (4 to 8%), alveolar type II (6 to 15%), endothelial (33 to 43%), and interstitial (32 to 43%) cells, which include fibroblasts and fibrocytes. The remaining cells are ciliated, glandular, and blood vessel cell types.

Type I cells line more than 95% of the alveolar surface. These cells contain little cytoplasm and few organelles, but because of their location and composition, are the parenchymal cells that are most susceptible to damage by toxicants.

Located at the corners of the alveoli and into the respiratory bronchioles are epithelial type II cells. Type II cells have dense, well-defined cytoplasmic structures including rough endoplasmic reticulum, Golgi apparatus, and multilamellated inclusions. These cells are active

metabolically and mitotically. Type II cells are believed to be the source of alveolar surfactant which serves to regulate surface tension-related events and keeps the alveoli maximally distended for gas exchange.

Within the interstitium and lying between endothelial and epithilial cells are mesenchymal cells. This cell type is comprised primarily of fibroblasts, which are surrounded by a connective tissue network. The fibroblast is responsible for the production of the intercellular substances which make up connective tissue such as collagen, elastin and ground substance. Other cell types in the lung include ciliated bronchiolar epithelium, nonciliated bronchiolar epithelium (Clara cells) and alveolar macrophages. The ciliated bronchiolar epithelium and alveolar macrophages are important parts of the major defense mechanisms available to the lung. Ciliated epithelial cells predominate in the trachea, bronchi, and larger bronchioles. These cells function to move a viscous fluid film containing deposited particles to the nasopharynx where it is expectorated or swallowed. Alveolar macrophages are the lung's phagocytic cells. These mobile cells are located in the alveolar lumena where they ingest foreign particles which are then degraded intracellularly or removed to the lymphatic system.

Dusts in the Lung

Dust consists of solid mineral or organic particles dispersed in air and arise from processes such as grinding, milling, or blasting of rocks or minerals or from decomposition of organic materials such as cotton fibers, pollens, and spores (Lee, 1985). Dusts are distinct from smokes, fumes, or vapors, although all of these categories can be considered under the general term "aerosols".

Mechanisms of Dust Deposition

Environmental or industrial dusts contain particles in a wide range of sizes and shapes. The toxicity of dust particles to the lung is highly dependent on the respirability of the particles, the nature of the particles, and the site of their deposition in the respiratory tract. Particle deposition depends on the shape, size, density, and aerodynamic properties of the particle as well as airway anatomy. Five mechanisms involved in dust deposition in the respiratory tract are interception, impaction, sedimentation, diffusion, and electrostatic precipitation.

As particles move into smaller and smaller airways, they reach a point where the distance to a surface is less than the particle dimensions. This process is called interception. Interception is mainly important for fibrous dust particles such as asbestos and fiberglass (Harris, 1976). Fiber length is probably the most important factor in

interceptive deposition. Inhaled air must follow a tortuous path through the nose or mouth to the branching airways of the lung.

Particles in the air, because of inertia, will tend to continue along their original path. When air changes direction, as in a bifurcating airway, a particle may be impacted on the surface. Impaction is determined by particle mass and air velocity.

Gravitational sedimentation is an important mechanism for the deposition of particles in the smaller bronchi, bronchioles and alveolar ducts where the airway is small and air velocity is low. If these conditions are met, a particle may sediment on the wall of the airway by gravitational pull. This process becomes less effective when the terminal settling velocity decreases below 0.001 cm/sec, corresponding to an aerodynamic diameter of 0.5 micrometer (Lee, 1985). The settling velocity is proportional to th density of the particle and the square of its diameter.

Submicron particles that are inhaled are continuously subjected to bombardment by surrounding gas molecules. This random movement of particles results in diffusion from one region of a gas volume to another. The diffusivity of a particle is inversely proportional to its diameter but independent of density. Diffusional deposition is an important process in the small airways and alveoli for particles with diameters smaller than 0.5 micrometers.

Most ambient particles have low charge or have reached charge equilibrium. Deposition due to charge is called electrostatic precipitation and is usually small in comparison to that of other mechanisms.

Deposition of Fibrous Particles

Deposition by interception becomes effective when fibers are approximately 6 micrometer in length and increases with increasing fiber length (Lee, 1985). Fibers with an aspect ratio (length/ diameter) less than five would be too short to be effectively deposited by interception. Longer fibers (aspect ratio > 5) tend to align parallel to the stream of airflow and deposit in the bifurcations of bronchioles. An increase in the fiber length also increases deposition by impaction and sedimentation. Fiber shape also plays an important role in deposition. The commonly curled shape of chrysotile asbestos makes it behave as a three-dimensional object with random alignment in the airways and allowing efficient deposition at airway bifurcations. Straight amphibole asbestos fibers tend to align with the airstream and thus allow deeper penetration into the respiratory tract than curled fibers.

Lung Clearance Mechanisms

The mechanisms of removal of particles that have deposited on the surfaces of the respiratory tract vary depending on the site of deposition (Menzel and McClellan,

1980). The efficacy of clearance of deposited materials is critical to their toxic potential. A material which is rapidly and more completely removed would have less potential to cause critical damage to the pulmonary tissues.

In the ciliated surfaced areas of the respiratory system, which extend from terminal bronchioles to the nose, the clearance mechanism is served by a mucous blanket covering the mucociliary escalator. The surface of the tracheal epithelia are covered with cilia and numerous microvilli which beat upward toward the nasopharyngeal region. The mucous blanket derives its mucin from the goblet cells which are associated with the ciliated epithelial cells and submucosal glands. The mucous is transported from the bronchioles and bronchi toward the nasopharyngeal region by the action of the cilia. Deposited particles are carried with the mucous to be expectorated or swallowed.

If a particle has been deposited in the pulmonary region, there are three mechanisms for its removal: 1) particles may be phagocytized by the alveolar macrophage population and cleared up the tracheobronchial tree via the mucociliary escalator, 2) particles can be phagocytized and removed via lymphatic drainage, or 3) the material may dissolve and be removed via the bloodstream or lymphatic drainage.

Intratracheal Instillation vs. Inhalation Exposure

In this study, intratracheal instillation of asbestos fibers was used. There are several advantages as well as some important disadvantages to this technique (Brain, et al, 1976). Intratracheal instillation involves administration of a liquid suspension of particles directly into the trachea of an animal. This technique is simple and very inexpensive compared to inhalation exposure. Another advantage of intratracheal instillation, which was of particular importance in this research, was that accurate and easily quantifiable doses could be administered and the hazards to laboratory personnel were minimal. However, disadvantages of this technique are 1) introduction of large-sized particles by an unnatural route, 2) avoidance of upper respiratory defense mechanisms, and 3) uneven patterns of particle distribution with preferential deposition in dependent portions of the lung. Also, species differences in particle distribution are decreased when intratracheal instillation is used (Brain et al., 1976). The research presented here used multiple instillations of fibers. The uniformity of particle distribution is increased when multiple instillations are used and thus more accurately models the aerosol inhalation route. Another important morphological consideration is that, in unskilled hands, it is possible to injure the tracheal mucosa and the quantity and quality of its

secretions by use of the intratracheal technique and thereby may alter the mucociliary transport system.

Pulmonary Responses to Toxic Agents

Menzel and McClellan (1980) divided the responses of the lung to toxic agents into 5 categories as follows:

(1) Irritation of the airways which results in bronchoconstriction and edema and can be compounded by secondary infection. Examples of agents which elicit this type of response are ammonia and chlorine.

(2) Damage to the cells lining the respiratory tract resulting in necrosis, increased vascular permeability, and intraluminal edema. Agents causing this type of response include phosgene and paraquat.

(3) Pulmonary fibrosis which may obliterate the respiratory capacity of the lung. This response is discussed in more detail in a later section.

(4) Airway constriction caused by allergic responses. This type of response can be caused by any agent causing a type I hypersensitivity reaction including pollens, fungal spores and a number of drugs.

(5) Oncogenesis leading to primary tumor formation.

Examples of agents causing this type of response are arsenic, other metals, and asbestos.

These responses are not mutually exclusive. Multiple responses may be elicited by a single agent, and the development of these responses may overlap in time course.

Enzymes in Lung Lavage Fluid

The analysis of bronchoalveolar fluid, by lung washing, has proven to be a useful tool as a rapid screen for lung injury from the inhalation of airborne toxicants. Several investigators have monitored changes in biochemical parameters and/or cell populations as indicators of pulmonary damage (Henderson, Damon, and Henderson, 1978; Henderson et al, 1978; Henderson, Rebar, and DeNicola, 1979; Henderson et al, 1980; DeNicola, Rebar, and Henderson, 1981; Roth, 1981; Kagan, Oghisho, and Hartmann, 1983; Glassroth et al, 1984). In vitro tests using a single cell type, although rapid and of relatively low cost, lacks the ability to monitor the integrated response of the whole animal to an inhaled material (Henderson, 1984).

Henderson (1984) has reviewed the possible lung lavage fluid parameters that are possible indicators of pulmonary damage. Elevated levels of serum proteins may indicate damage to the alveolar-capillary barrier. Mucous secretions may be increased in lavage fluid if the upper airways have been damaged or irritated. This response can be

detected by monitoring sialic acid levels, a component of mucin. Changes in the levels of different enzyme activities in lung lavage fluid can also indicate lung cell injury has occurred. Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many cell types of the body including the lung. If cells are lysed, or the cell membrane is damaged, LDH would be elevated in lung lavage fluid (Henderson, 1984). LDH activity has been reported to increase in response to many agent in the lung including oxygen, nitrogen dioxide, and cadmium. In response to oxidant injury, glucose-6-phosphate dehydrogenase, another cytosolic enzyme, has been shown to increase in lavage fluid. Lysosomal enzymes, such as acid phosphatase and beta-glucuronidase are released by polymorphonuclear leukocytes (PMNs) and macrophages during the process of phagocytosis. The increase in lysosomal enzyme activity could be indicative of increased phagocytosis or macrophage damage. Alkaline phosphatase is associated with the cell membrane and has been observed in type II cells by histochemical staining. Type II cell damage or increased secretions may cause elevated levels of alkaline phosphatase in lung lavage fluid. Angiotensin converting enzyme levels have been used as a measure of endothelial cell damage.

