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The University of Arizona, 1987

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THE PULMONARY INFLAMMATORY AND FIBROTIC RESPONSE INDUCED BY GLASS FIBERS

by

Leslie Royce Pustilnik

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

In Partial Fulfillment of the Requirement 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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Andrea K. Hubbard 12/8/87

ANDREA KAY HUBBARD
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Date

DEDICATION

To my parents,
who have always encouraged me to set high
goals for myself, and for their love and
support which has helped me to achieve them.

ACKNOWLEDGMENTS

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ABSTRACT

The present study was initiated to evaluate the pulmonary inflammatory and fibrotic responses induced by single and repeated exposures to glass fibers. Single and repeated intratracheal injections of glass fibers induced an acute inflammatory response which progressed to a chronic inflammatory and fibrotic response. Mice exposed to glass fibers in single or repeated doses demonstrated elevated numbers of eosinophils, neutrophils and macrophages and increases in cell-free protein in lung lavage fluid at five days post-exposure. These parameters, in addition to relative lung/body weight ratios and lung tissue hydroxyproline levels, were elevated in comparison to saline control animals at five weeks post-exposure. Although repeated exposures to glass fibers did not potentiate the cellular inflammatory response, they did induce a marked infiltration of eosinophils, a response not observed with either asbestos or silica exposures. These observations suggest that glass fibers may be more toxic to the lungs than previously thought.

INTRODUCTION

Pneumoconiosis, or "dusty lung disease", results from the exposure of the lungs to inorganic airborne particles. These particles, once inhaled, are deposited within the lungs in different areas depending on particle size. Larger particles sediment in the upper respiratory tract whereas smaller particles will reach the alveolar spaces. Many of the particles in the upper respiratory tract are cleared from the pulmonary tract by movement up the mucociliary escalator. Although clearance mechanisms usually provide an effective means of protecting the lungs from such environmental hazards, they may become overwhelmed, resulting in the progression of pulmonary damage. In addition, phagocytosis by macrophages and polymorphonuclear leukocytes contribute to particle clearance out of the lungs. These phagocytic cells also release inflammatory mediators and lysosomal enzymes which can cause damage to pulmonary tissue. Exposure of the lungs to natural and synthetic toxic particles have been reported to initiate pulmonary fibrosis (silica) as well as the development of neoplasia (asbestos). However, investigation is required to explore more fully the potential damaging effects of synthetic, man-made particles such as glass fibers.

Composition and Use of Particles

Silicon dioxide, a major component of the earth's crust, is ubiquitous in our environment. Silica particles are composed of a silicon-oxygen tetrahedra arranged in a nearly symmetrical manner with each oxygen being shared with a neighboring silicon atom (Harington, Allison and Badami, 1975). The danger of exposure to silica exists in many occupations, including stonecutting, mining, foundry working, quarrying and sandblasting (deShanzo, 1982). The pathogenicity of silica is initiated by hydrogen bonding of the particles with the phosphate ester groups of phospholipids in the cellular lysosomal and plasma membranes. This results in the distortion of the membranes and allows the release of hydrolytic enzymes and other intracellular constituents into the interstitium and alveolar spaces (Harington, Allison and Badami, 1975). In 1969, Heppleston proposed that damaged macrophages release factors capable of stimulating collagen formation. Adamson and Bowden (1984) suggest that macrophages and PMN release factors chemotactic for fibroblasts. Their investigations demonstrated an increase in fibroblast proliferation and activity, paralleled by an increase in total lung hydroxyproline content. Dauber et al (1980) and Heppleston (1982) also noted an increase in the hydroxyproline content of animal lungs exposed intratracheally to silica. In addition, Heppleston (1982) exposed rat macrophages to silica crystals for 24 hours, disintegrated the cells and removed the particles and cellular debris. The supernatant was transferred to a culture of chick embryo fibroblasts. Heppleston observed that the fibroblasts produced an

increased amount of hydroxyproline. When the experiment was repeated with titanium dioxide, a non-fibrotic dust, there was no increase in hydroxyproline formation.

Amosite asbestos is a hydrated silicate of iron and magnesium amphibole. Its atoms are organized in a tetrahedron with a silicon atom in the center and oxygen atoms at each of the four corners. These tetrahedra are arranged in double chains to form this amphibole fiber. Amosite is extremely stable and its industrial uses include electrical and thermal insulation, fire-resistant paints, asbestos cement products, textiles and as a major constituent of automotive clutch and brake linings (Harington, Allison and Badami, 1975). It is thought that asbestos exerts its cytolytic actions by trapping cell membrane glycoproteins into clusters through interactions between the ionized carboxyl groups of sialic acid and the magnesium hydroxide groups of asbestos. As a result, there is an increase in the cation permeability of the cell membrane and the cell undergoes osmotic lysis (Harington, Allison and Badami, 1975). Heppleston (1984) remarked on the involvement of cytoplasmic enzymes and the release of a macrophage fibroblast factor in the development of asbestos-induced fibrosis. The target cells for macrophage fibroblast factor are the interstitial fibroblasts, whose increased presence correlates with increased lung hydroxyproline production. Heppleston (1984) also suggests that the lesser fibrogenic effects of asbestos in comparison to silica is due to the lesser toxicity of asbestos to macrophages. Longer interaction of the fibers with cell structures may cause a slower release of

macrophage fibroblast factor, perhaps at lower concentrations, resulting in less prominent fibrosis.

Glass fibers are also composed of silicon-oxygen tetrahedra, but in a manner unlike the crystalline fashion of silica, or the chain structure of asbestos. The use of glass fibers has increased as an asbestos substitute, in such products as thermal and electrical insulation, filter papers and plastic reinforcements. Similar to silica and asbestos, the pathogenicity of glass fibers is thought to be based on the interaction between the particles and the cellular membrane constituents. However, the exact mechanism has yet to be ascertained (Harington, Allison and Badami, 1975).

Clinical Manifestations and Incidence of Pneumoconioses

The pneumoconioses are among the oldest of the occupational health hazards recorded in medical literature. In 1556, Agricola published a discussion on the mining and metal industries in which he described the suffering of the miners. He concluded that due to the conditions in the mines, and the high concentration of dust in the air, dust particles were entering the lungs producing difficult breathing and asthma (Hoover and Hoover, 1912). One hundred and fifty years after Agricola published his book, Bernardo Ramazzini published his own manuscript in 1700 describing the "maladies that attack stonecutters, sculptors and quarrymen", stating that the lungs of such workers were "found to be stuffed with small stones..." (Ramazzini, 1964).

In 1870, Visconte coined the term silicosis to describe the pathological condition resulting from the inhalation of dust (Hunter, 1969). By the end of the 19th century, the effects of occupational dust inhalation on the lungs were well recorded and its influence in the development of silicosis established.

The United States disregarded silicosis until the early 1900's. At this time, a series of studies revealed silica as a serious occupational health hazard. As a result, protective legislation and workmen's compensation laws began to be passed (Higgins et al, 1917; Hoffman, 1918). During the Depression, however, because the unemployed would chance exposure for an income, little pressure was exerted on industry to create safe working conditions. Perhaps one of the most publicized events was the Gauley Bridge incident in 1930-1931. The New Kanawha Power Company hired the Rinehart-Dennis Company to drill a tunnel through a mountain at Gauley Bridge, West Virginia, to divert water to a hydroelectric plant. According to testimony, the employers knew the rock was high in silica content, but took virtually no action to limit the exposure of the workers to the dust. Workers soon complained of shortness of breath and displayed other symptoms of silicosis. Of an estimated 2,000 workers employed over a 2 year period, 476 men died and eventually 1,500 were disabled (Congressional Record, 1936).

During the 1930's and 1940's, as the hazards of dust exposure became known, states began to establish industrial hygiene programs. In addition, laws were introduced that would provide compensation for

victims of silicosis. However, much damage had already been done. At the 1955 McIntyre-Saranac Conference on Occupational Chest Diseases, the Occupational Health Program of the Public Health Service presented a study covering the period 1950-1954 that revealed 10,362 reported or compensated cases of silicosis in 22 states. Twenty-two percent of these were dead, fifty percent were totally disabled and thirty percent were still working, looking for work or were of an unknown status (Trasko, 1956). Eventually, the incidence of disease did lessen following the initiation of dust control programs. In the United Kingdom, 721 cases of silicosis were compensated in 1957, with the number decreasing to 162 new cases in 1969 (Seaton, 1984). Between 1958 and 1961, the U.S. Public Health Service and the Bureau of Mines conducted a detailed survey of 76.6% of the workforce in 50 metal mines. There was an overall prevalence of silicosis in 3.4%, and one-third of the workers had complicated disease. They found little disease in workers employed less than 10 years, with the occurrence increasing to 3% after 20 years of employment, 12% at 30 years of work and 16.6% after 30 years of exposure (U.S. Public Health Service, 1961). The incidence of disease has declined in recent years, but is by no means a thing of the past. For example, in Illinois in 1981, two silica mills reported that 37% of its workers had silicosis, with 11% of these individuals exhibiting massive fibrosis (Banks et al, 1981).

Asbestos exposure has also been linked to pulmonary fibrosis as well as to carcinogenesis. In 1955, Doll examined workers in an asbestos textile plant in England. He noted that 113 men employed 20

years or more had a risk of developing lung cancer which was ten times more than expected in the general population. In the United States, most cases of asbestosis occur in people employed in asbestos textile fabrication or pipe insulation installation. Murphy et al (1971), reported that shipyard workers installing pipe insulation had an incidence of asbestosis that was eleven times that of a control population.

Asbestosis and silicosis share some disease characteristics such as shortness of breath on exertion and a slight cough. With continued exposure, the dypsnea and coughing increase in severity. As fibrosis spreads throughout the pulmonary parenchyma, the lungs become inelastic and stiff. Thickening of the alveolar septae causes difficulty in inflation of the lungs as well as impedes the transfer of oxygen into the blood. The heart works harder to pump blood through these stiff and inelastic lungs, and once enlarged and weakened, can lead to cardiac failure (Corn, 1980; Smith, 1955).

It has been suggested that the development of these pulmonary disorders are due more to the size, shape and durability of these particles than to their molecular composition. Stanton et al (1977) injected glass fibers of diverse type and dimensional distribution into the pleural cavity of rats. Fibers less than or equal to 1.5 microns in diameter and greater than 8 microns in length were found to yield the highest probability of pleural sarcoma formation. Thus, attention has turned towards the potential pathogenicity of glass fibers. These fibers are similar in composition and shape to asbestos and silica and

are now widely used as an asbestos substitute. Several epidemiological studies have been conducted on workers exposed to glass fibers. All reports indicate that glass fibers are not a health hazard, nor do they produce carcinogenic effects (Enterline and Henderson, 1975; Hill et al, 1973; Utidjian and de Treville, 1970). In 1969, the Bureau of Occupational Safety and Health, U.S. Department of Health, Education and Welfare concluded that "there appears to be no reason to class the manufacture of fibrous glass products as a hazardous occupation" (Johnson et al, 1969). In addition, a large scale study conducted by NIOSH in 1976 (Bayliss et al, 1976) investigated males employed 5 years or more in the manufacture of glass fiber. Investigators were unable to find a statistically significant increase in malignancy from glass fiber exposure. However, an increase in deaths from nonmalignant respiratory diseases after only ten years of exposure was noted. Gross, Harley and Davis (1974) examined the lungs of workers exposed to fiber glass dust for 16 to 32 years. They found no recognizable tissue response to the fiber glass. Moreover, the workers had no more fibers present in their lungs than the control population.

The negative findings of these epidemiologic studies can not, however, eliminate the pathogenicity of all glass fibers. New ultra fine fibers are now widely used, and have only been commercially produced in recent years. Since the latency period for the development of fibrosis and mesothelioma can be 20 years or longer, and with increased use and exposure to these fibers, additional investigations on the pathogenicity of these glass fibers are needed.

Animal Studies

Since the inhalation of silica, asbestos and potentially glass fibers, have been demonstrated to cause detrimental effects on the health of humans, many animal studies have been initiated to study the mechanisms of such effects on the respiratory system. Because of differences in experimental design in these studies, conflicting results have been reported by various investigators. Therefore, several variables must be considered when interpreting the results of these investigations. These variables include (1) the route of exposure to the particles; (2) the size distribution and doses of the particles administered; and (3) the parameters used to evaluate the pulmonary response.

Route of Exposure:

To examine the pathogenicity of particles such as glass fibers, the major routes of exposure are by inhalation, intrapleural injection and intratracheal instillation. In 1970, Gross et al, reported that a significant number of rats displayed a focal increased cellularity of alveolar walls and an accumulation of dust-filled macrophages in the alveoli following 2 years of exposure to high concentrations of glass fiber. Although, in most cases, there was no collagenization of alveolar septae, a few rats developed foci of septal collagenous fibrosis. Gross et al (1970b) also instilled glass fiber by the intratracheal route. They noted that injections of fibrous glass in rats produced polypoid masses of inflammatory tissue in smaller

bronchial divisions by 4 days post-exposure. However, these lesions disappeared by 1 year. These results prompted Gross and his colleagues to conclude that intratracheal injections of glass fibers produce mechanical trauma resulting in inflammatory foci which eventually resolved and disappeared.

Lee et al (1981), exposed rats, hamsters and guinea pigs by inhalation to amosite asbestos (3.1×10^6/liter, less than 5 microns in length) and glass fibers (0.7×10^6/liter, diameter 1.2 microns and less than 2 microns in length) for 90 days. At 20 days post-exposure, both glass fibers and asbestos provoked the infiltration of numerous neutrophils and caused alveolar proteinosis in rats and hamsters. By day 90, glass fiber exposed rats and hamsters displayed marked hyperplastic granular pneumocytes and dust-cell reactions which extended to the peripheral alveoli distant from the respiratory bronchioles. Although no fibrosis was observed, animals exposed to asbestos demonstrated a reticulin fiber network with thick, wavy collagenous fibers. By 6 months post-exposure to glass fibers or asbestos, resolution was apparent with a marked reduction in cellularity in all animals exposed to fibers. Moreover, there was no collagenized fibrosis in glass fiber dust-deposited sites. Conversely, there was an increase in collagen deposition in asbestos exposed animals. At 2 years post-exposure to glass fibers, there was negligible collagen deposition found in the interstitium where dust-cells had been trapped, whereas the asbestos granulomas were replaced with collagenized fibrotic lesions.