Lung pathogenesis can also be monitored by measuring the cellular content of the lung lavage fluid (Henderson, 1984). Early in an inflammatory response, an influx of PMNs would be expected while an increase in the macrophage

population occurs in persistent inflammation. Glassroth (1984) found that after intratracheal administration of 5 mg chrysotile asbestos to hamsters, the percentage of neutrophils was significantly increased up to 180 days.

Henderson (1984) explains that extracellular LDH levels are good indicators of nonspecific cell damage. Alkaline phosphatase activity in lavage fluid is also a good indicator of acute lung cell damage. The isozyme pattern of alkaline phosphatases can be determined by isoelectric focusing which could give some indication of the source of the enzyme and thus the site of injury.

Few studies have used lung lavage to follow the development of chronic lung disease. Beta-glucuronidase and LDH activities were shown to be significantly increased at 17-18 months in rats after exposure to chrysotile or crocidolite asbestos which indicated continuous injury after withdrawal from exposure (Kagan et al, 1983). More studies are needed to determine the usefulness of lung lavage fluid analysis in following the development of chronic lung disease.

Pulmonary Fibrosis

Pulmonary fibrosis is a severely debilitating disease which occurs after exposure to a wide variety of agents. A general term applied to the class of diseases in which pulmonary fibrosis is a central factor is "pneumoconiosis".

Fibrosis can be considered as an advantageous host response because of its value in repair of traumatic or destructive lesions (Bateman, Emerson, and Cole, 1981). A fibrogenic response also traps foreign particles and organisms which gain access to the lung from the environment. This response occurs when normal clearance mechanisms such as phagocytosis by macrophages and clearance via the mucociliary escalator have failed. Fibrosis is well tolerated in most organs and is a beneficial method of healing, but in the lung, a relatively small increase in the amount of fibrous tissue is associated with a considerable impairment of pulmonary function. The development of fibrosis in the lung cannot be thought of as a single process but is a final common pathway of many biologic responses to numerous inciting agents.

Collagen Biochemistry

Lung connective tissue normally is involved in the maintenance of the structural integrity and function of the lung. It is composed of collagen (60-70%), elastin (25-35%), and proteoglycans (5%) (Hollinger, 1985). Collagen is the most abundant protein in the adult mammalian lung, comprising 15 to 20% of the alveolar dry weight. It is well characterized in normal and fibrotic pulmonary tissue.

The primary structure of collagen, sometimes referred to as tropocollagen, is made up of three polypeptide chains,

called chains, each containing approximately 1100 amino acids coiled around each other to form a triple helical configuration. The polypeptide chains which make up tropocollagen have the repeating sequence (x-y-glycine)n, in which x and y are most commonly proline-hydroxyproline and lysine-hydroxylysine. The amino acids, hydroxyproline and hydroxylysine, are almost unique to collagen and are required for the formation of a stable triple helix. They are not incorporated into the polypeptide chain as such but are formed by hydroxylation of proline and lysine in the nascent polypeptide chain. The tropocollagen chains are secreted from the cell and aggregate into fibrils, which are stabilized by covalent crosslinking between lysine and hydroxylysine residues. Once the collagen has been crosslinked, it becomes rather insoluble and has high tensile strength.

Five different types of collagen have been identified in the lung, varying in their amino acid sequence and in the types of polypeptide chains making up the triple helix of tropocollagen. The most abundant and widely distributed in the lung is type I collagen. It is found in bronchi, blood vessels, and interstitium. Type I collagen is formed in fibroblasts and in type I epithelial cells. Type II collagen is found in cartilage and produced by chondrocytes in the cartilaginous tissue of the trachea and bronchi. Type III collagen is widely distributed in the lung interstitium and

accounts for 30% of the total lung collagen. It is formed in fibroblasts and type I epithelial cells, but differs from type I collagen in that is contains disulfide links. Collagen types IV and V are found in basement membrane, and are products of synthesis of epithelial and endothelial cells.

"Reticulin" is a histopathological term applied to fibers of connective tissue which are argyrophilic, or stain black with silver impregnation (Bateman et al, 1981). Reticulin contains both collagenous and non-collagenous components although its exact composition has not been determined. These fibers may adopt the staining characteristics of collagen after a time, possibly because of maturation of fibers. An increase in argyrophilic fibers may be the only light microscope feature of developing fibrosis.

Connective Tissue Accumulation

Fibrotic lung diseases can be either idiopathic or chemically induced (Hollinger, 1985). Numerous environmental agents have been shown to be associated with lung fibrosis. Agents which are known chemical factors in fibrosis include radiation (x-ray), inhaled inorganic dusts (e.g., silica, asbestos, beryllium), toxic gases (e.g., ozone, oxygen at high tensions, nitrogen dioxide), cigarette smoke, methotrexate, bleomycin, cyclophosphamide, and gold. Fibrosis-producing agents may act directly or indirectly,

being mediated through effector cells. In pulmonary fibrosis, the amount, type, and location of collagen becomes altered. The ratio of type I to type III has been shown to increase in fibrosis caused by certain agents. This response may occur with or without an increase in total collagen content (Last, 1985). The high tensile strength of type I collagen is consistent with decreased lung compliance that occurs with pulmonary fibrosis.

Asbestos-Related Pulmonary Fibrosis

Asbestos is a general term referring to a large group of naturally occurring hydrated mineral silicates of serpentine and amphibole groups. These groups share a fibrous shape although each have different physical and chemical properties. The serpentine chrysotile is the most important form commercially, representing over 90% of the asbestos used in the U.S. Economically important amphiboles include crocidolite and amosite, whereas anthophyllite, actinolite, and tremolite have little economic value because of their limited availability and low tensile strength.

The crystal structures of the two general asbestiform minerals, the amphiboles and serpentine, are very different. The amphiboles form very long bands of linked SiO_4-tetrahedra, four tetrahedra wide. These bands run parallel to the fiber axis and are linked by cations (mainly Mg and Fe). The serpentine chrysotile are also made up of SiO_4-

tetrahedra but are linked to form wide thin sheets where the apical oxygens are part of a Mg-(O,OH) octahedra. In chrysotile asbestos, the layers form concentric cylinders with very high length to width ratio and the length parallel to the fiber axis.

Asbestosis (pneumoconiosis as a result of asbestos exposure) is a bilateral progressive diffuse interstitial fibrosis. In man, it also involves calcification and fibrosis of the pleura, bronchogenic carcinoma, and mesothelial tumors.

Macrophages appear to play an important role in asbestos-induced fibrosis by a two-stage mechanism (Lee, 1985). The first stage is the fiber-macrophage interaction, and the second involves a macrophage interaction. Short fibers are phagocytized completely and are retained in phagocytic vesicles and secondary lysosomes of the macrophage for prolonged periods of time. These fibers are ultimately cleared from the lung or degraded within the macrophage. Large asbestos fibers, such as those of the serpentine form, are surrounded by several macrophages. Each end of the fiber may remain outside the cells (Allison, 1977). Prolonged stimulation of macrophages by asbestos fibers may be a stimulus for chronic lysosomal enzyme release. Lysosomal enzymes may directly stimulate the fibroblast, or fibroblasts may be stimulated by products of tissue destruction or mediators released during an inflammatory response.

Control of Collagen Accumulation

The importance of the development of antifibrotic drugs is evident when one considers the high frequency of occurrence of fibrosis in Western society and the increasing death rate resulting from end-stage fibrosis of various organs. Clinical treatment of the disease often involves the use of steroids and other immunosuppressive agents although no drug has proven effacacious in controlled clinical trials (Fulmer and Crystal, 1978). Treatment of fibrotic diseases remains largely supportive.

A number of agents have been tested in experimental models to prevent the development of fibrosis. Last (1985) arbitrarily divided these studies into those which have focused upon attempts to alter collagen synthesis or processing and upon anti-inflammatory and immunosuppressive agents.

Various toxic agents have been used to induce a pulmonary fibrotic response to study the mechanisms involved in the disease as well as to investigate potential antifibrotic agents. These include numerous studies involving the administration of bleomycin (Kelley, Newman, and Evans, 1980; Thrall et al., 1979; Phan, Thrall, and Williams, 1981; Stering et al, 1982), ozone (Hesterberg and Last, 1981), silica (Reiser and Last, 1981; Levene, Bye, and Saffiotti, 1967), butylated hydroxytoluene + oxygen (Haschek

et al., 1982), paraquat (Greenberg, Lyons, and Last, 1978), and asbestos (Glassroth et al., 1984).

Proline analogs, such as cis-hydroxyproline and L-3,4-dehydroproline, have been used to prevent or ameliorate induced lung fibrosis (Riley, Kerr, and Yu, 1984; Kelley, Newman, and Evans, 1980). These molecules become incorporated into nascent procollagen polypeptides and do not allow the formation of the triple helical configuation of tropocollagen. These changes delay the secretion of collagen from cells and enhance its degradation intracellularly (Uitto and Prockop, 1974). The therapeutic potential of the proline analogs has come under some question because of their toxicities (Madden et al., 1973) and the lack of specificity for inhibiting collagen biosynthesis (Chvapil et al., 1975).

Beta-amino-proprionitrile (BAPN) has been shown to prevent collagen accumulation in different animal models of pulmonary fibrosis (Levene et al, 1967; Riley et al, 1982). BAPN inhibits lysyl oxidase and thus crosslinking of the collagen chains. This action of BAPN would enhance the extracellular degradation of collagen.

Steroidal and non-steroidal anti-inflammatory drugs have been studied for their ability to prevent lung fibrosis in animal models. Hesterberg and Last (1981) and Phan et al (1981) have reported that methylprednisolone was able to prevent collagen synthesis and its accumulation in the lung in ozone- and bleomycin-induced fibrosis. High doses (8 mg)

of triamcinolone was shown to prevent increases in collagen synthesis and accumulation in bleomycin-treated rats (Sterling et al, 1982). The prevention of collagen accumulation by steroids is mediated by both direct and indirect actions. Steroids act directly by inhibiting prolyl hydroxylase activity and net collagen synthesis (Ponec et al, 1979). The immune and inflammatory systems regulate fibroblast function and since steroids are known to have anti-inflammatory activity (Fauci, 1979), these agents indirectly affect collagen metabolism.