Goldstein, Webster and Rendall (1984) also compared the effects of inhaled glass fiber to crocidolite asbestos. They exposed baboons by inhalation for 8 months to fibers with counts of 1125 fibers/cm^3. They observed in glass fiber exposed animals a thickening of respiratory bronchioles, an infiltration of histiocytes and fibroblasts as well as a moderately compact reticulin formation and a few collagen fibers. By 18 months, there was evidence of desquamative alveolitis and a loose network of reticulin fibers with a more marked development of fibrosis. Asbestos produced a morphologically similar, albeit more severe response.

Intrapleural injection of particles is another method used to determine their toxicity towards the lungs. Davis (1976), in mice, evaluated the response to glass fibers of varying diameters and lengths from 2 weeks to 18 months post-injection. These fibers had diameters of either 3.5 or 0.05 microns and lengths were either less than 20 microns, or of several hundred microns. He demonstrated that at doses of 10 mg/mouse, all fibers produced fibrosis when injected into the pleural cavity. Moreover, the degree of fibrosis varied with length. Short fibers, whether thick or thin, produced very small compact granulomas which never formed adhesions between the lungs, heart and chest wall. Although these granulomas eventually became fibrotic, only small amounts of collagen were produced since the size of these granulomas was also small. With the longer fibers, the effect was much more severe. Long fibers provoked the development of very large granulomas, forming firm adhesions between the lungs, diaphragm, heart

and chest wall. A small amount of collagen was detected within these granulomas as early as two weeks after injection. The deposition of collagen steadily increased with time until, in older animals, the lungs were firmly bound to the chest wall with masses of fibrous tissue. Davis concluded that these lesions were as severe as those seen after similar doses of chrysotile or crocidolite asbestos (Davis, 1971). Stanton et al (1977), also exposed animals intrapleurally with 17 kinds of fibrous glass which were of different dimensions, but of identical composition. They concluded that the dimension of the fibers demonstrated a strong correlation with malignant neoplasm development. Fibers greater than 8 microns in length and less than 1.5 microns in diameter were found to have the greatest propensity for inducing neoplasms.

Intratracheal instillation is another frequently used method of exposure, since it has been demonstrated that the distribution of dilute injections mimics the distribution of inhaled particles (Brain et al, 1976). In 1984, Bernstein and associates repeatedly (10 times) injected 0.5 mg of glass fiber intratracheally into rats. The histology of animals exposed by inhalation or intratracheal instillation displayed strong similarities in the distribution of fibers, either long or short (5 or 60 microns, 1.5 microns in diameter). Shorter fibers were cleared out of the lungs by macrophages whereas the longer fibers remained in the lungs. By 1 year post-exposure, the long fibers caused a focal increase in cellularity due to the mobilization of macrophages. No fibrosis however, was observed. In an earlier

study, Schepers (1955) injected guinea pigs intratracheally with glass wool which was 20 to 50 microns in length. He noted an abundant cellular infiltrate, hyperplasia and desquamation of the epithelial lining by one month post-exposure. By two months post-injection, isolated foci of intense cellular infiltration within the interstitium were seen. Again, no fibrosis was seen by 18 months post-exposure.

Size Distribution and Dose:

Kuschner and Wright (1976) examined the effects in guinea pigs of repeated intratracheal injections of glass fibers of varying length (3 injections of 4 mg each, 2 weeks apart). Interstitial fibrosis was observed around respiratory bronchioles and proximal alveoli in animals exposed to long fibers (92% longer than 10 microns, 0.1-1 microns in diameter). Long, very thin glass fibers (diameter less than 0.1 microns, 50% longer than 50 microns) produced a minimal, but definite lesion in the same area of the terminal bronchioles. Short fibers (less than 10 microns) of either diameter produced a macrophage inflammatory response, but no fibrosis. Inflammation induced by glass fibers was compared with that induced by crocidolite asbestos. Asbestos produced similar but qualitatively greater lesions. These observations tend to support the contention that the leakage of tissue damaging enzymes from phagocytes is the "ultimate inciter" of fibrosis and that the qualitative differences seen between responses generated by glass fibers or asbestos may be due to the less durable nature of glass fibers.

Feron et al (1985) and Van Graft (1986) both observed in hamsters the development of "silicotic granulomas" following single (10 mg) or repeated (1 mg every 10 days for 1 year) intratracheal instillations of glass fibers (5-20 microns in length, 0.5-1 microns in diameter). These granulomas, which were surrounded by a layer of alveolar epithelial cells, were the predominant lesion noted by both investigators. Collagen fibers were identified in these granulomas by 6 months.

Parameters Evaluated:

In addition to histopathology, other parameters may be evaluated to ascertain the pulmonary response to particle exposure. Levels of lactate dehydrogenase (LDH), alkaline phosphatase, neutral protease, acid protease, total protein and glucose-6-phosphate dehydrogenase as well as the total inflammatory cell number and cell type present in the lung lavage fluid have all been assessed. In addition, total lung hydroxyproline levels and wet lung weights have also been determined. Pickrell et al (1983) evaluated protein, LDH, alkaline phosphatase, neutral protease, glucose-6-phosphate dehydrogenase and acid protease in the lung lavage fluid of hamsters exposed intratracheally to crocidolite asbestos (count median diameter of 0.25 microns) or glass fibers (count median diameter of 0.1, 0.2, 2.3, 3 and 4.1 microns). All of these markers of inflammation were elevated above control levels. Asbestos and glass fibers with a count median diameter of 0.2 microns produced the most pronounced reactions as reflected in

macrophage aggregation, persistent increases in polymorphonuclear leukocytes (PMN), tissue soluble protein levels, pulmonary collagen deposition and fibrosis. All animals injected with glass fibers with a count median diameter of 0.1 microns died within 30 days of instillation. These animals demonstrated acute injury in the form of severe hemorrhage and edema in the lungs. These results suggest that fibers with small diameters are more pathogenic.

Sykes et al (1983a) evaluated LDH, protein levels and total inflammatory cell number in the lavage fluid, and hydroxyproline content of the lung tissue as indicators of pulmonary injury following the intratracheal administration of fibrous dusts to rats. Long (117 microns in length) and short (unspecified) UICC amosite asbestos and glass fibers (10-100 microns in length, 1.5 microns in diameter) were used in this study. At one day post-exposure, the acute response, as monitored by lavage LDH, cell numbers and dry lung weights were similar for both amosite and glass fiber injected animals. As compared to another inflammatory particle, silica provoked a 50% greater infiltration of PMN than did either glass fibers or asbestos (Sykes et al, 1983b). Amosite and silica injected animals were also examined at 50 and 100 days post-exposure to evaluate the subacute response. With both particles, cellular infiltration into alveolar spaces, increases in dry lung weight, lavage protein and LDH, and tissue hydroxyproline levels as well as the development of cellular nodules with collagen deposition, were seen by 50 days and were still increasing by 100 days post-exposure.

The most frequently used parameters for evaluating lung damage include histopathology, tissue hydroxyproline levels, lavage fluid protein and LDH levels and the number of inflammatory cells and cell types present in the lavage fluid. Evaluating sections of lung tissue under light microscopy provides information concerning the intensity and progression of the inflammatory response. The degree of this response, from a slight macrophage infiltrate induced by exposure to "nuisance dusts", to a more serious progression of the pulmonary reaction (e.g., fibrosis or neoplasia) induced by silica or asbestos can be evaluated histopathologically. Development of fibrosis is also measured by assessing elevated lung tissue hydroxyproline levels, which are indicative of collagen deposition. The contents of the lavage fluid indicate the inflammatory processes that may be involved in the pulmonary response to particle exposure. Elevation of the levels of cell-free lavage protein may be due to the secretion and/or leakage of cellular constituents into the alveolar space. The presence of LDH in the lavage fluid further indicates the rupture of the cell membrane and the death of the cell. Determination of the number of inflammatory cells lavaged from the alveoli of the lung is useful in assessing the chronology of pulmonary damage, since cellular infiltration is one of the earliest responses seen following particle exposure (Gee, 1980). Differentiation of these cells by cell type provides additional information as to the type of inflammatory response present, albeit acute (primarily PMN), subacute (PMN and macrophage) or chronic (primarily macrophage).

Since the results of these studies of glass fiber exposed animals are conflicting, more investigations are needed into the toxicity of fibrous dusts. Moreover, glass fibers are of special interest since their use in industry has increased in recent years, thereby increasing the potential for exposure for a large population.

Cellular Mechanisms in Pulmonary Inflammation

To understand fully the pulmonary response to particle exposure, the cellular mechanisms involved in pulmonary inflammation will be described. First, the particle is inhaled. Generally, particles with an aerodynamic diameter of 5-30 microns are deposited in the nasopharyngeal region. Sedimentation of particles 2-5 microns in diameter occurs in the tracheobronchiolar area whereas particles of aerodynamic diameters less than 1 micron will diffuse into the alveoli. The earliest cellular inflammatory response in the lungs is a rapid influx of granulocytic leukocytes, cells which are normally not present (Gee, 1980). This influx by granulocytes is followed by a large increase in the macrophage population. This initial phase of macrophage expansion is not dependent upon mitotic activity, but rather suggests the action of an alveolar-generated chemotactic factor (Bowden and Adamson, 1978). Later, the initial PMN response decreases while the macrophage response remains elevated, suggesting a mitogenic factor responsible for this second phase of macrophage expansion (Bowden and Adamson, 1978). Lee (1985) has proposed several possible sources of the extra alveolar macrophages that appear in response to a particle:

(1) the division of free macrophages; (2) migration into the alveolar spaces of blood monocytes; (3) the release of macrophages from the interstitium into the alveoli; or (4) the proliferation of interstitial macrophagic precursors with subsequent migration of daughter cells into the alveoli.

Upon ingestion of particles (1) the macrophage may transport the particle up the mucociliary escalator to be expectorated or swallowed, or via lymphatics, to lymphoid tissue; (2) the macrophage may die, releasing the particles and the cell's constitutive enzymes; (3) the cells may survive, provoked to release toxic products which can injure adjacent cells and tissue; (4) the macrophage may be stimulated to secrete mediators that alter the function and behavior of other cells. Upon phagocytosis, these particles are incorporated into a phagosome which merges with a lysosome containing proteolytic and other digestive enzymes. The membranes fuse, forming a single membrane-bound vesicle called a phagolysosome (Uber and McReynolds, 1982). If the particles are too large to be internalized, the lysosomal granules will fuse with incomplete phagosomes. Subsequent release of the enzymatic contents of the granules into the extracellular space acts either to initiate an inflammatory reaction or to amplify a pre-existing one by the local generation of chemotactic factors (Hunninghake et al, 1984). In addition to "frustrated phagocytosis", the particles themselves may interact with and disrupt the lysosomal and cell membranes. This results in the leakage of the particles and cellular constituents, or cell death by interfering with the cells' ability to exclude calcium.

This re-exposure of the lung to the particles perpetuates the sequence, producing an "inanimate infection" (Uber and McReynolds, 1982).

The activated macrophages and PMN contain powerful chemotactins. Once released, they will attract additional PMN, macrophages and fibroblasts into the lung (Hunninghake et al, 1984; Rola-Pleszczynski et al, 1984)). Even the products of ruptured macrophages have been found to enhance the recruitment of additional macrophages and phagocytes into the airways (Katsnelson and Privalova, 1984). Hunninghake et al (1984), noted that as the inflammatory response develops, the phagocytic cells lose their ability to secrete significant amounts of hydrolases. This suggests that the perpetuation of tissue injury requires the continued recruitment of circulating inflammatory cells into the lung, or reactivation and/or the proliferation of cells already present in the lung tissue. These additional inflammatory cells release hydrolytic enzymes which may, in addition to the direct action of the particles, cause injury to the pulmonary parenchyma. Macrophages, neutrophils and eosinophils all contain a membrane-associated NADPH oxidase. During cell stimulation, this oxidase is activated and functions as a significant source of reactive oxygen metabolites. These metabolites may cause increased vascular permeability, interstitial and intraalveolar edema and increased fibrin as well as the hydrolysis of tissue components (Fatone et al, 1987). The complement system has also been implicated in the pulmonary response to particle exposure. In 1977, Wilson, Gaumer and Salvaggio reported that amosite asbestos activated the alternative complement

pathway and generated factors chemotactic for PMN. Glass fiber and silica did not appear to generate complement derived chemotactic factors. However, in 1986, Callis and associates were able to demonstrate *in vitro* that silica crystals were capable of activating complement via the alternative pathway. In addition, it was reported that the fifth component of complement appeared to be responsible for a portion of the pulmonary inflammation induced by silica exposure. Callis et al (1986a) have also noted an involvement for T-lymphocytes in the development of particle-induced inflammation. Pernis and Vigliani (1982) proposed that exposure to fibrogenic dusts stimulated macrophages inducing the release of mediators, including the monokine, interleukin-1. In addition to stimulating fibroblasts and the production of collagen (Schmidt, Mizel and Green; 1981), interleukin-1 also activates nonspecifically T-lymphocytes. These activated T-lymphocytes release lymphokines, which further activate macrophages, thereby potentiating their inflammatory and immune functions, and perpetuating the inflammatory cycle. Included in these lymphokines is an eosinophil chemotactic factor which attracts these cells to the site of inflammation.