Pirfenidone

Pirfenidone (1-phenyl, 5-methyl, 2-pyridone) was developed as a nonsteroidal anti-inflammatory drug and used in this research to attempt to affect the fibrotic response induced by asbestos. The structure of pirfenidone is shown in Figure 1. It has been shown to be effective in acute tests for anti-inflammatory activity such as the carrageenan paw edema and adjuvant arthritis tests (Munroe, personal communication). In other studies, rats and dogs with chronic pneumonitis and interstitial pulmonary hyperplasia were given pirfenidone in the diet. The results showed significant reductions in interstitial hyperplasia and the accumulation of thick fibrous bands. Pirfenidone did not induce gastric mucosal ulcerations as do many other nonsteroidal anti-inflammatory drugs (Munroe, personal communication).

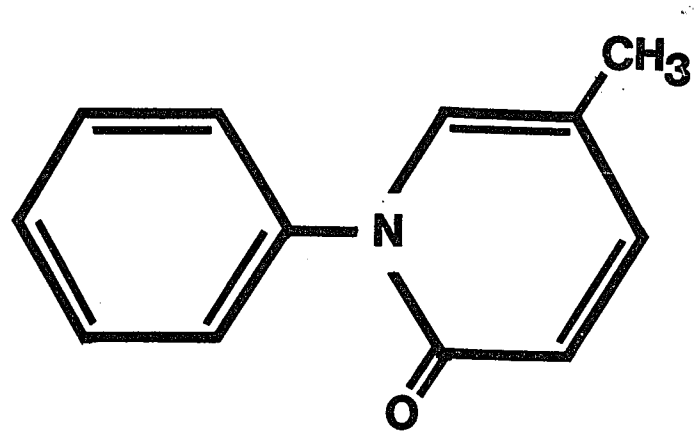

Figure 2. Structure of Pirfenidone
(1-phenyl, 5-methyl, 2-pyridone)

Statement of Research Problem

The objective in this research was to study the development of pulmonary fibrosis in hamsters induced by intratracheally instilled chrysotile asbestos and evaluate the efficacy of pirfenidone in the amelioration of the fibrosis. The objectives were accomplished by monitoring lung weights, enzymes in lung lavage fluid, biochemical indicators of collagen biosynthesis (prolyl hydroxylase activity) and collagen accumulation (hydroxyproline content of the lungs), and histopathology.

MATERIALS AND METHODS

Test Materials

Chrysotile asbestos fiber (Calidria, Lot RG-144), supplied by Hunterdon Pharmaceuticals, Inc., was obtained from Union Carbide. Asbestos suspensions were made in 0.9% NaCl.

Pirfenidone (Lot 4392) was supplied by Hunterdon Pharmaceuticals, Inc. Pirfenidone was not soluble in water or saline, but was slightly soluble in various oils. Homogenous dosing suspensions were prepared by adding a preweighed amount of pirfenidone to safflower oil and stirring overnight.

Cortisone acetate (Lot L-507), supplied by Hunterdon Pharmaceuticals, Inc., was obtained from Merck, Sharp, and Dohme Research Labs. Homogenous dosing suspensions were prepared by adding a preweighed amount of cortisone to safflower oil and stirring for a few hours.

Animals

Male and female Golden Syrian hamsters, age 6 weeks and weighing an average of 88 and 90 g respectively, were obtained from Charles River Breeding Laboratories, Inc. The hamsters were housed in polycarbonate cages on autoclaved

aspen bedding. Wayne Lab Blox (Allied Mills, Chicago, IL) and tap water were available ad libitum. The animals were isolated for acclimation and observation of ill health for 2 weeks prior to instillation of asbestos. The animals were housed in a temperature-controlled room at 72 $\pm$ 4^{o}F and maintained on a 12-hour light/dark cycle.

The animals were randomly assigned to the following test groups:

Group 1: No asbestos instilled, pirfenidone or cortisone.

Group 2: Asbestos instilled, no pirfenidone or cortisone.

Group 3: Asbestos instilled, 25 mg/kg pirfenidone given daily for 10 - 40 days beginning at 60 days after asbestos instillation.

Group 4: Asbestos instilled, 250 mg/kg pirfenidone given daily for 10 - 40 days beginning at 60 days after asbestos instillation.

Group 5: Asbestos instilled, 500mg/kg pirfenidone given daily for 10 - 40 days beginning at 60 days after asbestos instillation.

Group 6: Asbestos instilled, 1 mg cortisone given daily for 10 - 40 days beginning at 60 days after asbestos instillation.

Groups of hamsters were terminated for necropsy and measurement of biochemical and histological parameters according to the following schedule:

Days After Asbestos	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
30	6	10	0	0	0	0
45	6	10	0	0	0	0
60	6	10	0	0	0	0
70	6	10	10	10	10	10
80	6	10	10	10	10	10
90	6	10	10	10	10	10
100	6	10	10	10	10	10

In each group, half of the animals terminated were male and half were female.

Dosing Procedures

Hamsters were lightly anesthetized with 1% methohexital i.p. (Brevital, Eli Lilly & Co.) at a dose of 45 mg/kg. Chrysotile asbestos fiber (0.5 mg) was instilled intratracheally in 0.15 ml saline (Brain et al, 1976). This dosing procedure was repeated 2 times per week for 2 weeks. Each asbestos treated animal received a total dose of 2 mg asbestos.

Sixty days after asbestos instillation, hamsters in groups 3, 4, and 5 received 25, 250, and 500 mg/kg/day pirfenidone respectively, by gavage in a volume of safflower oil such that a 150 g hamster received 1.0 ml of the solution. Group 6 animals received 1 mg cortisone in

safflower oil. Pirfenidone- and cortisone-treated groups were given daily injections p.o. for 10 - 40 days.

Safe Handling Procedures

To minimize the risk of human exposure, asbestos fiber suspensions were prepared under a hood. Persons in contact with asbestos in any way wore a mask, gloves, and eye protection at all times.

Body Weights

Animals were weighed before each instillation of asbestos and then weekly throughout the study.

Necropsy

Each hamster was anesthetized with 12.5 mg sodium pentobarbital. Blood was taken by cardiac puncture. The animal was given more pentobarbital for euthanasia. An incision was made to expose the lung and trachea. The trachea was clamped using hemostats above the fifteenth cartilage ring distal to the bronchial bifurcation. The lungs and heart were removed intact with larynx and trachea. Sections of liver, heart, kidneys, pancreas, and stomach were removed and placed in formalin.

Lung Weight Determination

The lungs were dipped in cold saline to rinse and blotted dry on a damp towel. The lungs, with trachea intact, were weighed. After lavage, the trachea, esophagus, and

remaining connective and fatty tissues were removed and weighed. The corrected lung weight was determined by subtracting this value from the total combined weight of lung, trachea, esophagus, and connective tissues. The left diaphrammatic lobe was excised so that it remained inflated with saline. This lobe and sections of other lobes were used for histopathology. The right cardiac lobe of the lung was used to obtain wet lung weight/dry lung weight ratios. This lobe was weighed to the nearest 0.0001 g and dried for approximately 4 days in an oven at 80°C so that the weights were within 0.0005 g on two consecutive days.

Lavage Procedure

A polyethylene catheter (18 G) with syringe attached was introduced into the trachea and tied in place. The lungs were inflated with 5 ml saline. While inflated, the left diaphrammatic lobe was tied off with a single ligature. The remaining lobes were lavaged twice with 5 ml saline. Immediately after its collection, the lavage fluid was poured into a plastic centrifuge tube which was placed on ice. The lavage fluid samples were centifuged at 400 X g for 20 minutes at 4°C. The cell pellet was discarded and the supernatant was stored at 4°C.

Evaluation of Enzymes in Lung Lavage Fluid

Lactate dehydrogenase (LDH). The assay for LDH in bronchoalveolar lavage fluid was performed according to Sigma

Technical Bulletin #340-UV (1983) and utilizing an assay kit from Sigma Chemical Company (St. Louis, MO) designed to determine LDH in serum samples. Lavage fluid was added instead of serum in the Sigma procedure. In this procedure, the activity of LDH was determined by monitoring the rate at which the substrate, pyruvate, is reduced to lactate. The reduction of pyruvate is coupled with the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH). NADH has a maximal absorbance at 340nm so that the reduction in absorbance due to NADH oxidation can be monitored spectrophotometrically.

$$\text{Pyruvate} + \text{NADH} \text{ -------> } \text{Lactate} + \text{NAD}^+$$

In duplicate for each animal, NADH and a 0.05 ml aliquot of lavage fluid were added to a test tube. Pyruvate was added and absorbance at 340nm recorded as a function of time using a Beckman Acta C II spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The rate of decrease of A340 was used to calculate the units of LDH per ml of lavage fluid.

Alkaline phosphatase. The assay for alkaline phosphatase in bronchoalveolar lavage fluid was performed according to Sigma Technical Bulletin #104 (1982), utilizing an assay kit from Sigma Chemical Company. Upon hydrolysis of p-nitrophenol phosphate by phosphatase, p-nitrophenol and inorganic phosphate are formed. When made alkaline, p-nitrophenol is

converted to a yellow complex which is readily measured at 420nm. The level of alkaline phosphatase activity is measured by the intensity of the yellow color produced when alkali is added. The Sigma procedure, designed for serum was modified by substituting 0.1 ml lavage fluid for serum and bringing the range of the standard curve down to 0.25 Sigma units of alkaline phosphatase activity per ml lavage fluid. The thirty minute incubation method was used. A standard curve was run along with lavage fluid samples. Absorbance at 420nm was measured using a Gilford Stasar II (Gilford Instrument Laboratories, Oberlin, OH). Units of alkaline phosphatase per ml lavage fluid were determined from the standard curve.

Acid phosphatase. The assay for acid phosphatase in lung lavage fluid was performed according to Sigma Technical Bulletin #104 (1982) utilizing an assay kit from Sigma Chemical Company. The acid phosphatase assay is based on the same principle as the assay for alkaline phosphatase. Lavage fluid was substituted for serum and the standard curve was always run along with the lavage fluid samples. Absorbance at 400nm was measured using a Gilford Stasar II spectrophotometer. Units of acid phosphatase per ml lavage fluid were determined from the standard curve.