Many researchers have noted that the predominant lesion following dust exposure, is granuloma formation in the peribronchiolar area (Lee et al, 1981; Davis, 1976; Bernstein, Drew and Kuschner, 1980; Dauber et al, 1980; Feron et al, 1985; Lugano, Dauber and Daniele, 1982; van Graft et al, 1986). Bowden and Adamson (1984, 1985) demonstrated the translocation of asbestos and silica particles towards

peribronchial connective tissue. They suggested that the pathways of dust clearance predisposed the focal aggregation of particles in these areas. In addition, the concentration of toxic particles at these specific locations may be an important factor in the initiation and development of fibrosis. The presence of inert particles triggers an accumulation of inflammatory cells, or alveolitis. This accumulation appears to provide the ideal environment to foster the formation of compact focal granuloma around the particles. The granuloma are composed primarily of inflammatory cells derived from mononuclear phagocytes (Hunninghake et al, 1984). The activated macrophage subsequently releases potent chemotactins for fibroblasts, which attracts them to the sites of inflammation and stimulates them to undergo cell division. Dauber et al (1980), Heppleston (1982) and Lugano et al (1984) also demonstrated that silica stimulates macrophages to release a factor that enhances the production of collagen by fibroblasts. Thus, with the increase in fibroblast number in response to damage to the lung parenchyma caused by the release of hydrolytic mediators, collagen production increases and the development of fibrosis ensues (Hunninghake et al, 1984).

Role For the Eosinophil in Pulmonary Inflammation

The eosinophil constitutes a very small proportion of the white blood cells present in the circulation and is rarely seen in the lungs (Davis et al, 1984). However, several investigators have noted large numbers of these cells in the lungs of animals exposed to asbestos and

glass fibers. In 1955, Schepers intratracheally injected guinea pigs with 20-50 micron glass wool. By one month post-exposure, the histopathology revealed an abundance of eosinophils within the alveolar and bronchiolar lumina and their walls. Rola-Pleszczynski and associates (1981) noted a large number of eosinophils in the lavage fluid of sheep given six monthly intratracheal injections of asbestos. Van Graft et al (1986) examined the lungs of hamsters seven days after a single intratracheal injection of glass fibers (5-20 microns in length, 0.5-1 microns in diameter). Numerous eosinophils were evident in the histopathologic sections. The recruitment of eosinophils into the lungs may be due to a variety of mechanisms. Mast cells release products chemotactic for eosinophils such as histamine and eosinophil chemotactic factor (Anwar and Kay, 1977). Since mast cells are located near the mucous membranes lining the respiratory tract, they have the potential to interact with particles. In addition, mast cells have receptors for complement components and after activation, secrete inflammatory mediators. Alternately, mast cells may be activated by direct physical damage or by factors produced by neutrophils (Dale and Foreman, 1984). Other factors chemotactic for eosinophils include T-cell derived prostaglandins, leukotrienes and PMN chemotactins derived from macrophages.

Although little is known about the potential role of the eosinophil in particle-induced pulmonary inflammation, studies in animals suggest that these cells may contribute to parenchymal damage caused during inflammation. Davis et al (1984) demonstrated that

eosinophils exhibited spontaneous cytotoxicity for human lung fibroblasts, epithelial and mesothelial cells. They also reported that antioxidants suppressed a significant amount of injury, suggesting that a reactive oxidant species was involved. Eosinophils are capable of generating hydrogen peroxide and superoxide anion. In 1987, Petreccia, Nauseef and Clark reported that after a 10 minute incubation with opsonsized zymosan, eosinophils produced 2-3 times the amount of superoxide anion than neutrophils. Furthermore, this respiratory burst was sustained longer. Even unstimulated eosinophils had greater resting superoxide anion levels than neutrophils, which had no detectable levels. Since the lung parenchyma is relatively deficient in intracellular antioxidants, eosinophils may damage the tissue through the release of oxygen radicals (Cantin et al, 1983).

Eosinophils also contain a major basic protein which is toxic for parasites such as Schistosoma mansoni (Butterworth et al, 1979). The major basic protein has also been found to damage lung epithelial and tracheal tissue (Frigas, Loegering and Gleich, 1980; Frigas et al, 1981). In addition, Hastie et al (1987), observed that human eosinophil major basic protein reduced the beat frequency of tracheal explants as well as decreased the zone of ciliary activity. Because of this inadequate ciliary clearance, exposure of the lungs to such damaging substances as inflammatory mediators and particles is prolonged. The capacity of eosinophils to kill Schistosoma mansoni larvae is potentiated by the release of a factor which enhances eosinophil cytotoxicity from activated monocytes and T-lymphocytes. Lenzi, Mednis and

Dessein (1985) subsequently postulated that these enhancing factors increased the damage caused to tissues by the inflammatory mediators released by activated eosinophils.

RATIONALE AND OBJECTIVES

In recent years, there has been an increasing public awareness of the potential occupational health hazards to workers exposed to asbestos substitutes such as glass fibers. Although many studies have been conducted on the pathogenicity of these fibers, no definite conclusions have been drawn as to potential health problems suffered by workers as a result of exposure to glass fibers. Previous work on the toxicity of glass fibers (Risendal et al, 1987) has shown that the inflammatory and fibrotic changes seen in the lungs following a single exposure to glass fiber are resolved by 15 weeks. However, in the workplace, workers are repeatedly exposed to these fibers. Therefore, it is important to determine the effects of repeated exposures on the lungs. Thus, three Specific Aims comprise this project:

1. To evaluate the acute pulmonary inflammatory response following single and repeated exposures to glass fibers.
2. To assess the chronic inflammatory and fibrotic response of the lungs after single and repeated exposures to glass fibers.
3. To compare the inflammatory and fibrotic effects of glass fiber exposure to effects elicited by other fibrous and crystalline particles known to evoke damage.

To accomplish these goals, a well developed murine model of pneumoconiosis (Callis et al, 1983) was used to assess several parameters of acute and chronic inflammation following exposure to

three particles. Glass fiber was the test particle, silica was examined as a crystalline inflammatory and fibrotic particle and amosite asbestos was used as a comparative inflammatory and fibrotic fiber. To evaluate the acute inflammatory response, changes in the cell number and cell types present in lung lavage fluid were determined as well as the amounts of extracellular protein and plasminogen activator in the lung lavage fluid. In addition to these parameters, changes in the wet lung weight and the total lung hydroxyproline content were examined to assess the chronic pulmonary response to these particles.

METHODS

Animals

Male and female mice 8 to 12 weeks of age with body weights of 15-20 grams were obtained from Jackson Laboratories, Bar Harbor, ME. Body weights of these mice remained between 20-30 grams throughout the experiment. All mice used were of the C57BL/6 strain, a known high responder to the instillation of silica crystals (Callis et al, 1983). Animals were housed in the Division of Animal Resources, Arizona Health Sciences Center in a room maintained at 25^{o} C on a 12 hour light/12 hour dark cycle. Cages were equipped with filter bonnets to minimize respiratory infection, and animals were provided with food and water ad libitum.

Intratracheal Instillation

Intratracheal instillation is a well accepted method used for the exposure of animal lungs to many soluble or particulate compounds (Brain et al, 1976). This technique allows for the delivery of a defined quantity of particles (0.25 or 0.75 mg/mouse) into the lungs and enables the investigator to follow a specific time course (five days or five weeks) to monitor the chronology of response. In addition, the distribution of dilute particle instillations have been shown to mimic the distribution of particles following inhalation (Bernstein et al, 1982).

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital [40 mg/kg (Abbott Laboratories, N. Chicago, IL)] and the trachea exposed by dissection. Particles were suspended in sterile saline [0.9% (American McGaw, Irvine, CA)] and instilled in a dose volume of 0.1 ml (Callis et al, 1983) directly into the lungs using a 1 cc tuberculin syringe (Becton Dickinson & Co., Rutherford, NJ) with either a 23 gauge (glass fiber and asbestos) or a 25 gauge needle (silica). Control animals received 0.1 ml of sterile saline alone. Animals lay at a 45^{0} angle during the injection process. Following injection, the overlying skin was closed with 9 mm stainless steel surgical Autoclips (Becton Dickinson & Co., Parsippany, NJ). In some groups of mice, doses were repeated at 5 days and again at 10 days following the first exposure whereas other groups received a single exposure. Animals were euthanitized 5 days or 5 weeks following the final exposure (Figure 1). The cellular inflammatory response was evaluated in the lavage fluid by cell number, cell type, cell-free protein and extracellular plasminogen activator content. To measure collagen deposition, total lung hydroxyproline content was also assessed at the 5 week time point. Increases in wet lung weight normalized to body weight were also measured at 5 weeks post-exposure. If death occurred as a result of the injections, it was usually within 24 hours. Approximately 80% of all silica and 60% of all asbestos and glass fiber injected animals survived this procedure. Survivors remained healthy and maintained appropriate body weight throughout the period of observation.

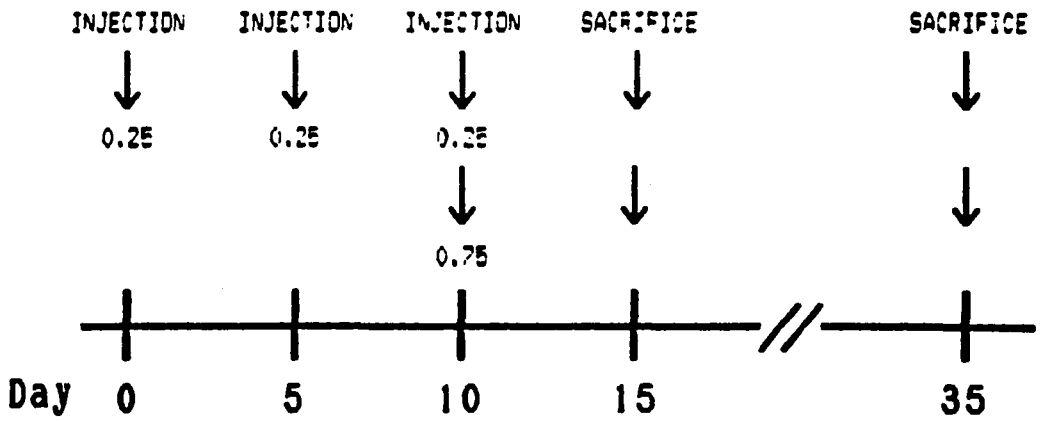

Figure 1. Effect of Glass Fibers on Pulmonary Inflammation and Fibrosis at Five Days and Five Weeks Post-Exposure in the Mouse: Dosing Regimen. 0.25= 0.25 mg/0.1 ml; 0.75= 0.75 mg/0.1 ml.

Particles: Particulate suspensions were prepared in concentrations of 2.5 mg/ml or 7.5 mg/ml in sterile saline to deliver a dose of 0.25 or 0.75 mg in a 0.1 ml injection volume to each animal. Glass fibers (JM-code 100 microfiber resin-free, 0.1 microns in diameter, 8 microns in length) were prepared for injection by J.A. Pickrell, Lovelace Toxicology Institute, Albuquerque, NM (Carpenter et al, 1983). Silica crystals (α-quartz, Min-U-Sil 5, Pittsburgh Paint and Glass Corporation) less than 5 microns in diameter were used for comparison as a crystalline inflammatory and fibrotic control. Amosite asbestos fibers (generously provided by Mr. Mitchell Rosner, University of Arizona, Tucson, AZ) were used in comparison as a fibrous inflammatory and fibrotic control. Three doses of 0.25 and one dose of 0.75 mg/mouse were chosen for use in this investigation based on previous reports showing the production of a measurable inflammatory response by these amounts without death from severe acute pulmonary injury (Callis et al, 1983; Pickrell et al, 1983).

Measurement of Pulmonary Inflammatory Response

Lung Lavage Fluid: Lungs of animals were lavaged in situ with 1 ml sterile saline. A 3 ml reservoir, 25 cm above the work area, was allowed to drip slowly into 15 cm of polyethylene tubing, 5 mm in diameter. A 3-way stopcock regulated the flow through a 20 gauge needle into 10 cm of intramedic tubing, 0.86 mm in diameter, which fed directly into a small incision in the trachea (Callis et al, 1983).

The lavage fluid was retrieved and centrifuged at 4^{0} C, 400 x g for 5 minutes to separate the cells from the supernatant (Sorvall RT6000 Centrifuge, DuPont). The cell-free supernatant was portioned into aliquots and frozen at -20^{0} C until the protein and plasminogen activator content of each sample could be determined.

Cell Number: The cell pellet was resuspended in 0.1 ml of sterile saline containing 5% bovine serum albumin [BSA (Sigma Chemical Co., St. Louis, MO)]. Fifty microliters of this suspension was diluted with 0.05 ml 1% crystal violet stain and counted on a hemocytometer under a light microscope at 400x (Callis et al, 1983). Total cell number is expressed as cells per ml of lavage fluid.

Cell Differentials: The BSA/saline solution (0.15 ml) was added to the remaining suspension of cells which were pelleted onto a glass slide in a Shandon Cytospin 2 centrifuge for 6 minutes at 400 x g. The resulting cell smear was air dried for 10 minutes, stained with Wright-Giemsa stain (Cambridge Chemical Products, Inc., Ft. Lauderdale, FL) for 10 seconds and rinsed with deionized water for 20 seconds. Five hundred cells per slide were counted under a light microscope at 400x and differentiated based on nuclear morphology and cytoplasmic granules (Callis et al, 1983). Data are expressed as cell number per ml of lavage fluid.

Microscopic Evaluation of Lavage Cells: As a visual demonstration of the cell types enumerated, photomicrographs are presented of the lung lavage cells at 400x after centrifugation onto a glass slide and staining with Wright-Giemsa stain. All photomicrographs presented are from animals given three injections of saline, glass fibers, asbestos or silica, five days after exposure. Based on nuclear morphology and cytoplasmic granules, the cells were differentiated as macrophages, neutrophils or eosinophils.

Protein: The total protein content of the cell-free supernatant was determined by the method developed by Lowry et al (1951). A protein standard of 1 mg/ml BSA (Miles Scientific, Naperville, IL) in saline was serially diluted with saline for the standard curve. Lavage samples from particle injected animals were diluted 1:2 with saline. Lavage fluid from saline injected animals was not diluted. This colorimetric assay measures the amino acids tryptophan and tyrosine with a sensitivity range of 0.005 to 0.2 mg protein. Data are expressed as mg of protein per ml of lavage fluid.

Plasminogen Activator: The plasminogen activator content of the cell-free supernatant was determined as described by Overwien, Neumann and Sorg (1980). Experimental samples and the positive control, urokinase [Sigma Chemical Co., St. Louis, MO; activity 15-25 units per mg protein; 4 mg in 1 ml phosphate-buffered saline 0.01 N, pH 7.2], in 0.225 ml volume were incubated with 0.04 ml plasminogen (Sigma; 1 mg in

2 ml phosphate-buffered saline) and 0.013 ml of the chromophore D-Val-L-Leu-L-Lys-p-nitroanilide (Sigma; 2 mg in 1 ml phosphate-buffered saline) in a microtiter plate for 2.5 hours at 37° C. Plates were read in a Titertek Multiskan MC ELISA reader at 405 nm with a reference wavelength of 490 nm. This colorimetric assay measures the cleavage of plasminogen in the presence of the chromophore. Values are expressed as percent of the positive urokinase control.

Measurement of Pulmonary Fibrotic Response

Lung Indices: Increases in wet lung weight as calculated by the following equation (Allen, Moore and Stevens, 1977) were used as a general indication of pulmonary inflammation and fibrosis:

$$\frac{\text{lung weight/body weight particle injected mouse}}{\text{lung weight/body weight saline injected syngeneic mouse}}$$

Therefore, a ratio of one is representative of a normal lung index. Data are expressed as indices.

Total Lung Hydroxyproline Content: Collagen deposition was determined by assessing the total hydroxyproline content of the lungs. Lungs were excised, minced with scissors, placed in 10 ml 6 N hydrochloric acid and autoclaved for 90 minutes. The hydrolysate was filtered and evaporated under air. The resulting residue was resuspended in 10 ml deionized water and the pH adjusted to between 6 and 7 with 1 N sodium hydroxide. The sample is analyzed colorimetrically for the imino acid, hydroxyproline, using the method described by Woessner (1961). For

each assay, a hydroxyproline standard (Sigma; 25 mg in 250 ml of 0.001 N hydrochloric acid) was diluted to 5 ug/ml in deionized water. Serial 2-fold dilutions were made for a standard curve. Samples from particle injected animals were diluted 1:16 with deionized water and samples from saline injected animals were diluted 1:8, also with deionized water. Following the addition of 1 ml chloramine T solution (Sigma; 1.41 g in 20 ml deionized water, 30 ml methyl cellosolve; Sigma) to 2 ml of each sample, tubes were vortexed and allowed to stand at room temperature for 20 minutes. One ml perchloric acid (JT Baker Chemical Co.; 27 ml diluted to 100 ml with deionized water) was added to each tube and mixed well. After 5 minutes, 1 ml p-dimethylaminobenzaldehyde (Sigma; 20 g in 100 ml methyl cellosolve) was added, mixed vigorously and incubated in a 60^{0} C water bath for 20 minutes, protected from light. The tubes were then cooled for 20 minutes in a room temperature water bath and read at 557 nm (Gilford Stasar II Spectrophotometer). Hydroxyproline values are expressed as micrograms of hydroxyproline per pair of lungs.

Statistical Analysis

Data are expressed as means +/- standard error with n values between 5 and 12 for each data point. Statistical significance between data points was determined by the ANOVA one-way test. Significance of data points within the same particle treatment group was determined by the Student's T test, and the Neuman-Keuls ad hoc test was used to determine significance of data points between treatment groups. All statistical tests were conducted with a significance level of $p < 0.05$.

RESULTS

In a murine model of pneumoconiosis, the pulmonary inflammatory and fibrotic response to single (0.75 mg/mouse) and repeated (3 doses of 0.25 mg/mouse at 5 day intervals) exposures to various particles was assessed. The cellular inflammatory response was evaluated by changes in the number and types of inflammatory cells present in the lung lavage fluid, whereas the non-cellular inflammatory response was evaluated by measuring the inflammatory mediator, plasminogen activator, and the total protein content in the lung lavage fluid. These parameters were evaluated five days following exposure to assess the acute inflammatory response found to peak at this time (Stefaniak and Hubbard, 1987), and at five weeks post-exposure to assess the chronic inflammatory response. In addition to these parameters, the total lung hydroxyproline content and wet lung weight/body weight ratio were also evaluated five weeks following exposure to quantify the chronic inflammatory and fibrotic response in the lungs. Three particles were used in this study: glass fiber, the experimental particle; silica, used as a comparative inflammatory and fibrotic crystal; and amosite asbestos, used as a comparative inflammatory and fibrotic fiber. In addition, animals were exposed to repeated doses of saline alone as a vehicle control.

Cellular Inflammatory Response

Inflammatory Cell Number in the Lavage Fluid:

The acute cellular inflammatory response to the instillation of particles or saline was evaluated five days after exposure by determining the total number of inflammatory cells present in the lung lavage fluid (Figure 2). At five days post-exposure, all particles, regardless of dosing regimen, induced a cellular inflammatory response that was significantly greater than the injection of saline alone. Although glass fiber exposure induced a cellular response that was greater than that evoked by saline, there was no significant difference between animals receiving single or repeated exposures. The cellular inflammatory response induced by repeated injections of any of the three particles was similar. However, a single injection of glass fiber induced a significantly greater cellular response than did a single injection of either asbestos or silica.

At five weeks post-exposure (Figure 3), with one exception, the cell number in the lavage fluid, regardless of particle or dosing regimen, was still significantly above levels in animals receiving saline alone. The one exception was a single injection of asbestos that did not induce a sustained cellular infiltration above that seen in saline injected mice. There was no difference in the total cell number between animals receiving either single or repeated injections of glass fibers or asbestos. Injections (single or repeated) of either glass fibers or silica elicited a comparable cellular inflammatory

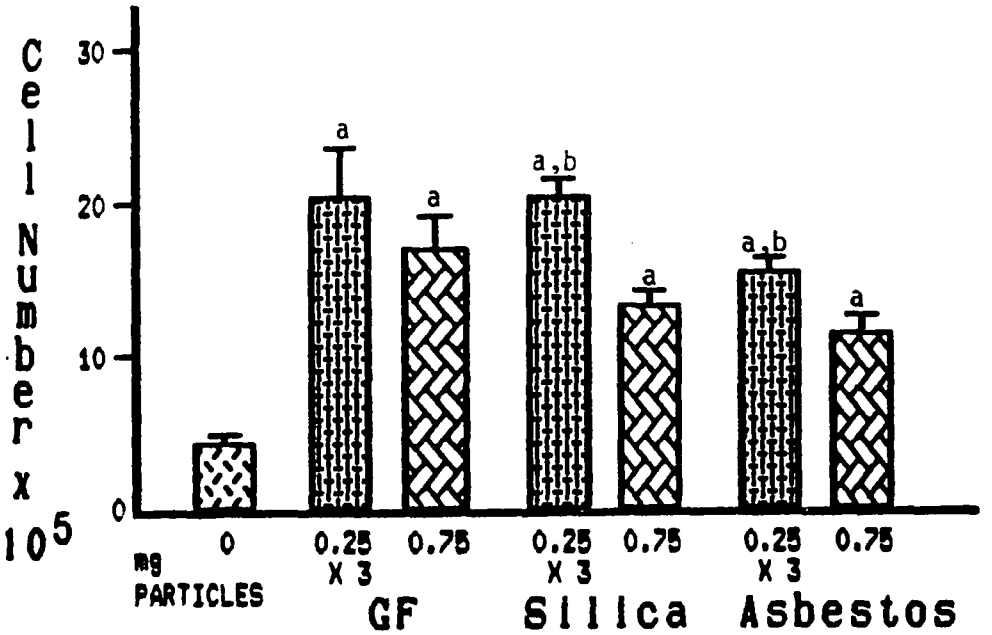

Figure 2. Effect of Glass Fibers on Pulmonary Inflammation at Five Days Post-Exposure in the Mouse: Inflammatory Cells/ml of Lavage Fluid. Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

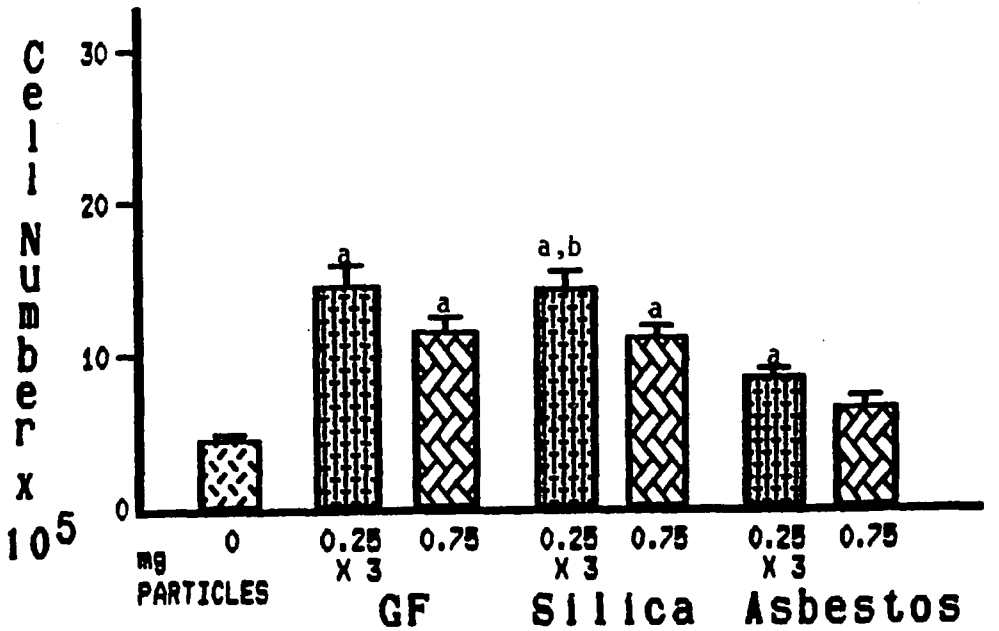

Figure 3. Effect of Glass Fibers on Pulmonary Inflammation at Five Weeks Post-Exposure in the Mouse: Inflammatory Cells/ml of Lavage Fluid. Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

response. Both of these particles evoked an inflammatory response that was significantly greater than that induced by either repeated or single injections of asbestos. Thus, it appears that exposure to these particles, in either single or repeated injections, provokes a significant cellular infiltration that, with the exception of a single injection of asbestos, is still evident at five weeks post-exposure. Overall, repeated injections of glass fibers did not potentiate the acute or chronic cellular inflammatory response as assessed by total cell number.

Inflammatory Cell Type in the Lavage Fluid:

The inflammatory cells present in the lavage fluid were differentiated as either eosinophils, neutrophils or macrophages based on nuclear morphology and cytoplasmic granules. The presence of neutrophils and eosinophils in the lavage fluid indicate an acute inflammatory response (Gee, 1980) whereas an increase in the number of macrophages in the lavage fluid is indicative of a more chronic inflammatory response (Callis et al, 1985). At five days post-exposure, it was found that glass fibers, whether injected once or repeatedly, induced a significant increase in all three cell types above that seen with saline injected animals (Figure 4a). In addition, a marked eosinophilic response was noted following exposure to glass fibers. These eosinophils are readily identified by the presence of red granules in the cytoplasm (Figure 6b). The eosinophilic response was significantly greater following repeated exposures than after a

single exposure. Since saline injected animals had no eosinophils present in the lavage fluid, only neutrophils and macrophages were enumerated (Figure 6a). Although animals exposed to glass fibers had elevated numbers of both neutrophils and macrophages, there was no difference between animals receiving single or repeated injections in either of these cell types. Similar to glass fibers, injections of asbestos also elicited an eosinophilic response (Figures 4b and 6c). However, there was virtually no effect by either single or multiple injections of silica on the number of eosinophils present (Figures 4c and 6c).

It appears then, that five days after exposure to any of the three particles, an inflammatory response is elicited and characterized by an infiltration of a large number of neutrophils, macrophages and, in the case of glass fibers and asbestos, eosinophils. In addition, repeated exposures to glass fibers results in significantly greater numbers of eosinophils whereas repeated exposures to asbestos causes a significant increase in the number of macrophages and repeated injections of silica induces an increase in the number of both neutrophils and macrophages above that seen following a single injection.

At five weeks post-exposure, animals given single or repeated injections of glass fibers continued to demonstrate a significantly elevated number of neutrophils, eosinophils and macrophages in the lung lavage fluid above that seen in the saline injected controls (Figure 5a). Repeated injections of glass fibers elicited a neutrophilic

response that was significantly greater than that seen following a single exposure. However, this enhancement by repeated injections was not observed in the numbers of eosinophils or macrophages. Again, single and repeated exposures to glass fibers elicited a marked eosinophilic infiltration that was significantly greater than any eosinophil response induced by either silica or asbestos (Figures 5a, 5b, 5c). Thus, by five weeks post-exposure to single or repeated injections of any of the three particles, the inflammatory response was still evident as reflected in elevated numbers of neutrophils, macrophages and, in the case of glass fibers, eosinophils. In addition, repeated injections of glass fibers and asbestos resulted in significantly greater numbers of neutrophils whereas repeated injections of silica resulted in a greater number of macrophages.