Biochemical Parameters

Prolyl Hydroxylase Activity Determination

Preparation of labelled protocollagen substrate. Tritium labeled protocollagen was prepared from chick embryos as modifications from Hutton, Tappel, and Udenfriend (1966) and Peterkofsky and DiBlasio (1975). Frontal and thoracic bones (approximately 2 g) from 13 to 15 day old chick embryos were incubated at 37°C with 200 uCi of L-[3,4- H]proline in incubation media containing 0.154M NaCl, 0.1M CaCl2.2H2O, 0.154M KCl, 0.154M MgSO4, 0.12M HEPES pH 7.4, and 2.29M glucose and in the presence of 5mM ascorbic acid, ampicillin, and 1mM 2,2'-dipyridyl for 3 hours. The pulse-labeled bones were washed and rinsed with copious amounts of cold saline and homogenized in 0.5M acetic acid with a Polytron (Brinkmann Instruments, Rexdale, Ontario) twice at 15 second intervals. The resulting suspension was allowed to stir overnight at 4°C then centrifuged at 15,000 X G for 60 minutes. The supernatant was dialyzed with stirring with several changes of 0.01M acetic acid at 4°C for 2-3 days. The supernatant contained the labeled protocollagen and was stored in small aliquots at -18°C. Linear formation of hydroxylated collagen was established as a function of incubation time and enzyme concentration for the substrate preparations.

Preparation of 9000g supernatant. The right diaphrammatic lobe of the lung was minced and washed in

saline then homogenized twice for 15 second intervals with a Polytron in three volumes of cold 1.15% KCl-0.05M Tris-HCl buffer (pH 7.4). The resulting homogenate was centrifuged at 9,000 x g for 15 minutes. The supernatant protein concentration was measured as described by Lowry (1951) with bovine serum albumin as the protein standard.

Prolyl hydroxylase assay. The activity of prolyl hydroxylase was estimated by the release of tritiated water via the hydroxylation of labeled protocollagen (Peterkofsky and DiBlasio, 1975). The standard assay mixture consisted of 0.025 ml of a cofactor solution containing 40mM Tris-HCl, 1.0mM -ketoglutarate, 1.0 mM sodium ascorbate, 0.2mM ferrous ammonium sulfate, 0.5mM dithiothreitol, 0.4 mg/ml catalase, and 2.0 mg/ml bovine serum albumin, 0.025 ml (approximately 31,000 dpm) labeled protocollagen, and 0.020 ml of the 9,000g supernatant containing the prolyl hydroxylase activity. Incubation was performed at 37°C for 25 minutes and was terminated with 0.4 ml trichloroacetic (6.25%), followed by 0.025 ml of bovine serum albumin (15 mg/ml) as a coprecipitant. After centrifugation at 2000 x g for 4 minutes, the supernatant was eluted through a 0.5 x 2 cm column of washed BioRad AG50W-X8 strong cation exchange resin (BioRad Laboratories # 142-1441, Richmond, CA). The precipitate was resuspended in 0.5 ml of 5% trichloroacetic acid and resedimented. The supernatant was again applied to the column followed by washing with 0.5 ml deionized water.

The effluent was dissolved in 10 ml of Aquasol (New England Nuclear, Boston, MA) and counted in a Beckman LS2800 scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Hydroxyproline Determination

The hydroxyproline content of the lungs was measured spectophotometrically as described by Woessner (1961). A lobe was weighed and minced and then hydrolyzed in 6N HCl at 110°C for 18 hours in a Pyrex culture tube with a Teflon^{R}-lined screw cap. The hydrolysate was neutralized with 2N and 0.1N NaOH and diluted with deionized water to 15 ml. The diluted hydrolysate was filtered and a 1 ml aliquot was used in the assay. One ml of 0.05M Chloramine-T was added to each tube and shaken to mix. This mixture was allowed to stand for 20 minutes then 1 ml 3.15M perchloric acid was added, the tube vortexed and allowed to stand at least 5 minutes. One ml of p-dimethylaminobenzaldehyde was added and mixed by inversion. The tubes were heated for 20 minutes in a 60°C water bath then cooled in tap water. The color was read spectrophotometrically at 561nm using a Gilford Stasar II spectrophotometer.

Histopathology

The grading of inflammatory lesions and counting of collagen and reticulin fibers was performed by Dr. Susan E. Wilson, DVM, in the Division of Animal Resources, College of

Medicine at the Arizona Health Science Center.

After fixation of organs in 10% buffered formalin for 48 hours, sections of each organ were embedded in paraffin. Five micron sections were made and stained with hematoxylin and eosin. Collagen stains (Masson's Trichrome and Gomori's Method for Reticulin) were performed on sections of lung from each animal.

Histological Evaluation of Inflammation and Fibrosis

Hematoxylin and eosin-stained sections were graded for inflammatory changes. These changes were graded qualitatively on a scale of 1 to 5 to indicate changes ranging from minimal to severe respectively. At 90 and 100 days after asbestos instillation, a range of up to 7 was used to indicate increased severity of lesions over those observed at earlier time points. Individual scores for each inflammatory parameter were added to yield one score for the organ. The parameters scored for inflammatory changes in the lung, kidney, and liver are shown in figures 3, 4, and 5, respectively.

Because of the importance of monitoring changes in fibrosis in this study, and the problems associated with qualitative grading systems, two new methods of evaluating the presence of collagen were devised:

1. Large collagen fibers, numbers per high power field.

   The number of large collagen fiber bundles (which stain

1) General increase in macrophages
2) Multifocal desquamative alveolitis
3) Chronic bronchiolitis
4) Lymphoid hyperplasia
5) Perivascular cuffing with lymphocytes
6) Increase in alveolar wall thickness
7) Consolidation
8) Bronchial exudates in general
9) Pneumocyte hyperplasia
10) Proliferative bronchiolitis

Figure 2. Histological Parameters for Lung Inflammation Scoring

1) Pyknosis of collecting duct nuclei
2) Congestion
3) Tubular dilation
4) Regenerating tubular epithelium
5) Casts
6) Fibrosis in glomeruli and interstitium
7) Chronic inflammation
8) Glomerular tufts thickened

Figure 3. Histological Parameters
for Kidney Lesion Scoring

1) Periportal infiltrates
2) Biliary hyperplasia
3) Chronic hepatitis
4) PMN infiltrates
5) Congestion
6) Fibrosis

Figure 4. Histological Parameters for Liver Lesion Scoring

blue with trichrome) were counted for each of 7, 400x fields. The average number was obtained for an index. The fibers were counted by scanning randomly selected fields using a Marbel Blood-Cell Counter. This score gives an average of both severity and distribution of fibrosis by quantitative means (Wilson, personal communication).

2. Large reticulin fibers, number per high power field. Seven randomly selected fields with evidence of fibrosis were evaluated for numbers of large reticulin fibers (stain black with Gomori's method). This evaluation differs from #1 in that only affected fields were counted. Counting was done as described for #1.

RESULTS

Wet Lung Weight to Body Weight Ratios

Means ± SEMs for wet lung weight to body weight ratios in male and female groups are presented in tables 1 and 2, respectively.

30 Days. The ratios for male and female asbestos-instilled animals were significantly increased over their respective nontreated control groups.

45 Days. The ratios for male and female asbestos-instilled animals were significantly increased over their respective nontreated control groups.

60 Days. The ratios for the asbestos-instilled animals of both sexes were increased but only the increase in the male ratio was statistically significant. The significant increase in the male ratio is due to a decrease in the wet lung weight of the animals in the male group.

70 Days. The ratios for male asbestos-instilled groups were increased significantly over the nontreated control group. No changes in the ratios were observed in pirfenidone or cortisone treated groups from the asbestos-instilled only groups at 70 days. There were no differences in female groups at this time point.

Table 1

MALES

Wet Lung Weight/Body Weight Ratios[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	$6.5\pm.41^{A}$	$5.7\pm.21^{A}$	$3.8\pm.40^{A}$	$4.8\pm.05^{A}$	$4.8\pm.46^{A}$	$4.6\pm.20^{A}$	$5.1\pm.24^{A}$
2	$8.8\pm.17^{B}$	$8.2\pm.72^{B}$	$7.2\pm.59^{B}$	$7.3\pm.30^{B}$	$8.5\pm.38^{BC}$	$7.2\pm.36^{B}$	$8.0\pm.76^{BC}$
3	--	--	--	$7.2\pm.56^{B}$	$7.6\pm.38^{BC}$	$10\pm.65^{C}$	$8.2\pm.47^{BC}$
4	--	--	--	$6.7\pm.52^{B}$	9.5 ± 1.5^{C}	8.6 ± 1.0^{BC}	$9.5\pm.49^{C}$
5	--	--	--	--[3]	$7.7\pm.45^{B}$	$7.7\pm.85^{B}$	$8.1\pm.83^{BC}$
6	--	--	--	$6.2\pm.33^{AB}$	$6.4\pm.51^{AB}$	$6.6\pm.24^{B}$	$6.5\pm.22^{AB}$

[1] Values represent the mean (10^{-3}) $\pm$ SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

Table 2

FEMALES

Wet Lung Weight/Body Weight Ratios[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	$6.7\pm.18^{A}$	$5.5\pm.06^{A}$	$5.7\pm.65^{A}$	$5.4\pm.14^{A}$	$4.9\pm.59^{A}$	$5.1\pm.78^{A}$	$5.4\pm.13^{A}$
2	$8.6\pm.44^{B}$	$9.0\pm.65^{B}$	$7.1\pm.65^{A}$	$7.0\pm.30^{A}$	$8.6\pm.81^{B}$	$7.3\pm.56^{A}$	$7.4\pm.60^{AB}$
3	--	--	--	$6.6\pm.15^{A}$	$7.5\pm.28^{B}$	$7.4\pm.52^{A}$	$8.6\pm.28^{B}$
4	--	--	--	$7.1\pm.34^{A}$	$7.9\pm.34^{B}$	$7.1\pm.28^{A}$	$7.0\pm.38^{AB}$
5	--	--	--	--[3]	$6.9\pm.06^{B}$	$7.4\pm.56^{A}$	$7.1\pm.39^{AB}$
6	--	--	--	$6.3\pm.39^{A}$	$7.3\pm.52^{B}$	$6.7\pm.25^{A}$	$6.7\pm.49^{AB}$

[1] Values represent the mean (10^{-3}) $\pm$ SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

80 Days. The ratios for all asbestos-instilled groups of both sexes were significantly increased over the respective nontreated control groups. The male group treated with cortisone was not significantly different from nontreated or asbestos-instilled only group. There were no significant differences in the ratios for pirfenidone or cortisone treated groups from the asbestos-instilled only groups although the ratios in the female animals treated with pirfenidone tended to decrease at this timepoint.

90 Days. The ratios for the male asbestos-instilled animals were significantly increased over the nontreated group. The ratio for the male group given 25 mg/kg pirfenidone (group 3) was significantly increased over the group given asbestos only. No other differences were seen in male groups given pirfenidone. No differences in the ratios were observed in the female groups at 90 days.