In addition to the strong eosinophil response, the neutrophil response evoked by single or repeated injections of glass fiber was also significantly greater than that resulting from asbestos exposure. Repeated exposures to glass fibers induced a significantly greater number of macrophages in the lavage fluid than did repeated exposures to asbestos. Moreover a single injection of glass fibers induced a greater number of macrophages than a single injection of either asbestos or silica. It would appear then, that glass fibers induced a lasting neutrophilic and macrophage response. During acute inflammation, repeated exposures to glass fibers induced a greater eosinophilic influx than did a single exposure (Figures 4a and 6b). This effect was not observed during chronic inflammation (Figure 5a).

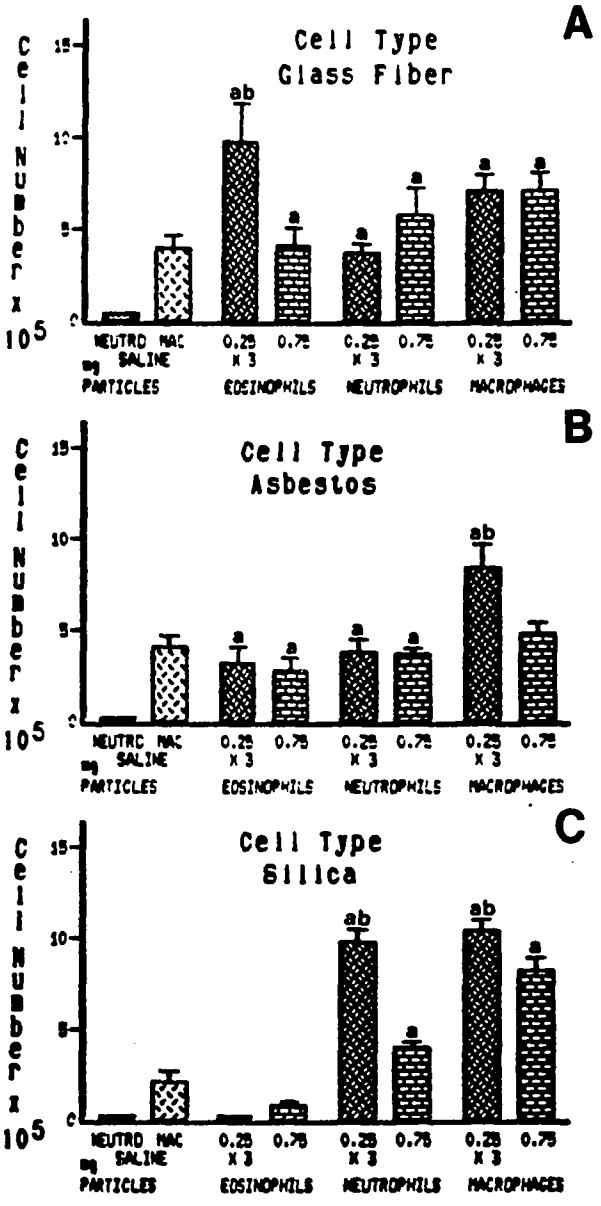

Figure 4. Effect of Glass Fibers on Pulmonary Inflammation at Five Days Post-Exposure in the Mouse: Types of Inflammatory Cells/ml of Lavage Fluid in Animals Exposed to A) Glass Fibers; B) Asbestos; C) Silica. Cells were differentiated as neutrophils (neutro), macrophages (mac) or eosinophils. Doses are: 0= saline controls; 0.25 x 3=33 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

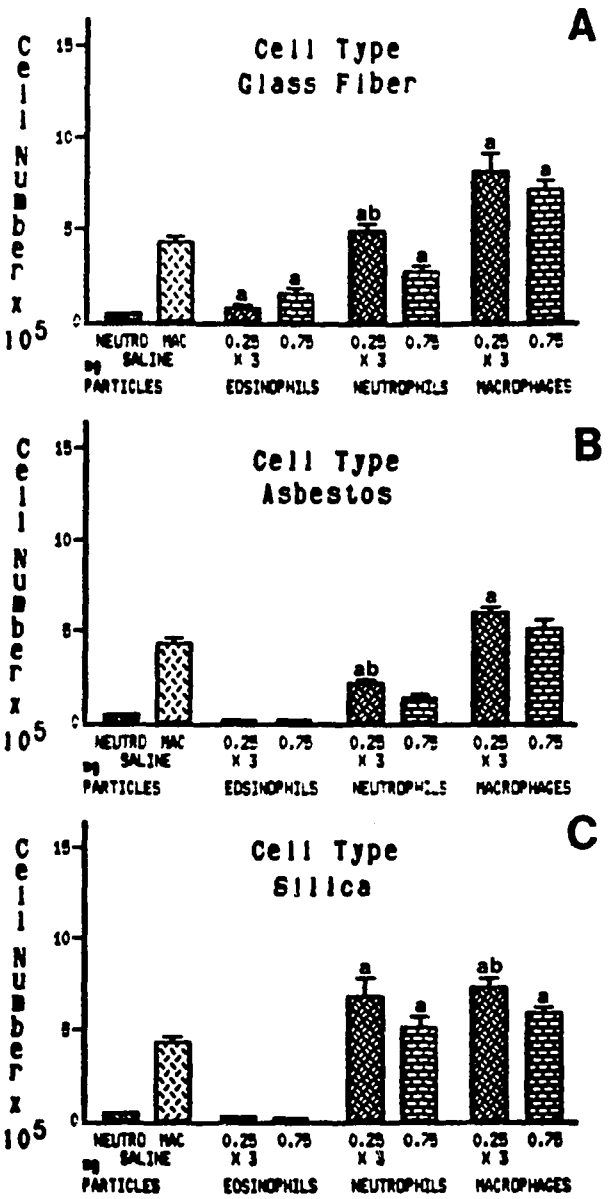

Figure 5. Effect of Glass Fibers on Pulmonary Inflammation at Five Weeks Post-Exposure in the Mouse: Types of Inflammatory Cells/ml of Lavage Fluid in Animals Exposed to A) Glass Fibers; B) Asbestos; C) Silica. Cells were differentiated as neutrophils (neutro), macrophages (mac) or eosinophils. Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

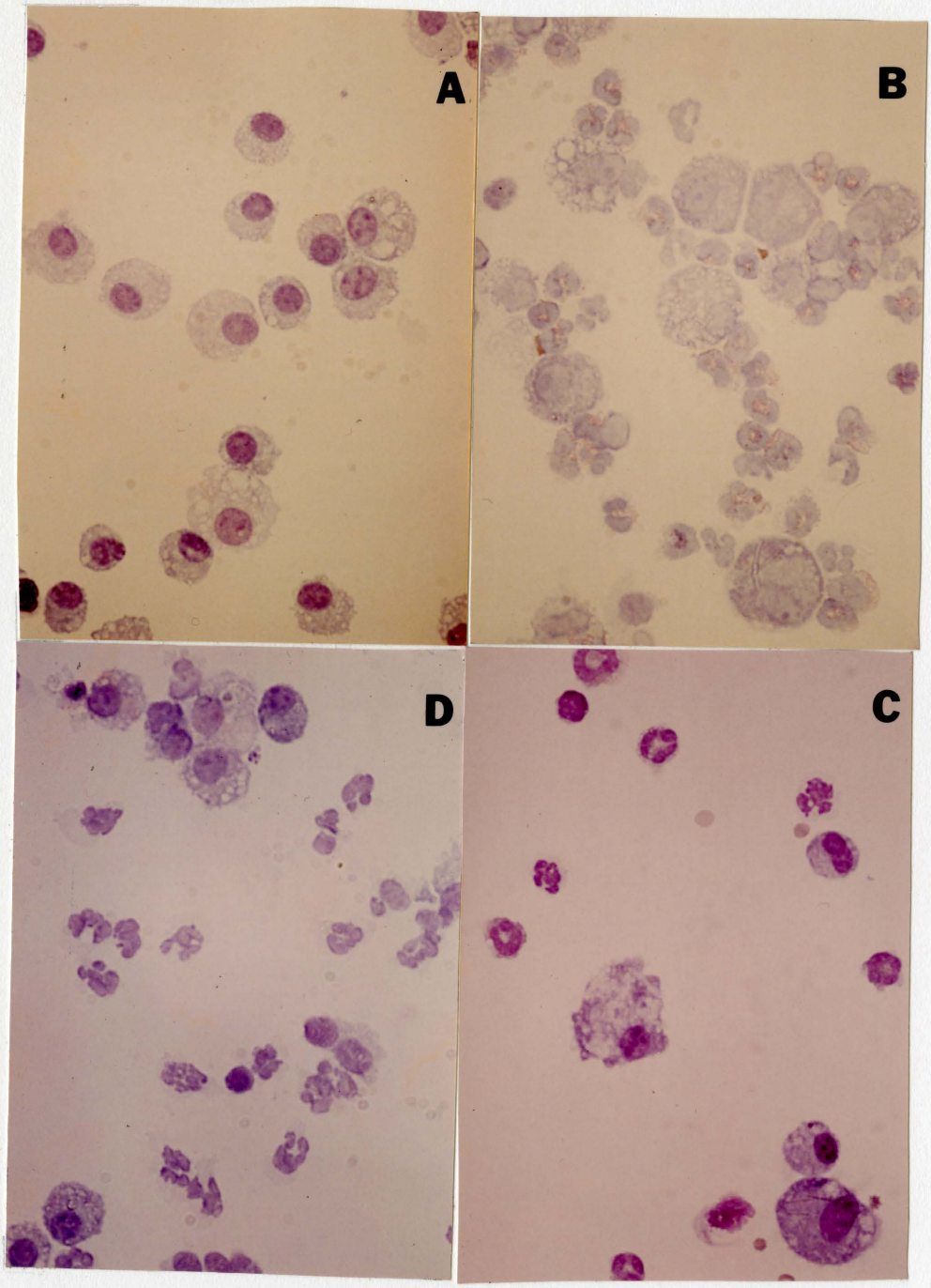

Figure 6. Effect of Glass Fibers on Pulmonary Inflammation at Five Days Post-Exposure in the Mouse: Photomicrographs (400x) of Lavage Cells of Animals Given 3 Exposures of 0.25 mg/0.1 ml at Five Day Intervals of A) Saline; B) Glass Fibers; C) Asbestos; D) Silica. Cells were stained with a differential Wright-Giemsa stain.

Instead, at five weeks post-exposure, there was a shift from the eosinophil and macrophage response seen at five days post-injection to a neutrophil and macrophage response.

Non-Cellular Inflammatory Response

Total Protein Content of Lavage Fluid:

Damage to the epithelial cells of the lung, increased interstitial permeability and/or cell lysis or enzyme release from inflammatory cells may account for elevations in the level of extracellular protein present in the cell-free lung lavage fluid (Gross, 1968). The protein content in the lung lavage fluid at five days following exposure to any of the three particles was significantly elevated over that seen in saline injected animals in all cases except with a single exposure to asbestos (Figure 7). Only animals repeatedly exposed to glass fibers had significantly greater levels of protein than those receiving a single exposure.

Repeated injections of either glass fibers or silica induced protein levels that were comparable and significantly greater than that elicited by repeated injections of asbestos. On the other hand, a single injection of glass fibers induced protein levels comparable to a single injection of asbestos, but significantly less than that induced by a single injection of silica.

At five weeks post-exposure, the protein levels in the lavage fluid of animals receiving repeated injections of any of the three particles, and those receiving single injections of either glass fibers

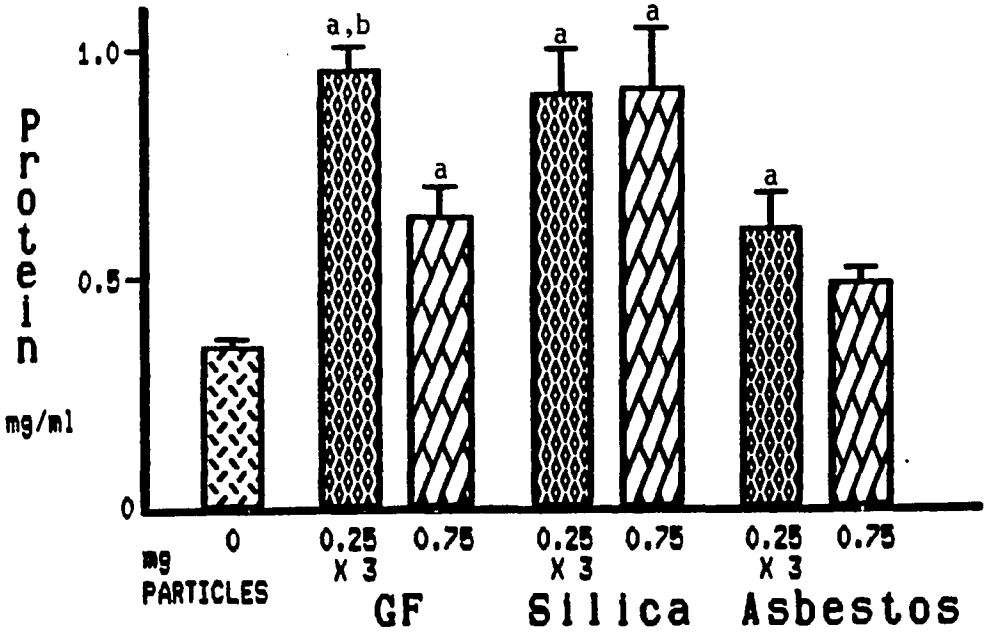

Figure 7. Effect of Glass Fibers on Pulmonary Inflammation at Five Days Post-Exposure in the Mouse: Total Protein (mg per ml of lavage fluid). Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

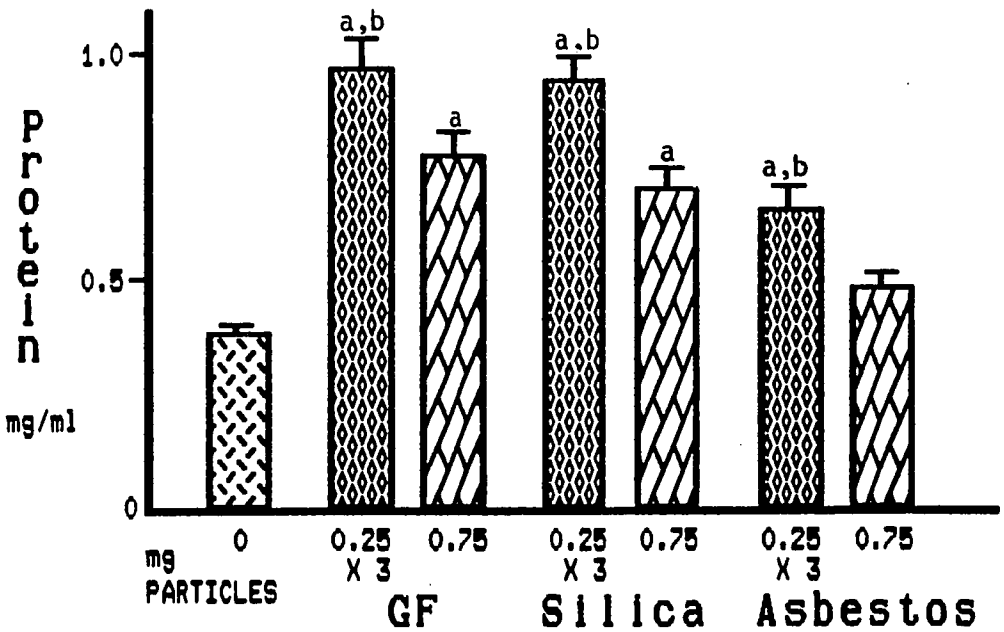

Figure 8. Effect of Glass Fibers on Pulmonary Inflammation at Five Weeks Post-Exposure in the Mouse: Total Protein (mg per ml of lavage fluid). Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

or silica, were all elevated above saline levels (Figure 8). At five weeks post-exposure, all animals receiving repeated injections of any of the three particles had protein levels significantly greater than those animals receiving a single exposure. In addition, the lavage fluid protein levels of animals given a single exposure to glass fibers were more comparable to animals exposed once to silica than to animals exposed once to asbestos. This similarity between glass fibers and silica was also observed in animals receiving multiple injections of the particles. Thus, during both the acute and chronic stages of inflammation, repeated exposures to glass fibers resulted in greater protein levels than did a single exposure (Figures 7 and 8). This enhanced increase in protein levels seen five weeks after exposure to glass fibers was more similar to the response elicited by silica than to the response resulting from asbestos exposure.

Plasminogen Activator Content of Lavage Fluid:

In addition to total protein content, the activity of the neutral protease, plasminogen activator, was also measured to evaluate the inflammatory response to these particles (Figure 9). Release of plasminogen activator is highly correlated with macrophage activation (Unkeless, Gordon and Reich, 1974; Gordon, Unkeless and Cohn, 1974), and this enzyme also possesses chemotactic activity for mononuclear cells and neutrophils (Ward, 1976; Kaplan, Goetzl and Austen, 1973). Only a single injection of silica induced an elevation in the lung lavage fluid plasminogen activator content that was significantly

greater than that in saline control animals (Figure 9). However, repeated injections of glass fibers induced a significantly greater level of this enzyme above that seen after a single injection. Thus, it appears that the plasminogen activator level in the lavage fluid is not affected by a single exposure to either glass fibers or asbestos, or by repeated exposures to any of the particles investigated in this study. Curiously, repeated exposures to glass fibers induced a level of plasminogen activator that was significantly greater than that induced by a single injection.

The plasminogen activator activity in the lung lavage fluid five weeks after single or repeated exposures to either glass fibers or silica was significantly greater than levels seen in saline controls (Figure 10). There was, however, no difference in plasminogen activator levels between animals receiving single or repeated injections of either glass fibers or silica. Moreover, both particles evoked comparable levels of plasminogen activator. It appears then, that multiple injections of glass fibers induced more of this inflammatory mediator during acute inflammation than did a single injection. And, the activity of this neutral protease, if induced by glass fibers or silica, was still measurable five weeks following exposure (Fig. 10).

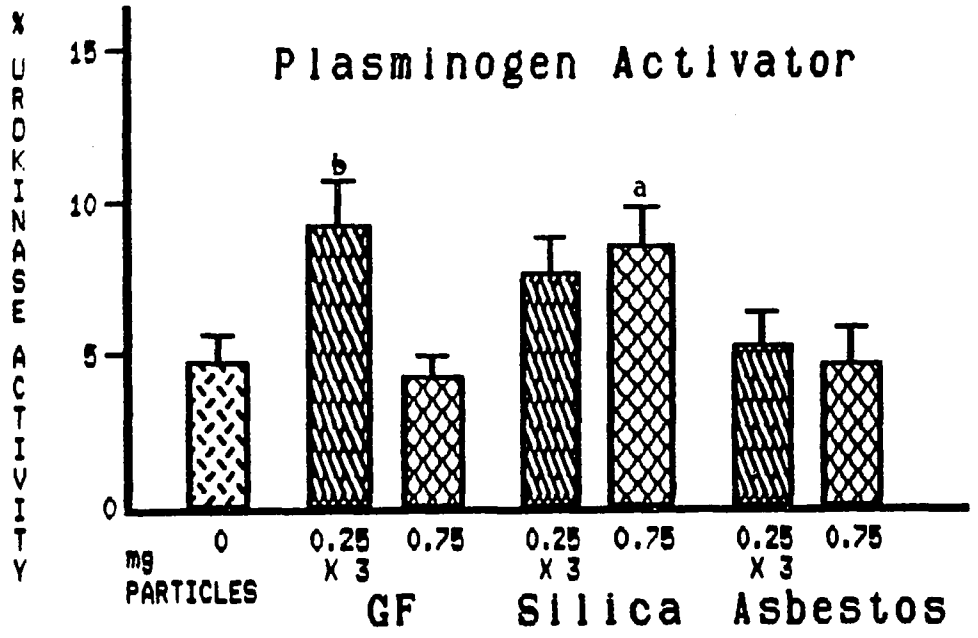

Figure 9. Effect of Glass Fibers on Pulmonary Inflammation at Five Days Post-Exposure in the Mouse: Plasminogen Activator Content of Lavage Fluid. Data are expressed as % of urokinase activity. Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

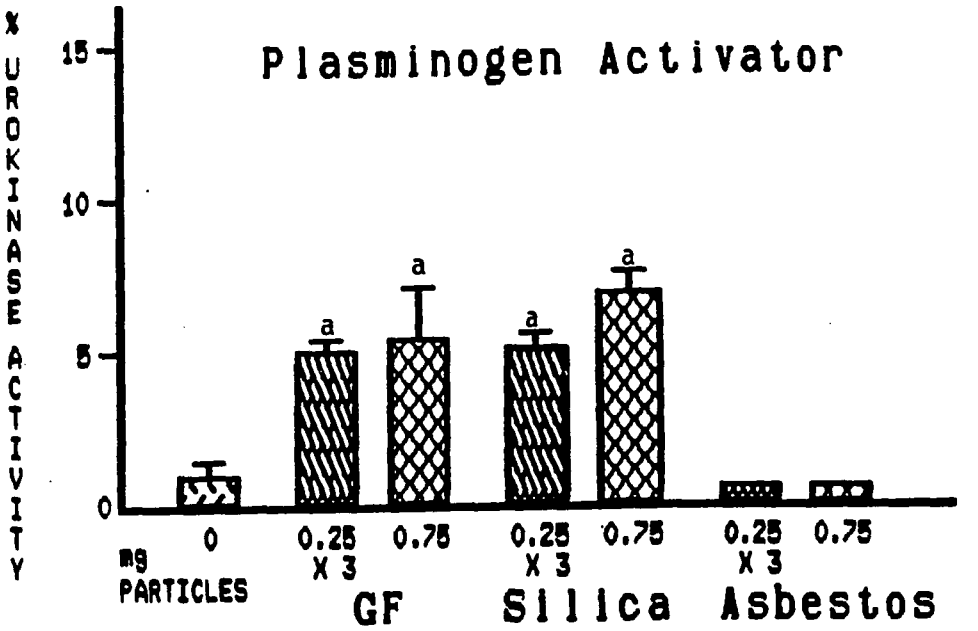

Figure 10. Effect of Glass Fibers on Pulmonary Inflammation at Five Weeks Post-Exposure in the Mouse: Plasminogen Activator Content of Lavage Fluid. Data are expressed as % of urokinase activity. Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

Parenchymal Tissue Response

Lung Indices:

Increases in the wet lung weight/body weight ratio of mice exposed to lung toxicants may be attributed to the influx of inflammatory cells, edema and/or collagen deposition (Callis et al, 1985). Measurements of this parameter were made at five weeks post-exposure to reflect any increase in wet lung weight due to chronic changes (i.e., inflammatory cell influx or replication and collagen deposition). At five weeks post-exposure, repeated injections of either glass fibers or asbestos and a single injection of glass fibers induced a significant increase in the lung indices above that seen in saline controls (Figure 11). Silica exposures, either single or repeated, had no effect on this parameter at this time (Figure 11).

Both repeated and single injections of glass fibers caused an increase in lung indices that was significantly greater than that seen in animals exposed to repeated or single injections of silica. Additionally, repeated injections of either glass fibers or asbestos gave comparable index values. Thus, it appears that glass fibers, given in either single or repeated exposures, caused the greatest increase in the lung weight/body weight ratio. Only repeated injections of the asbestos fibers caused an increase in this parameter above values seen in animals receiving a single exposure.

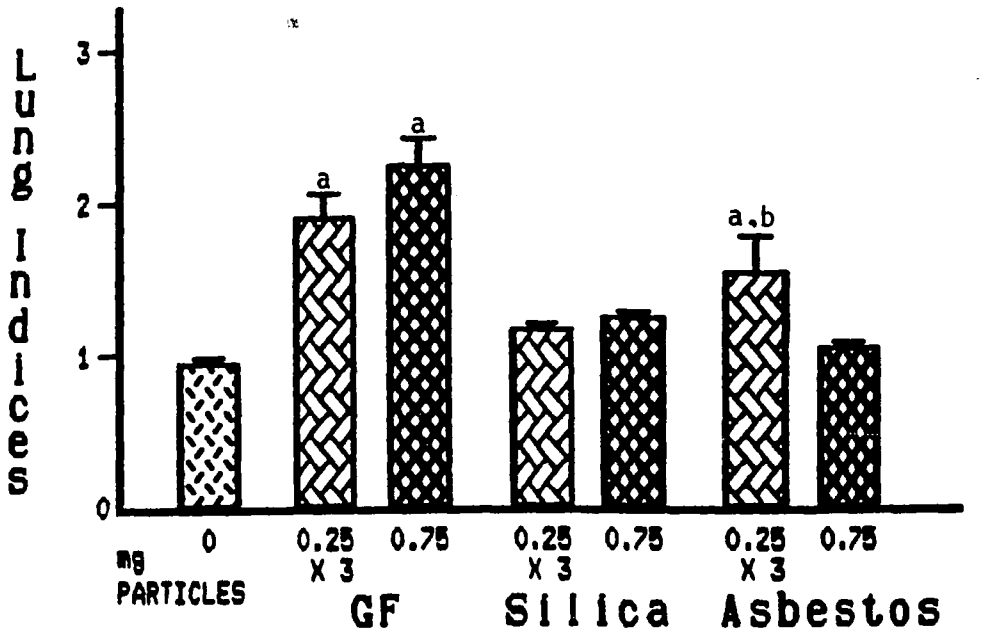

Figure 11. Effect of Glass Fibers on Pulmonary Inflammation and Fibrosis at Five Weeks Post-Exposure in the Mouse: Lung Indices (wet lung weight/body weight). Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

Total Lung Hydroxyproline Content:

Increases in the hydroxyproline content of the lungs are a reflection of the formation of collagen in the lungs (Woessner, 1961). This parameter was measured at five weeks post-exposure since previous data indicated little change between silica and saline injected animals at five days post-exposure (Callis, 1983). At five weeks post-exposure, repeated exposures to any of the three particles and single exposures to either glass fibers or asbestos elicited a significant increase in the lung hydroxyproline content (Figure 12).

Single or repeated exposures to glass fibers induced hydroxyproline levels that were significantly greater than levels elicited by single or repeated exposures to either asbestos or silica. As seen in the wet lung weight/body weight ratios (Figure 11), glass fibers appear to have greatest effect on the lung hydroxyproline content among these three particles. There was, however, no enhancement in lung hydroxyproline content by repeated exposures to glass fibers. It appears then, that five weeks after exposure to glass fibers in either single or repeated doses, lung hydroxyproline levels are elevated.