100 Days. All male asbestos-instilled groups had ratios which were significantly increased over the nontreated group. No other differences were observed. In female groups, the ratios for the group given pirfenidone (25 mg/kg) were significantly increased over nontreated controls. No other differences were observed among female groups. Overall, the cortisone-treated groups (both male and female) seemed to have decreased wet lung weight to body weight ratios at all time points as compared to groups given

asbestos only. These differences were shown not to be statistically different.

Dry Lung Weight to Body Weight Ratios

Means ± SEM's for dry lung weight to body weight ratios are presented in tables 3 and 4 for males and females respectively.

30 Days. The ratios for the asbestos-instilled male and female animals were increased over non-treated controls. This increase was only statistically significant in the female animals, whereas increased dry lung weights in the male control group caused increased ratios and thus a less than significant increase in the group 2 ratios.

45 Days. The ratios for the asbestos-instilled animals, both male and female, were significantly increased over the non-treated controls.

60 Days. The lung dry weight to body weight ratios were increased in asbestos-instilled animals of both species although this increase was statistically significant in male animals.

70 Days. Although the dry lung weight to body weight ratios tended to increase in asbestos-instilled animals at 70 days, these changes were not statistically significant.

80 days. The male group instilled with asbestos and given pirfenidone (500 mg/kg) had significantly increased ratios over the non-treated control group. No other

Table 3

MALES

Dry Lung Weight/Body Weight Ratios[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	7.3 ± 3.1^{A}	$2.4\pm.46^{A}$	$3.7\pm.56^{A}$	$4.9\pm.33^{A}$	$3.3\pm.12^{A}$	$4.2\pm.46^{A}$	$3.6\pm.57^{A}$
2	12.6 ± 1.6^{A}	11.4 ± 1.7^{B}	7.6 ± 1.0^{B}	9.2 ± 1.9^{A}	6.9 ± 1.0^{AB}	$6.4\pm.47^{A}$	6.1 ± 1.1^{B}
3	--	--	--	7.6 ± 1.5^{A}	7.2 ± 1.8^{AB}	15.0 ± 4.2^{A}	$6.7\pm.71^{B}$
4	--	--	--	$6.5\pm.60^{A}$	8.7 ± 2.4^{AB}	7.9 ± 1.3^{A}	$8.8\pm.63^{B}$
5	--	--	--	--[3]	9.4 ± 3.2^{B}	$6.0\pm.60^{A}$	$7.5\pm.61^{B}$
6	--	--	--	$6.1\pm.40^{A}$	6.6 ± 1.4^{AB}	6.7 ± 1.4^{A}	$6.3\pm.96^{AB}$

[1] Values represent the mean (10^{-4}) $\pm$ SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

Table 4

FEMALES

Dry Lung Weight/Body Weight Ratios[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	5.9±.20^{A}	4.2±.48^{A}	5.8±.90^{A}	5.3±.54^{A}	4.0±.38^{A}	4.9±1.5^{A}	4.4±.45^{A}
2	12.7±1.9^{B}	11.9±1.6^{B}	8.0±.67^{B}	6.3±1.4^{A}	9.1±1.5^{A}	8.2±.93^{A}	6.2±.79^{A}
3	--	--	--	7.1±.66^{A}	5.5±.51^{A}	8.3±1.0^{A}	6.6±.67^{A}
4	--	--	--	7.5±.53^{A}	6.5±.51^{A}	7.5±.06^{A}	4.9±.48^{A}
5	--	--	--	--[3]	5.9±.48^{A}	6.5±1.2^{A}	6.0±1.0^{A}
6	--	--	--	5.9±.37^{A}	7.2±.64^{A}	8.2±2.1^{A}	5.1±.69^{A}

[1] Values represent the mean (10^{-4}) ± SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

significant differences were observed among other groups at 80 days.

90 Days. Significant changes in the male or female ratios were not observed at 90 days after instillation of asbestos.

100 Days. The ratios of asbestos-instilled only male animals (Group 2) and asbestos-instilled male animals given pirfenidone (Groups 3, 4, and 5) were significantly increased over non-treated controls. No significant differences in the ratios were seen among female groups at 100 days.

Enzymes in Lung Lavage Fluid

The units used in presenting the results of the lung lavage studies are Sigma units of enzyme activity per ml of lavage fluid.

Lactate Dehydrogenase

The results of the assay for LDH in lung lavage fluid of male and female groups are presented in tables 5 and 6, respectively. Group results are presented as mean $\pm$ SEM for each time point. Analysis for LDH levels was performed at each termination day. A Sigma unit of LDH as defined in Sigma Technical Bulletin #340-UV will cause a decrease in the absorbance at 340nm of 0.001 per minute at 25^{o}C in a 3-ml reaction mixture in a cuvet of 1-cm lightpath. Sigma units of LDH activity can be converted to international units (IU) by multiplying by 0.48.

Table 5

MALES

Lactate Dehydrogenase Activity in Lung Lavage Fluid[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	61 ± 20^{A}	119 ± 23^{A}	159 ± 8^{A}	81 ± 17^{A}	75 ± 0^{A}	102 ± 17^{A}	31 ± 16^{A}
2	185 ± 35^{B}	165 ± 15^{A}	312 ± 101^{A}	360 ± 66^{B}	113 ± 17^{A}	144 ± 21^{A}	120 ± 17^{A}
3	--	--	--	417 ± 151^{B}	163 ± 61^{A}	182 ± 26^{A}	76 ± 7^{A}
4	--	--	--	200 ± 78^{AB}	269 ± 76^{A}	195 ± 47^{A}	178 ± 74^{A}
5	--	--	--	--[3]	182 ± 140^{A}	350 ± 224^{A}	68 ± 39^{A}
6	--	--	--	188 ± 62^{AB}	129 ± 40^{A}	288 ± 87^{A}	105 ± 31^{A}

[1] Values represent the mean (Sigma units/ml lavage fluid) $\pm$ SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

Table 6

FEMALES

Lactate Dehydrogenase Activity in Lung Lavage Fluid[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	79 ± 10^{A}	101 ± 12^{A}	162 ± 20^{A}	80 ± 5^{A}	54 ± 3^{A}	75 ± 3^{A}	38 ± 19^{A}
2	371 ± 53^{B}	162 ± 56^{A}	295 ± 64^{B}	115 ± 18^{A}	82 ± 14^{A}	117 ± 12^{A}	94 ± 21^{A}
3	--	--	--	53 ± 5^{A}	92 ± 34^{A}	137 ± 15^{A}	69 ± 6^{A}
4	--	--	--	108 ± 36^{A}	111 ± 46^{A}	246 ± 99^{A}	58 ± 16^{A}
5	--	--	--	--[3]	206 ± 165^{A}	135 ± 14^{A}	83 ± 21^{A}
6	--	--	--	116 ± 12^{A}	152 ± 76^{A}	212 ± 87^{A}	97 ± 30^{A}

[1] Values represent the mean (Sigma units/ml lavage fluid) $\pm$ SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

30 Days. LDH levels were significantly increased in asbestos-instilled male and female animals (Group 2) over non-treated contols (Group 1). The LDH levels in group 2 female animals were also significantly increased over the group 2 males.

45 Days. The increases in LDH levels in the lung lavage fluid of asbestos-instilled animals was not statistically significant.

60 Days. The levels of LDH were significantly increased in asbestos-instilled female animals over the non-treated control group. Although not statistically significant, the LDH level in group 2 males was increased over the nontreated control group at 60 days.

70 Days. LDH levels in the lung lavage fluid for the male asbestos-instilled only animals (group 2) and male animals given asbestos and treated with 25 mg/kg pirfenidone (group 3) were significantly increased over non-treated controls (group 1). Male animals given 250 mg/kg pirfenidone or 1 mg cortisone were not different than non-treated or asbestos-instilled only groups. There were no differences in LDH levels among female groups.

80 to 100 Days. There were no significant differences in LDH levels between treatment groups of either sex at the 80, 90, and 100 day timepoints.

Alkaline phosphatase

The results from the assay for alkaline phosphatase in lung lavage fluid are presented as treatment group means ± SEM for male and female groups for each timepoint in tables 7 and 8. The alkaline phosphatase assay was done on alternate termination days (30, 60, 80, and 100 days). A Sigma unit of alkaline phosphatase activity is defined in Sigma Technical Bulletin #104 as that amount of enzyme activity that will liberate 1 umole of p-nitrophenol per hour at 37°C.

30 Days. Alkaline phosphatase levels in male and female asbestos-instilled groups were significantly increased over the nontreated groups. The levels in the female asbestos-instilled group were significantly increased over the respective male group.

60, 80, and 100 Days. There were no significant differences in alkaline phosphatase levels in lavage fluid between groups at 60 and 80 days. At 100 days, the levels of alkaline phosphatase in lavage fluid were undetectable in all groups.

Acid Phosphatase

The results of the assay for acid phosphatase levels in lung lavage fluid are presented as treatment group means ± SEM for each time point for males and females in Tables 9 and 10. The assay for acid phosphatase activity was performed on alternate sacrifice days (30, 60, 80, and 100 days).

Table 7

MALES

Alkaline Phosphatase Activity in Lung Lavage Fluid[1]

Group[2]	Days After Asbestos Instillation 30	60	80	100
1	0.29±.05^{A}	0.49±.02^{A}	0.39±.04^{A}	ND
2	0.47±.07^{B}	0.69±.06^{A}	0.48±.03^{A}	ND
3	--	--	0.48±.05^{A}	ND
4	--	--	0.93±.27^{A}	ND
5	--	--	0.59±.13^{A}	ND
6	--	--	0.54±.09^{A}	ND

[1] Values represent the mean (Sigma units/ml lavage fluid) ± SEM of 3-5 animals (ND = level below lower limit of sensitivity). The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus pirfenidone (25mg/kg); 4:asbestos plus pirfenidone (250 mg/kg; 5:asbestos plus pirfenidone (500 mg/kg); 6:asbestos plus cortisone (1 mg/day).

Table 8

FEMALES

Alkaline Phosphatase Activity in Lung Lavage Fluid[1]

Group[2]	Days After Asbestos Instillation 30	60	80	100
1	0.25±.05^{A}	0.52±.01^{A}	0.41±.04^{A}	ND
2	1.34±.14^{B}	0.73±.15^{A}	0.44±.04^{A}	ND
3	--	--	0.47±.02^{A}	ND
4	--	--	0.39±.02^{A}	ND
5	--	--	0.61±.18^{A}	ND
6	--	--	0.48±.03^{A}	ND

[1] Values represent the mean (Sigma units/ml lavage fluid) ± SEM of 3-5 animals (ND = level below lower limit of sensitivity. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus pirfenidone (25 mg/kg); 4:asbestos plus pirfenidone (250 mg/kg) 5:asbestos plus pirfenidone (500 mg/kg); 6:asbestos plus cortisone (1 mg/day).

Table 9

MALES

Acid Phosphatase Activity in Lung Lavage Fluid[1]

	Days After Asbestos Instillation			
Group[2]	30	60	80	100
1	$0.15 \pm .02^{A}$	$0.12 \pm .01^{A}$	$0.24 \pm .01^{A}$	ND
2	$0.18 \pm .04^{A}$	$0.19 \pm .03^{A}$	$0.21 \pm .03^{A}$	ND
3	--	--	$0.25 \pm .05^{A}$	ND
4	--	--	$0.28 \pm .05^{A}$	ND
5	--	--	$0.24 \pm .05^{A}$	ND
6	--	--	$0.27 \pm .02^{A}$	ND

[1] Values represent the mean (Sigma units/ml lavage fluid) $\pm$ SEM of 3-5 animals (ND = level below lower limit of sensitivity). The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus pirfenidone (25 mg/kg); 4:asbestos plus pirfenidone (250 mg/kg); 5:asbestos plus pirfenidone (500 mg/kg); 6:asbestos plus cortisone (1 mg/day).

Table 10

FEMALES

Acid Phosphatase Activity in Lung Lavage Fluid[1]

	Days After Asbestos Instillation			
Group[2]	30	60	80	100
1	$0.18 \pm .02^{A}$	$0.16 \pm .05^{A}$	$0.21 \pm .02^{A}$	ND
2	$0.29 \pm .04^{A}$	$0.15 \pm .03^{A}$	$0.21 \pm .01^{A}$	ND
3	--	--	$0.21 \pm .02^{A}$	ND
4	--	--	$0.21 \pm .02^{A}$	ND
5	--	--	$0.24 \pm .06^{A}$	ND
6	--	--	$0.21 \pm .02^{A}$	ND

[1] Values represent the mean (Sigma units/ml lavage fluid) $\pm$ SEM of 3-5 animals (ND = levels below lower limit of sensitivity). The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus pirfenidone (25 mg/kg); 4:asbestos plus pirfenidone (250 mg/kg); 5:asbestos plus pirfenidone (500 mg/kg); 6:asbestos plus cortisone (1 mg/day).

There were no significant differences in acid phosphatase levels in the lavage fluid at any time point. Levels of acid phosphatase in lavage fluid were not detectable at 100 days after the instillation of asbestos.

Biochemical Parameters

Prolyl Hydroxylase Activity

Prolyl hydroxylase activity in the lung was determined as a measure of collagen synthesis. The results from this determination are presented in tables 11 and 12 for male and female groups, respectively.

The prolyl hydroxylase activity in the lung of asbestos-instilled male animals was significantly increased over non-treated controls at 30 and 60 days. The values for prolyl hydroxylase activity at 45 days post-instillation were abnormally high. Since the 45 day animals lung homogenates were run through the prolyl hydroxylase assay well before the other groups, these differences may be due to the increased radioactivity of the substrate. No other differences in this parameter were observed at other time points. Pirfenidone and cortisone treatment of asbestos-instilled animals did not affect prolyl hydroxylase activity.

Hydroxyproline Content

To investigate the development of pulmonary fibrosis and the efficacy of pirfenidone and cortisone in modulating the fibrotic response, lung collagen was estimated by the

Table 11

MALES

Prolyl Hydroxylase Activity[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	1.9±.40^{A}	8.6±1.6^{A}	2.4±.07^{A}	4.0±.05^{A}	2.5±.33^{A}	3.5±.27^{A}	3.1±.65^{A}
2	4.4±.60^{B}	6.5±.59^{A}	3.6±.07^{B}	4.3±.51^{A}	4.7±.23^{AB}	4.2±.44^{A}	3.8±.60^{A}
3	--	--	--	4.5±.26^{A}	4.2±.35^{AB}	3.2±.61^{A}	3.8±.31^{A}
4	--	--	--	5.2±.42^{A}	5.5±1.4^{B}	3.9±.58^{A}	3.6±.30^{A}
5	--	--	--	--[3]	3.4±.31^{A}	3.3±.24^{A}	5.1±.42^{A}
6	--	--	--	4.0±.42^{A}	3.2±.30^{A}	4.1±.45^{A}	4.0±.72^{A}

[1] Values represent the mean (dpm released X 10^3/mg lung protein) ± SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

Table 12

FEMALES

Prolyl Hydroxylase Activity[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	2.8±1.1[A]	5.0±.34[A]	2.7±.24[A]	4.1±.45[A]	3.3±.89[A]	4.0±.31[A]	4.9±.27[A]
2	3.9±.18[A]	5.5±.67[A]	3.0±.09[A]	4.3±.38[A]	3.1±.41[A]	4.6±.47[A]	4.5±.27[A]
3	--	--	--	4.3±.82[A]	3.0±.61[A]	4.7±.56[A]	5.0±.70[A]
4	--	--	--	4.7±.18[A]	2.7±.44[A]	4.9±.80[A]	3.6±.10[A]
5	--	--	--	--[3]	3.3±.52[A]	4.9±.94[A]	4.2±.44[A]
6	--	--	--	5.7±.32[A]	3.1±.68[A]	5.2±.21[B]	3.3±.51[A]

[1] Values represent the mean (dpm released X 10^3/mg lung protein) ± SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

quantitation of total lung hydroxyproline. The results of lung hydroxproline content determination for male and female groups are presentedin tables 13 and 14.

The hydroxyproline content of the lungs was significantly increased over non-instilled controls in male and female animals at 30 days post-instillation. The increases in hydroxyproline content observed at 45 and 60 days were not significant statistically.

70 Days. No significant changes were observed in lung hydroxyproline content among male groups. The hydroxyproline content of the female group treated with pirfenidone (25 mg/kg) were significantly increased over other female groups treated with pirfenidone and the non-treated control group.

80 Days. Males treated with pirfenidone (250 mg/kg) had significantly increased lung hydroxyproline content over nontreated control males. The other male groups were increased over the control group but these increases were not statistically significant. Lung hydroxyproline of asbestos-instilled only females was significantly increased over the non-treated control group at 80 days. No significant changes from asbestos-instilled only females were observed in female groups given pirfenidone or cortisone.

90 Days. The lung hydroxyproline content was significantly increased in group 3 males over non-treated controls. No other differences were seen in this parameter

Table 13

MALES

Hydroxyproline Content in Lung[1]

Group[2]	Days After Asbestos Instillation 30	45	60	70	80	90	100
1	0.33±.03^{A}	0.50±.14^{A}	0.43±.06^{A}	0.73±.03^{A}	0.31±.13^{A}	0.58±.03^{A}	0.64±.07^{A}
2	0.51±.06^{B}	0.64±.04^{A}	0.62±.04^{A}	1.07±.23^{A}	1.00±.25^{AB}	0.85±.14^{A}	0.90±.15^{A}
3	--	--	--	1.00±.10^{A}	0.64±.12^{AB}	1.51±.10^{B}	1.11±.07^{A}
4	--	--	--	0.89±.25^{A}	1.18±.10^{B}	1.05±.10^{AB}	1.11±.12^{A}
5	--	--	--	--[3]	0.80±.28^{AB}	0.99±.16^{AB}	1.00±.08^{A}
6	--	--	--	0.71±.42^{A}	0.83±.12^{AB}	0.84±.21^{A}	0.88±.19^{A}

[1] Values represent the mean (mg hydroxyproline/lung) ± SEM of 3- 5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

Table 14

FEMALES

Hydroxyproline Content in Lung[1]

Days After Asbestos Instillation

Group[2]	30	45	60	70	80	90	100
1	0.32±.03^{A}	0.61±.01^{A}	0.71±.13^{A}	0.76±.16^{A}	0.57±.13^{A}	0.61±.04^{AB}	0.81±.07^{A}
2	0.48±.01^{B}	0.79±.07^{A}	0.70±.10^{A}	0.92±.13^{A}	1.39±.25^{B}	1.13±.16^{B}	0.87±.11^{A}
3	--	--	--	1.62±.10^{B}	0.93±.08^{AB}	0.84±.14^{AB}	1.19±.03^{A}
4	--	--	--	0.90±.20^{A}	1.09±.20^{AB}	1.11±.04^{AB}	0.90±.13^{A}
5	--	--	--	--[3]	0.72±.17^{AB}	0.43±.17^{A}	1.27±.12^{A}
6	--	--	--	1.16±.08^{AB}	1.03±.15^{AB}	0.72±.15^{AB}	1.21±.10^{A}

[1] Values represent the mean (mg hydroxyproline/lung) ± SEM of 3-5 animals. The data in each column were analyzed by two-way ANOVA and the means compared using the Student-Newman-Kuel's post-hoc test. Any two means in a column with different superscripts are significantly different ($p < 0.05$).

[2] Group 1:untreated controls; 2:asbestos; 3:asbestos plus low pirfenidone; 4:asbestos plus medium pirfenidone; 5:asbestos plus high pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

among other male groups at 90 days. A significant decrease in the hydroxyproline content in the lungs of female animals treated with 500 mg/kg pirfenidone from the female group that was only instilled with asbestos while other groups were unchanged.

100 Days. No differences were observed in lung hydroxyproline content in male or female groups at 100 days.

Histopathology Studies

The histological evaluation of inflammatory lesions in the various organs studied was based on the grading of hematoxylin and eosin stained sections using light microscopy.

Evaluation of Changes in the Lung

At thirty days after asbestos instillation, no significant lesions were in the lungs of nontreated animals. Pneumonitis was present in animals treated with asbestos. The inflammatory response consisted primarily of macrophages. Alveolar walls were thickened with pneumocytes and inflammatory cells. Increased lymphoid tissue was in peribronchial and perivascular aggregates. Syncytial giant cells were present in many alveoli. No differences were seen between males and females.