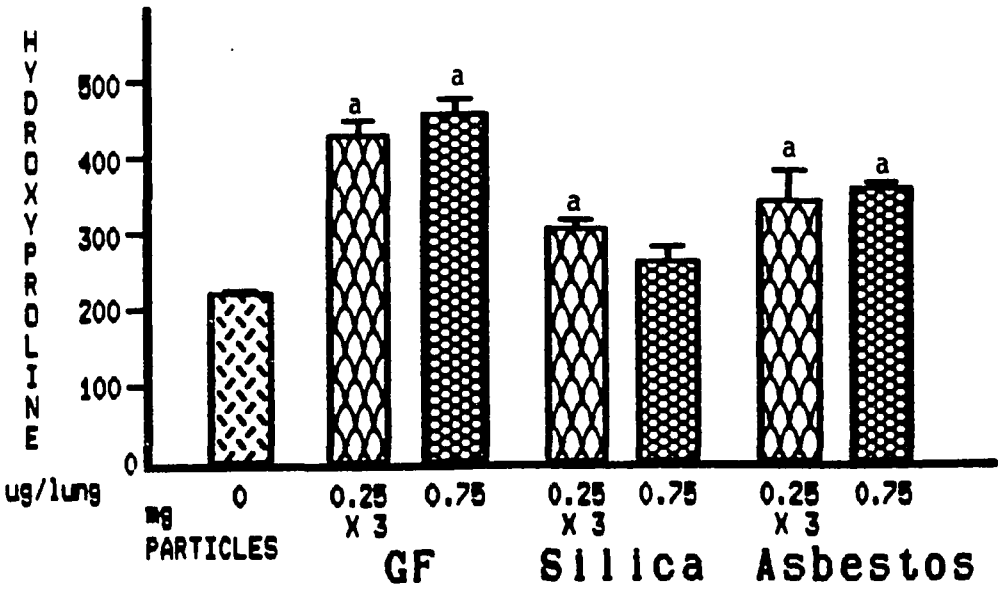

Figure 12. Effect of Glass Fibers on Pulmonary Fibrosis at Five Weeks Post-Exposure in the Mouse: Lung Hydroxyproline Content. Data are expressed as micrograms/lungs. Doses are: 0= saline controls; 0.25 x 3= 3 repeated exposures of 0.25 mg/0.1 ml each, 5 days apart; 0.75= a single exposure of 0.75 mg/0.1 ml. GF= glass fibers, a= significance over control, b= significance in comparison to single injections of the same particle. N= 5-12 for each test group.

Summary of Results

Potentiation by Repeated Exposures:

Indicator	Glass Fibers	Asbestos	Silica
Cell Number (acute)	-	+	+
Cell Number (chronic)	-	-	+
Eosinophil (acute)	+	-	-
Eosinophil (chronic)	-	-	-
Neutrophil (acute)	-	-	+
Neutrophil (chronic)	+	+	-
Macrophage (acute)	-	+	+
Macrophage (chronic)	-	-	+
Total Protein (acute)	+	-	-
Total Protein (chronic)	+	+	+
Plasminogen Activator (acute)	+	-	-
Plasminogen Activator (chronic)	-	-	-
Lung Indices (chronic)	-	+	-
Hydroxyproline (chronic)	-	-	-

DISCUSSION

The pulmonary inflammatory response to single or repeated exposures to glass fibers was investigated *in vivo* using a well-developed murine model of pneumoconiosis (Callis et al, 1983). In addition, this study used silica as a comparative crystalline inflammatory and fibrotic control, as well as amosite asbestos, as a comparative inflammatory and fibrotic fiber. All particles were within respirable size range, and of the pathogenic dimensions suggested by Stanton et al (1977).

Acute Pulmonary Inflammatory Response to Single or Repeated Exposures to Glass Fibers

The rapid influx of large numbers of inflammatory cells into the lung five days following exposure to glass fibers denotes the provocation of an acute inflammatory response. Both single and repeated injections of glass fibers induced comparable numbers of inflammatory cells present in the lung lavage fluid (Figure 2). Thus, repeated exposures to glass fibers did not potentiate the total cellular inflammatory response above that seen with a single dose of the equivalent amount of fiber. When these inflammatory cells were differentiated on the basis of cell type, it was interesting to note that exposure to glass fibers elicited a markedly enhanced eosinophilic response during acute inflammation (Figures 4a and 6b). In fact,

repeated exposures to glass fibers evoked a significantly greater eosinophil response than did a single exposure. Glass fiber exposed animals also demonstrated elevated levels of both neutrophils and macrophages in the lung lavage fluid (Figures 4a and 6b). However, repeated exposures did not enhance the number of these inflammatory cells present (Figure 4a). Thus, the increase in cell number observed following exposure to glass fiber can be attributed to an infiltration of all three cell types. However, repeated exposures, as opposed to a single exposure of equivalent dose, only appeared to influence the influx of eosinophil inflammatory cells rather than neutrophil and macrophage populations (Figure 4a).

Similar to increasing inflammatory cell numbers, glass fiber exposure elicited a significant increase in the level of cell-free protein present in the lung lavage fluid five days after exposure (Figure 7). In fact, both lavage protein and plasminogen activator levels were significantly greater after repeated injections than after a single exposure of the equivalent dose (Figures 7 and 9).

The effects of glass fiber exposure on the acute pulmonary cellular inflammatory response has been addressed by other investigators. Lugano et al (1982) observed a marked neutrophilic response followed by a massive accumulation of pulmonary macrophages in the lavage fluid of guinea pigs seven days after a single intratracheal injection of glass fibers. Van Graft et al (1986) examined the lungs of hamsters after a single intratracheal injection of glass fibers. He also remarked on the shift from a massive neutrophil infiltration to a

primarily macrophage response at three days post-exposure. None of these studies examined the acute pulmonary inflammatory response to repeated exposures to glass fibers. Thus, differences in the acute cellular pulmonary response between animals exposed once or multiple times to glass fibers had not yet been discussed.

Several investigators have reported the presence of large numbers of eosinophils in the lungs after fiber exposure. Van Graft et al (1986) frequently encountered eosinophils in the histopathological evaluation of the lungs of hamsters seven days after exposure to a single injection of glass fibers. In 1955, Schepers noted an abundance of eosinophils in the lungs of guinea pig two months after a single intratracheal injection of glass fibers. Rola-Pleszczynski et al (1981) studied the effects of repeated (monthly) asbestos exposures in the lungs of sheep. They reported the presence of a significant number of eosinophils in the lavage fluid three months following the first injection. Thus, although the presence of eosinophils in the lungs of glass fiber injected animals has been observed before, their role in fiber-induced pulmonary inflammation has yet to be discerned.

The pulmonary non-cellular inflammatory response to glass fiber exposure has also been investigated previously. Lee et al (1981) reported the presence of protein in the lungs of guinea pigs exposed for 90 days by inhalation to glass fibers. Sykes et al (1983a) noted an increase in the protein level in the lavage fluid of rats seven days after exposure. Although these studies found elevated lavage protein, this work is the first to report increased lavage protein in the acute inflammatory response following repeated exposures to glass fibers.

Chronic Pulmonary Inflammatory and Fibrotic Response to Single or Repeated Exposures to Glass Fibers

Previous investigators (Risendal et al, 1987) have reported that the pulmonary inflammatory and fibrotic responses to glass fibers are eventually resolved. Therefore, the chronic inflammatory response, as evidenced by the persistence of elevated numbers of monocytic inflammatory cells in the lung lavage fluid, was evaluated five weeks following exposure to glass fibers (Figure 3). Repeated exposures to glass fibers did not enhance the total number of inflammatory cells present during the chronic response. Similar to the acute inflammatory response measured at five days, the increase in total inflammatory cell number at five weeks can be attributed to elevated numbers of eosinophils, neutrophils and macrophages (Figure 5a). However, the eosinophilic infiltration which occurred during the acute response and was potentiated by repeated exposures, had, by five weeks post-exposure, diminished. The predominant granulocyte present during the chronic inflammatory response was the neutrophil (Figure 5a). The influx of neutrophils was significantly enhanced at this time by repeated exposures to glass fibers. Thus, it appears that both single and repeated exposures to glass fibers induces a chronic cellular inflammatory response (Figures 3 and 5a). In addition, the chronic, later response to repeated exposures to glass fibers had converted from an eosinophil/macrophage to a neutrophil/macrophage infiltrate.

Again, the elevated number of inflammatory cells in the lavage fluid corresponds with the heightened levels of cell-free protein in

the lavage fluid. These protein levels in the lavage fluid were still significantly elevated above levels in saline injected mice five weeks after glass fiber exposure (Figure 8). Repeated exposure to glass fibers significantly enhanced levels of lavage protein above that seen after a single exposure. This enhanced effect of repeated exposures on total protein may be due to other sources of lung protein (ie. cell lysis, edema) since plasminogen activator levels were not enhanced by repeated exposures (Figure 10).

Glass fiber exposed animals demonstrated an increase in lung index that was significantly above that of saline injected animals (Figure 11). Repeated exposures to glass fibers did not potentiate the increase in wet lung weight. This increase in lung index may be attributed to increases in the lung tissue hydroxyproline content (or indicates collagen deposition) since lung hydroxyproline content values were also elevated at this time, but not enhanced by repeated exposures to glass fibers (Figure 12). In addition, the increase in lung indices may also be attributed to elevated protein levels and/or the influx of inflammatory cells (Figures 3, 5a and 8).

The persistence in the increased number of inflammatory cells in the lavage fluid during chronic inflammation from glass fiber exposure has also been observed by others. Several investigators have noted an enduring elevation in PMN and macrophage number in the lungs of animals given a single intratracheal injection of glass fibers. This elevation, evident at one month post-exposure (Schepers, 1955; Van Graft, 1986), persisted in some cases for up to 18 months (Van Graft et

al, 1986; Bernstein et al, 1984). Repeated exposures to glass fibers have also been reported to induce a lasting infiltration of inflammatory cells into the alveoli. Kuschner and Wright (1976) noted that the macrophage reaction induced by three intratracheal injections of glass fibers continued for two years. Pickrell et al (1983) evaluated the lungs of hamsters given two injections of glass fibers. They noted pronounced aggregations of macrophages at one and 3.5 months post-exposure in addition to persistent increases of PMN and macrophages at 11 months post-injection. Similarly, Bernstein et al (1984) reported an enduring macrophage response in rats one year after ten intratracheal injections of glass fibers. Comparable to studies of the acute response, no comparisons were made between animals given single or repeated exposures.

Sustained elevations in cell-free protein levels have been reported previously by other investigators. Pickrell et al (1983) noted increased levels of such proteins as LDH and alkaline phosphatase in the lungs of hamsters one and 3.5 months after the administration of two intratracheal injections of glass fibers. Lee et al (1979) also reported the presence of alveolar proteinosis. Histological evaluation of the lungs of guinea pigs, hamsters and rats revealed alveolar proteinosis after 90 days of inhalation exposure to glass fibers. However, this proteinosis disappeared by one year post-exposure. As before, no comparisons have been made between animals given single or repeated exposures.

Increases in the lung index (wet lung weight/body weight) of experimental animals following glass fiber exposure have been reported previously. In a study similar to this investigation, Corsino et al (1987) noted a significant increase in the wet lung weight/body weight ratio of mice five weeks after exposure to a single intratracheal injection of glass fibers. In contrast, Pickrell et al (1983) found no significant difference above saline in the wet lung weight relative to body weight of hamsters at either one or 3.5 months after the intratracheal instillation of glass fibers.

Collagen deposition, in response to pulmonary injury induced by glass fiber exposure, has been demonstrated in experimental animals. Pickrell et al (1983) noted an elevated collagen content in hamsters at one and 3.5 months after two intratracheal injections of glass fibers. Similarly, Wright and Kuschner (1977) reported the presence of fibrotic lesions in guinea pigs two years after three intratracheal injections of glass fibers. None of these investigators, however, compared the results of single exposures with those of repeated exposures.

Comparison of the Inflammatory Responses to Single or Repeated Exposures to Glass Fibers and Asbestos

Acute Inflammatory Response:

It was demonstrated in this investigation that exposure to asbestos produced a cellular inflammatory response that, similar to glass fiber exposure, was significantly greater than in saline injected animals at five days post-exposure (Figure 2). In addition, repeated

exposures to either glass fibers or asbestos induces the infiltration of similar numbers of inflammatory cells whereas only asbestos will potentiate this cellular response by repeated exposures.

Animals given injections of either glass fibers or asbestos demonstrated numbers of eosinophils that were significantly elevated above those seen in saline injected animals (Figures 4a and 4b). In addition, repeated injections of glass fibers potentiated the eosinophil infiltration whereas repeated injections of asbestos did not (Figures 4a and 4b). Exposures (single or repeated) to either glass fibers or asbestos provoked the influx of comparable numbers of macrophages and neutrophils. Although exposures to either glass fibers or asbestos provokes an eosinophilic infiltration, repeated exposures to glass fibers potentiates the eosinophil response whereas repeated exposures to asbestos potentiates the macrophage response (Figures 4a and 4b).

Repeated exposures to glass fibers significantly enhanced the amount of cell-free protein present in the lavage fluid whereas repeated exposures to asbestos did not. In addition, the cell-free protein levels in animals exposed repeatedly to glass fibers were significantly greater than in animals exposed repeatedly to asbestos (Figure 7). In contrast, animals given single exposures to either glass fibers or asbestos demonstrated comparable cell-free protein levels. The plasminogen activator levels in the lavage fluid of either glass fiber or asbestos exposed animals were also comparable to each other (Figure 9). In fact, the plasminogen activator levels were not

significantly greater than those in saline injected animals. However, in glass fiber exposed animals, the potentiation by repeated exposures of the cell-free protein level was paralleled by a potentiation of the plasminogen activator level (Figures 7 and 9). Thus, although it appears that glass fiber and asbestos exposure results in elevated cell-free protein levels, enhancement of the protein level by repeated exposures is only seen in glass fiber injected animals. In addition, only in glass fiber exposed animals can the elevation in cell-free protein level be attributed, in part, to the presence of plasminogen activator in the lavage fluid.

Chronic Inflammatory and Fibrotic Response:

Upon evaluation of the chronic (five weeks) cellular inflammatory response, it was noted that exposure to glass fibers (single or repeated) and repeated exposures to asbestos provoked a cellular influx that was significantly greater than that induced in saline injected animals (Figure 3). Moreover, the cellular responses evoked by injections of glass fibers were significantly greater than those induced by injections of asbestos. In neither case was the total number of inflammatory cells present in the lavage fluid potentiated by repeated exposures. Thus, it appears that exposures to glass fibers evokes a more enduring cellular inflammatory response than exposures to asbestos.