At 45 days post-instillation, pneumonitis was present in all asbestos-treated animals as described for 30 days but more cells were present. Reticulin fibers were increased and

the early deposition of mature collagen fibers was observed.

The severity of pneumonitis in asbestos-instilled animals was increased at 60 days from that observed at 30 and 45 days, with a general increase in macrophages and multifocal, desquamative alveolitis and bronchiolitis. The presence of increased reticulin and mature collagen fibers was observed.

At 60 days after the instillation of asbestos, treatment was started with pirfenidone (groups 3,4, and 5) and cortisone (group 6). To evaluate lung changes at 70 to 100 days, incidence tables were constructed for each animal at sacrifice in terms of inflammation, the number of collagen fibers per high power field (HPF), and the number of reticulin fibers per HPF.

Inflammation Scores. A summary table for the mean inflammation scores for males are presented in Table 15. The results for the female animals are presented in Appendix A.

The severity of the inflammatory response in asbestos-instilled animals reached a maximum at 80 days. The inflammatory scores then remained relatively constant through 100 days in the asbestos instilled only males (group 2) with mean scores of 25.2, 25.7, 25.4 for the 80, 90, and 100 day time points respectively. Group 2 females showed more variation with scores for 70 to 100 days of 20.2, 22.6, 28.2, and 21.3 respectively.

Table 15

MALES

Average Total Lung Inflammation Score

Days After Asbestos Instillation

Group[2]	70	80	90	100
2	18.6 (12-28)	25.2 (13-32)	25.7 (10-35)	25.4 (15-36)
3	21 (16-30)	16 (8-21)	24 (16-34)	26 (3-37)
4	12 (5-20)	18 (2-26)	29 (13-44)	11 (5-17)
5	--[3]	24 (21-26)	16 (4-28)	16 (5-28)
6	13 (6-23)	22 (7-28)	24 (10-28)	30 (20-36)

[1] Values represent the average total inflammation score. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

Group 3 males showed no consistent pattern in the inflammatory scores, being about equal to group 2 male scores between 70, 90, and 100 days, and lower than group 2 at 80 days. Inflammatory scores for group 3 females were not consistent, being found lower, equal, and higher than group 2 females.

At the 70, 80, and 100 day time points , group 4 males' inflammatory scores were decreased from group 2. Group 4 females had scores that were not different than group 2 between 70 and 100 days.

Inflammatory scores for group 5 males were lower than group 2 at 90 and 100 days, and about equal to group 2 at 80 days. No male animals were present at 70 days. Scores for group 5 females were consistently higher than group 2 females.

Inflammatory scores for group 6 males showed no consistent pattern throughout the treatment period. Females in group 6 tended to have lower scores than group 2.

Collagen Fibers per High Power Field. Mean values for male groups at 70 - 100 days are presented in table 16. A summary table for the female groups is given in Appendix A.

There was no consistent pattern in the number of collagen fibers per high power field (HPF) in either male or female group 2 animals. Scores were higher at 70 and 100 days than at 80 and 90 days.

TABLE 16

MALES

Collagen Fibers per High Power Field[1]

Group[2]	Days After Asbestos Instillation			
	70	80	90	100
2	23 (21-26)	17 (11-23)	17 (13-20)	24 (21-27)
3	24 (18-26)	16 (12-25)	18 (13-26)	17 (12-22)
4	15 (13-19)	15 (8-21)	17 (11-25)	22 (12-29)
5	--[3]	13 (7-18)	17 (15-18)	17 (8-24)
6	17 (13-20)	15 (9-25)	19 (14-31)	18 (10-25)

[1] Values represent the average number of collagen fibers per 400X field. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

Group 3 males showed approximately equal scores at 80 - 100 days, with scores ranging from 16-18. These scores were not different from asbestos-instilled only males (group 2). The number of collagen fibers per HPF in group 3 females were lower than group 2 at 70 days but were not different than group 2 females at 80-100 days.

Group 4 males showed only a slight decrease in the number of collagen fibers per HPF from group 2 males, having lower scores at 70, 80, and 100 days and equal to group 2 at 90 days. Group 4 females were not different from group 2.

Group 5 males had lower numbers of collagen fibers per HPF than group 2 males at 80 and 100 days. Larger numbers of fibers than group 2 females were found in group 5 females.

Group 6 males had lower numbers of fibers than group 2 at 70 and 100 days, while females in group 6 had consistently more fibers than group 2.

Reticulin fibers per high power field. The mean scores for males by group are given in Table 17. A summary table for females appears in Appendix A.

The pattern of scores for reticulin fibers in asbestos-instilled (group 2) animals was not consistent throughout the study. Group 2 males had higher numbers of fibers at 70 and 100 days as did group 2 females.

Group 3 males had lower numbers of fibers than group 2 at 80 and 100 days and were equal group 2 at 70 and 90

Table 17

MALES

Reticulin Fibers per High Power Field[1]

Group[2]	Days After Asbestos Instillation 70	80	90	100
2	41.4 (28-56)	27.4 (16-37)	27.3 (20-41)	34.6 (25-45)
3	41.5 (31-51)	20.6 (15-24)	29.4 (18-42)	26.4 (12-40)
4	25.7 (10-40)	22.6 (17-29)	18.2 (4-33)	18.3 (5-22)
5	--[3]	18.0 (11-20)	25.5 (22-29)	25.3 (16-31)
6	32.4 (21-49)	23.2 (20-66)	33.0 (25-39)	38.8 (18-66)

[1] Values represent the mean number of reticulin fibers per high power field. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

[3] All animals in this group died prior to sacrifice.

days. Group 3 females had lower numbers of fibers than group 2 at 70 and 100 days and slightly higher numbers at 80 and 90 days.

The numbers of reticulin fibers per high power field in group 4 males were decreased from group 2 at all time points. Group 4 females showed decreased numbers of fibers from group 2 at 100 days.

Group 5 males had decreased numbers of reticulin fibers from group 2 males at all time points evaluated while group 5 females had decreased numbers of fibers at 70, 80, and 100 days.

Group 6 males showed decreased numbers of reticulin fibers at 70 and 80 days, but the numbers were higher than group 2 males at 90 and 100 days. The numbers of reticulin fibers was decreased at 70, 80, and 100 days in group 6 females.

Evaluation of Changes in the Kidney

An inflammatory response was observed in the kidneys of male and female asbestos-instilled animals from 80 to 100 days. The lesions were those of chronic glomerulonephritis and interstitial nephritis. Sclerosis of the glomerular tufts and the interstitial tissues was also present during this period. The kidneys of female animals were more severely affected than males. A summary table of the female kidney scores is shown in table 18 for 80, 90, and 100 days.

Table 18

FEMALES

Average Total Kidney Lesion Score

Days After Asbestos Instillation

Group[2]	80	90	100
2	11.6 (0-25)	13.6 (2-19)	14.0 (8-23)
3	3.8 (0-15)	2.2 (1-4)	6.3 (1-15)
4	6.2 (1-16)	8.3 (5-12)	5.3 (4-11)
5	4.0 (1-13)	3.3 (0-9)	8.8 (0-16)
6	9.6 (2-20)	5.2 (1-10)	7.6 (1-21)

[1] Values represent the average total kidney lesion score for the lung. Hematoxylin-eosin sections from 3-5 animals were examined. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

Pirfenidone and cortisone treatment appeared to decrease the severity of the inflammatory lesions in the kidney at 80-100 days. Although no dose-response relationship was observed, the kidney lesion scores were considerably lower in pirfenidone and cortisone treated groups during this period. Since statistical analysis was not performed on these results, the significance of the changes in kidney lesion scores due to pirfenidone and cortisone has not been established. However, I believe the changes are meaningful.

Evaluation of Changes in the Liver

An effect attributable to asbestos was observed beginning at 80 days in the livers of asbestos-instilled animals. Females were affected more than males. The lesions observed were those of chronic hepatitis with fibrosis of portal triads and biliary hyperplasia. A summary table of the average female liver lesion scores for the various treatment groups at 80-100 days is presented in table 19.

Cortisone treatment appeared to decrease the severity of liver lesions. At each time point, the group treated with cortisone (group 6) had averages that were considerably lower than asbestos-instilled only animals (group 2).

There was no consistent effect in pirfenidone-treated groups. Some improvement was observed in females when all groups are observed together for each time point.

Table 19

FEMALES

Average Total Liver Lesion Score

Days After Asbestos Instillation

Group[2]	80	90	100
2	9.4 (6-12)	7.2 (1-11)	8.7 (4-15)
3	8.3 (8-11)	4.0 (2-6)	4.0 (1-10)
4	9.2 (6-12)	6.0 (3-8)	5.2 (3-9)
5	5.0 (4-6)	6.0 (1-10)	10.3 (7-13)
6	6.2 (1-12)	2.6 (2-5)	5.4 (4-9)

[1] Values represent the average total liver lesion score for the lung. Hematoxylin-eosin sections from 3-5 animals were examined. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

Statistical analysis was not performed on these results so whether the differences in lesion scores are real is not known.

DISCUSSION

Attempts to influence fibrotic connective tissue accumulation have focused on agents which impair collagen synthesis and processing and on those agents which alter the course of the inflammatory events which precede fibrosis. Prostaglandins are thought to play a modulating or homeostatic role in the events characterizing chronic inflammation and thus may influence the fibrotic response (Arrigoni-Martelli, 1985). The discovery of the involvement of prostaglandins in inflammation and the ability of nonsteroidal anti-inflammatory drugs to inhibit prostaglandin synthesis has led to the appearance of a large number of compounds of different chemical structures. Pirfenidone, a nonsteroidal anti-inflammatory drug, was used in this research to attempt to modulate the fibrotic response in the lung caused by asbestos instillation.

The levels of lung lavage fluid enzymes were measured to follow the course of lung injury due to asbestos. Asbestos-instilled animals showed significant elevations in the levels of LDH and alkaline phosphatase activities at 30 days post-instillation. Significant increases in LDH were also observed in asbestos-instilled animals at 60 and 70 days. Kagan et al (1983) found significantly elevated levels

of LDH in rats at 17-18 months after inhalation exposure to 10.7 mg/m^3 chrysotile asbestos for 6 hr/day, 5 days/wk for 91 consecutive days. The increases in LDH activity in lavage fluid in this research and other studies reflect pulmonary cell damage due to asbestos exposure. The elevations in enzyme activities were observed at varying times removed from initial exposure of the lung to asbestos fiber. These findings provide further evidence of persistent structural damage to the lung cells and chronic inflammatory response caused by asbestos fibers deposited on airway and alveolar surfaces.

To further characterize the development of lung injury and ultimately pulmonary fibrosis, wet lung weight to body weight and dry lung weight to body weight ratios were determined. Increases in both ratios were seen at 30-60 days. These findings give a general indication of an inflammatory response occurring in the lung. The increases in these ratios may indicate a massive influx of inflammatory cells during this period in response to asbestos exposure as well as the accumulation of edematous fluid. These findings are consistent with the histopathological observations of severe pneumonitis at these time points. Glassroth et al (1984) showed, at 4 days after the instillation of 5 mg of chrysotile asbestos into the lungs of hamsters, the development of a patchy bronchopneumonia which progressed into nearby alveolar tissues. During this period,

neutrophils and macrophages filled the terminal airways and extensively infiltrated adjacent alveoli.

The collagen synthesis rate, as measured by prolyl hydroxylase activity, and the collagen content of the lung, as estimated by hydroxyproline content, were also significantly increased at 30 days after asbestos instillation. Prolyl hydroxylase activity then returned to control levels at 60 days. Early collagen accumulation in the lung may also increase the wet and dry weights of the lung. The collagen synthesis rate in lungs exposed to fibrogenic agents has been shown to increase during early phases of acute lung injury. Giri et al (1983) showed that prolyl hydroxylase activity increased as early as 2 days after bleomycin instillation while the maximum response of the enzyme occurred at 14 days. Little is known about the early changes in the activity of this enzyme after exposure of animals to asbestos. Assays for lysyl oxidase, another enzyme necessary for the post-translational modification of collagen, tend to show an increase in rate during the acute lung injury and inflammation phase followed by a decrease towards control values during the chronic, fibrotic phase (Seigel et al, 1978). The significant increase in prolyl hydroxylase activity observed in this research at 30 days may reflect decreased levels from those which may have been seen if measurements of its activity were taken earlier after the instillation of asbestos. It would be important to study the

early changes in lung lavage fluid enzymes, lung weights, and collagen parameters to further understand the development of the fibrotic response. Acute infiltration of neutrophils and macrophages as well as increases in collagen content may have caused increases in lung weights earlier than 30 days. Larger increases in collagen synthesis rates may also occur earlier in the course of the lung's response to asbestos.

Pirfenidone and cortisone treatment began at 60 days after asbestos instillation. Evaluation of the effects of these drugs on lung weights, biochemistry, and histology was performed at 70-100 days. Enzyme activities (LDH and alkaline phosphatase) in lung lavage fluid and the collagen synthesis rate (prolyl hydroxylase activity) had returned to control levels and no significant changes were observed attributable to pirfenidone or cortisone treatment. The inflammatory response peaked at 80 days post-instillation and remained relatively constant thereafter. Lung wet weight and dry weight to body weight ratios tended to remain elevated in the asbestos instilled groups over non-treated controls. The hydroxyproline content of the lung also tended to be increased throughout the study period in animals given asbestos and fibrosis was confirmed histologically. The fibrotic state as measured by lung weight ratios and hydroxyproline content was not altered after treatment with pirfenidone. Cortisone tended to decrease the wet lung

weight to body weight ratios in males and females although changes in other parameters were not observed.

The histological findings on the effects of pirfenidone in the lung are not definitive. The lung inflammation scores, although variable, tended to decrease in males given pirfenidone. Groups 4 and 5 males (given 250 and 500 mg/kg pirfenidone) had lower inflammation scores than did group 3 males (given 25 mg/kg pirfenidone). Female animals did not respond to pirfenidone treatment and inflammation scores were at various timepoints increased over animals given only asbestos.

Decreases in the numbers of collagen and reticulin fibers were observed in male animals treated with pirfenidone but not in females. The male animals that received the higher doses of pirfenidone (groups 4 and 5) showed larger decreases in the numbers of fibers than the lower dose group (group 3). Males in the cortisone-treated group showed decreased numbers of collagen fibers at 70, 80, and 100 days and reticulin fibers at 70 and 80 days.

An effect attributable to asbestos was observed beginning at 80 days in the kidneys and livers of primarily the female animals. Glomerulonephritis and interstitial nephritis with sclerosis of the glomeruli and interstitial areas was observed in the kidney. Chronic hepatitis with fibrosis of the portal triads and biliary hyperplasia was observed in the liver. These findings are notable since the

systemic effects of inhaled or intratracheally instilled asbestos have not been reported. These effects may be related to the long-term immunologic stimulation caused by asbestos. Another explanation for the effects is that asbestos fibers may have been cleared via the mucociliary escalator and swallowed. The fibers could cross the intestinal wall and then be transported to the liver and kidney in the circulation. Kidney lesion scores were decreased in the groups treated with pirfenidone while pirfenidone's effect in the liver was not consistent. Cortisone treatment also tended to decrease kidney lesions.

The lack of conclusive evidence showing the efficacy of pirfenidone in ameliorating the fibrotic response in the lung due to asbestos exposure may be due to a number of important factors in the experimental design. A major factor in this project was the timing of the start of pirfenidone treatment. It may have been possible to inhibit the early events in acute inflammation if pirfenidone was administered to the animals within a few days after the instillation of asbestos. By inhibiting some of the cellular responses in acute inflammation, it may have been possible to influence the chronic inflammation and fibrosis. Kehrer and Witschi (1981) found that administration of 4 mg/kg indomethacin on days 1-6 after exposure to fibrogenic doses of butylated hydroxytoluene and oxygen increased survival and decreased the accumulation of collagen in mice at 14 days, while mice

given indomethacin on days 4-6 only had no beneficial effect. Rats given a single injection of 1.5U bleomycin intratracheally then immediately started on treatment with 2 mg/kg/day indomethacin for 60 days showed a marked reduction in the intensity of inflammatory changes and the accumulation of collagen in the lung (Thrall et al, 1979). In a study done in our laboratory, rats given a single injection of 0.5U bleomycin intratracheally and given 250 mg/kg/day pirfenidone orally on days 6-13 and sacrificed on day 21 had collagen levels that were markedly reduced from animals given only bleomycin and equal to animals instilled with saline (unpublished results).

Bleomycin administration into the lung may be the model of choice for anti-fibrotic drug screening as the resulting pulmonary fibrosis seems to accurately model the interstitial disease which occurs in humans (Last, 1985) and can be rapidly induced. Instillation of asbestos may take long periods of time to produce a severe fibrotic response (Glassroth et al, 1984), whereas bleomycin has been shown to significantly increase collagen levels in the lung in 2-3 weeks after its instillation (Hesterberg et al, 1981). The persistence of asbestos in the lung makes it a constant inflammatory stimulus, and thus, modulation of inflammation and fibrosis with pirfenidone may be difficult.

A larger dose of asbestos instilled into the lungs may have caused an increased severity of fibrosis earlier

although this benefit may have been compensated by increased mortality. A larger number of animals may also have increased the significance of the results of this research since there was a great interanimal variability in the responses to both asbestos and pirfenidone.

The results obtained in this research certainly warrant further study into the feasibility of using pirfenidone or other nonsteroidal anti-inflammatory agents to treat fibrosis. Especially interesting were the results in the kidney and liver in those groups of animals given asbestos and pirfenidone. Other studies have not investigated the systemic effects of asbestos or other particulates which are inhaled or instilled into the lung. Further study into this area may be important in assessing the toxicities of various inorganic particulates.

It would be necessary to investigate the effects of pirfenidone on other animal models of pulmonary fibrosis, look at longer and shorter time points after administration of a fibrogenic agent, begin therapy with pirfenidone early in the acute inflammatory response as well as other timepoints in the course of the lung's response to injury, and use larger numbers of animals before definitive statements could be made about the efficacy of pirfenidone in influencing fibrosis. Treatment of fibrosis as a disease entity does seem to be a feasible goal.

APPENDIX A

Female Lung Histological Score Tables

FEMALES

Average Total Inflammation Score

Days After Asbestos Instillation

Group[2]	70	80	90	100
2	20.3 (9-32)	22.6 (14-30)	28.2 (20-33)	21.3 (10-32)
3	8.0 (5-11)	27.5 (21-30)	19.0 (6-37)	18.0 (13-25)
4	26.0 (21-28)	21.2 (17-27)	31.3 (24-36)	23.6 (5-39)
5	19.0 (9-27)	24.3 (15-34)	33.7 (29-39)	28.8 (18-37)
6	13.6 (7-23)	24.6 (8-38)	20.6 (6-31)	16.8 (9-25)

[1] Values represent the average total inflammation score for the lung. Hematoxylin-eosin sections from 3-5 animals were examined. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

FEMALES

Collagen Fibers per High Power Field[1]

Group[2]	Days After Asbestos Instillation			
	70	80	90	100
2	19.8 (11-26)	12.8 (10-16)	16.4 (14-18)	18.6 (13-30)
3	14.5 (14-15)	12.8 (10-16)	16.0 (12-22)	19.3 (18-20)
4	22.6 (18-30)	14.2 (6-19)	18.0 (16-20)	16.4 (12-21)
5	15.8 (11-25)	15.5 (12-21)	19.0 (18-20)	23.0 (17-27)
6	16.4 (11-22)	16.6 (7-23)	18.2 (11-22)	19.8 (17-22)

[1] Values represent the mean number of collagen fibers per high power field. Sections from 3-5 animals were examined. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

FEMALES

Reticulin Fibers per High Power Field[1]

Group[2]	Days After Asbestos Instillation			
	70	80	90	100
2	36.5 (24-46)	21.6 (14-32)	25.4 (23-29)	44.0 (33-51)
3	30.0 (24-36)	22.0 (17-27)	27.8 (15-38)	38.0 (30-47)
4	34.0 (26-38)	21.4 (20-23)	24.3 (20-30)	23.4 (11-34)
5	31.6 (19-47)	19.3 (13-24)	32.0 (25-36)	27.8 (20-35)
6	26.0 (18-35)	21.0 (15-29)	34.0 (20-45)	23.4 (15-37)

[1] Values represent the mean number of reticulin fibers per high power field. Numbers in the parentheses represent the range. Statistical analysis was not performed.

[2] Group 2:asbestos only; Group 3: asbestos plus low dose pirfenidone; 4:asbestos plus medium dose pirfenidone; 5:asbestos plus high dose pirfenidone; 6:asbestos plus cortisone.

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