Injections of glass fibers, either single or repeated, resulted in the infiltration of eosinophils, neutrophils and macrophages that

was still significantly elevated above saline levels at five weeks post-exposure (Figure 5a). In contrast, only repeated injections of asbestos evoked a lasting elevation in neutrophil and macrophage numbers present in the lavage fluid (Figure 5b). Comparable to the total inflammatory cell numbers present in the lavage fluid, single or repeated exposures to glass fibers induced greater eosinophil, neutrophil and macrophage responses than did single exposures to asbestos (Figures 5a and 5b). However, repeated injections of either glass fibers or asbestos enhanced the numbers of the neutrophil population. This is in contrast to the acute (five day) response where repeated exposures to glass fibers potentiated the eosinophil response (Figure 4a) and repeated exposures to asbestos potentiated the macrophage response (Figure 4b). Therefore, it seems that the increase in inflammatory cell numbers induced by exposure to glass fibers, over that seen in asbestos exposed animals, can be attributed to increases in all three inflammatory cell types (eosinophils, neutrophils and macrophages). In addition, repeated exposures to either glass fibers or asbestos appear to have the greatest influence on the neutrophil population at five weeks post-exposure.

Similar to the acute response, glass fiber exposure (single or repeated) and repeated exposures to asbestos resulted in cell-free protein levels that were significantly elevated above saline values (Figures 7 and 8). Moreover, the levels of cell-free protein induced by glass fiber exposure were significantly greater than those induced by asbestos exposure (Figure 8). However, repeated exposures to either

glass fibers or asbestos enhanced the amount of cell-free protein present in the lung lavage fluid. A portion of the cell-free protein in glass fiber exposed animals may be attributed to the presence of plasminogen activator in the lavage fluid (Figure 10). Plasminogen activator levels in glass fiber exposed animals were significantly greater than those in either saline or asbestos injected animals as the lavage fluid of asbestos exposed animals had no measurable level of plasminogen activator. Whereas it appears that repeated exposures to glass fibers potentiates the amount of cell-free protein present, this increase is not due to increased levels of plasminogen activator.

In accordance with the cell number (Figure 3) and cell-free protein (Figure 8) data, repeated exposures to either glass fibers or asbestos and single exposures to glass fibers increased the lung index significantly above that seen in saline injected animals (Figure 11). Whereas repeated injections of either glass fibers or asbestos induced comparable lung index values, a single injection of glass fiber elicited greater lung index values than did a single injection of asbestos (Figure 11). These increases in the lung index values may be due, in part, to the deposition of collagen in the lung, as evidenced by elevated lung tissue hydroxyproline values. Glass fiber and asbestos exposed animals demonstrated hydroxyproline levels that were significantly elevated above those in saline injected animals (Figure 12). Hydroxyproline levels were not enhanced by repeated exposures to either glass fibers or asbestos. However, injections of glass fibers resulted in hydroxyproline levels that were significantly greater than

those elicited by injections of asbestos. Thus, it seems that the increase in hydroxyproline observed after glass fiber or asbestos exposure may contribute to the increase in lung index, although, elevated protein levels and the influx of inflammatory cells may also be a factor.

The acute inflammatory responses to glass fibers have often been compared with the responses generated by asbestos exposure. Sykes et al (1983a) investigated the acute response to intratracheal instillations of either glass fibers or asbestos, and the subacute response to repeated (three) instillations of asbestos. Similar to the results found in this study (Figures 4a and 4c), the number of PMN recovered from the lung lavage fluid at seven days post-exposure was comparable in either asbestos or glass fiber exposed animals. In contrast, the number of macrophages in the lavage fluid in fiber exposed animals was not elevated above those of saline controls. However, Gross et al (1970) noted a large infiltration of both macrophages and neutrophils four days following a single intratracheal injection of either glass fibers or asbestos in rats. In agreement with data found in this experiment, Sykes et al (1983a) also noted elevations in cell-free protein at seven days post-exposure in either glass fiber or asbestos exposed rats.

In the evaluation of the chronic inflammatory and fibrotic responses to glass fibers in comparison to those of asbestos, Sykes et al (1983a) reported that at 50 and 100 days, only a small number of inflammatory cells were present in the lungs of rats exposed to either

glass fibers or asbestos. In contrast, in the present study, animals given single or repeated exposures to glass fibers or a single exposure to asbestos had elevated numbers of inflammatory cells at five weeks post-exposure (Figure 3). Similarly, Rola-Pleszczynski et al (1981) reported the presence of pulmonary neutrophilia in sheep at 18 months after 12 months of weekly intratracheal instillations of asbestos. Pickrell and associates (1983) also reported elevations in granulocyte numbers one month after the injection of either glass fibers or asbestos although it was also noted that there was no increase in macrophage numbers. However, elevated macrophage numbers were noted by Bernstein et al (1980) 17 weeks after the instillation of glass fibers. In addition, Wright and Kuschner (1977) noted large numbers of macrophages in the lungs of guinea pigs up to two years after 2-6 intratracheal injections of either glass fibers or asbestos. Elevated cell-free protein levels in the lavage fluid have also been observed in the evaluation of the chronic inflammatory response to particles. Sykes et al (1983a) reported that after three injections of asbestos, protein levels were elevated by 50 days and continued to increase with time. Pickrell et al (1983) also noted an elevation in the lavage fluid cell-free protein one month after exposure to either glass fibers or asbestos. In contrast to the present study, an increase in the pulmonary wet lung weight was induced by asbestos exposure, but not by glass fibers. Sykes et al (1983a) also noted an increase in lung weight (dry) 30 days after asbestos exposure that remained elevated for the duration of the study (100 days). Pickrell et al (1983) also

assessed collagen levels 11 months after exposure to glass fibers or asbestos. Both fibers increased the pulmonary tissue collagen. In contrast to the present experiment (Figure 12), however, asbestos produced a more severe response. Sykes et al (1983a) evaluated the fibrotic response to asbestos, but not glass fibers. Like Pickrell et al (1983), Sykes et al (1983a) reported elevated tissue hydroxyproline levels at 50 days post-exposure that continued to rise until the termination of the study at 100 days. Wright and Kuschner (1977) also demonstrated fibrosis in guinea pigs two years after repeated (2-6) injections of either glass fibers or asbestos. Again, it was reported that asbestos induced a qualitatively greater response.

Pulmonary Inflammatory and Fibrotic Response to Single or Repeated Exposures to Silica

Acute Inflammatory Response:

Similar to glass fibers, exposure to silica, either single or repeated, provoked an acute (five days) cellular inflammatory response that was significantly greater than that elicited by injections of saline alone (Figure 2). However, in contrast to glass fibers, repeated injections of silica enhanced the total number of inflammatory cells present in the lung lavage fluid. Whereas repeated exposures to glass fibers or silica induced the infiltration of comparable numbers of inflammatory cells, a single exposure of glass fibers provoked a significantly greater cellular inflammatory response than did a single injection of silica (Figure 2). There was a wide variation in the

numbers of the different inflammatory cell types elicited in the cellular inflammatory responses to glass fibers or silica. The marked eosinophil infiltration observed after glass fiber exposure (single or repeated [Figure 4a]) was absent in silica exposed animals (Figure 4c). The numbers of neutrophils and macrophages elicited by repeated exposures to silica were significantly greater than those in animals given repeated injections of glass fibers. On the other hand, a single exposure to glass fibers induced a greater neutrophil influx than did a single exposure to silica whereas the number of macrophages present was comparable. Therefore, it appears that the potentiation of cell number seen after repeated exposures to silica can be attributed primarily to the influx of neutrophils and macrophages whereas the overall increase in cells seen after glass fiber exposure is due to an increase in all three cell types. In addition, exposure to glass fibers evokes a strong eosinophil response that is not observed in silica exposed animals.

The cell-free protein level in the lavage fluid of silica exposed animals, like that of glass fiber exposed animals, was significantly greater than in saline injected animals (Figure 7). Unlike glass fibers, repeated exposures of silica did not enhance the cell-free protein level although repeated injections of either glass fibers or silica induced comparable cell-free protein levels. Plasminogen activator levels were significantly greater than saline levels only in animals given a single injection of silica (Figure 9). However, only in glass fiber exposed animals was the plasminogen

activator level significantly enhanced by repeated exposures. Consequently, it appears that the plasminogen activator levels induced by a single exposure to silica may contribute to the elevation in the cell-free protein levels in the lavage fluid whereas the elevated protein level seen after a single exposure to glass fibers is probably due to additional sources of protein (ie. cell lysis, edema). In contrast, the elevation in cell-free protein in animals repeatedly injected with glass fibers was paralleled by an increase in plasminogen activator (Figures 7 and 9) whereas repeated exposures to silica had no such enhancing effect.

Chronic Inflammatory and Fibrotic Response:

Similar to the acute cellular inflammatory response (Figure 2), the number of inflammatory cells present in the lavage fluid of either glass fiber or silica exposed animals was still elevated above saline controls at five weeks post-exposure (Figure 3). In addition, the potentiation of the increase in cell number by repeated exposures to silica observed at five days post-exposure was still evident at five weeks post-exposure. There was no enhancement by repeated exposures seen in the inflammatory cell numbers of glass fiber exposed animals.

By five weeks post-exposure, the marked eosinophil response seen in animals injected repeatedly with glass fibers at five days (Figure 4a) had subsided (Figure 5a). In addition, the potentiation of cells by repeated exposures shifted from eosinophils in the acute response, to neutrophils in the chronic response. The enhancement in

macrophage number by repeated injections of silica seen at five days post-exposure (Figure 4c) was still evident at five weeks post-exposure (Figure 5c). Although silica induced a significantly greater number of neutrophils, glass fibers provoked a greater eosinophil infiltration. It appears then, that at five weeks post-exposure, glass fiber exposed animals still have elevated numbers of eosinophils whereas silica exposed animals are lacking in this response. Additionally, in the chronic cellular inflammatory response, repeated exposures to glass fibers seem to have an enhancing effect on the neutrophil population. In contrast, repeated injections of silica enhance the macrophage population in the chronic cellular inflammatory response.

The cell-free protein levels in the lavage fluid of either glass fiber or silica exposed animals (single or repeated) were significantly elevated above those of saline injected animals (Figure 8). In addition, exposures (single or repeated) to either glass fibers or silica induced comparable protein levels. In fact, repeated exposures to either glass fibers or silica significantly enhanced the cell-free protein levels above those evoked by a single injection of equal dose (Figure 8). Although repeated exposures to glass fibers or silica did not potentiate the amount of plasminogen activator in the lavage fluid, injections of these particles, either single or repeated, elicited similar plasminogen activator levels (Figure 10). Thus, it may be concluded that glass fibers and silica have comparable effects on the cell-free protein and plasminogen activator content in the lavage fluid five weeks post-exposure.

In contrast to glass fiber exposures, silica exposures, single or repeated, did not increase the lung wet weight/body weight ratio above those of saline controls (Figure 11). In addition, exposures to glass fibers increased the lung indices significantly above those of animals exposed to silica. There was no potentiation of increases in the lung indices by repeated exposures of either particle. A single injection of silica also failed to induce tissue hydroxyproline levels that were significantly greater than saline controls (Figure 12), correlating well with the lack of an increase in the lung index values (Figure 11). Unlike silica, exposures to glass fibers, either single or repeated, resulted in hydroxyproline levels that were significantly elevated above those in saline controls. These hydroxyproline levels, like the lung index values, were significantly greater than those in animals exposed singly or repeatedly to silica. Again, similar to the lung index values, there was no enhancement of the tissue hydroxyproline levels by repeated exposures to either glass fibers or silica. Consequently, it appears that the increase in the lung wet weight/body weight ratio after exposure to glass fibers (single or repeated) may be attributed, in part, to the increased production of hydroxyproline in the lung. Additionally, the lack of an increase in the lung indices of silica exposed animals may be partially due to the low levels of hydroxyproline produced.

As with asbestos, the pulmonary inflammatory and fibrotic effects of silica exposure has been studied extensively. In agreement with the acute cellular inflammatory response data presented in this

study (Figure 2), Callis et al (1985), Adamson and Bowden (1984) and Lugano, Dauber and Daniele (1982) all reported elevated numbers of inflammatory cells in the lavage fluid of animals within a few days (3-7 days) of silica exposure. The types of cells elicited in the acute response are also consistent between these studies and the present experiment (Figure 4c). Lugano, Dauber and Daniele (1982) reported that although the number of neutrophils in the lavage fluid of mice given an intratracheal injection of silica was greatest at one day post-exposure, there was a transition to a macrophage infiltrate by four days post-exposure. Similarly, Adamson and Bowden (1984) noted a rapid influx of PMN into the lungs of mice given an intratracheal injection of silica that reached a peak at one day. This PMN influx also converted to a macrophage infiltrate by seven days. Callis et al (1985) noted much the same response in mice after the intratracheal instillation of silica particles. Exposure to the silica particles induced a prominent neutrophil infiltration three days after injection which shifted to mononuclear cells by day seven. The acute non-cellular response to silica has also been evaluated by other investigators, and many of their results concur with those found in this study. Callis et al (1985) noted elevated cell-free protein levels in the lavage fluid at seven days post-exposure. In addition, Adamson and Bowden (1984) reported elevated levels of the lysosomal proteins glucosaminidase and glucuronidase in the lavage fluid on day two post-exposure. The levels of these enzymes remained elevated throughout the study. In the present study, markedly elevated cell-free protein

levels were also demonstrated by five days post-exposure (Figure 7).

The chronic inflammatory and fibrotic pulmonary responses to silica exposures have also been well-documented by other investigators. Increases in the total number of inflammatory cells in the lavage fluid after silica exposure has been reported by Callis et al (1985) at both one and three months post-exposure; by Sykes et al (1983b) at 50 and 100 days post-exposure; and by Adamson and Bowden (1984) at one, two, three, four and five months post-injection. These increases in inflammatory cell number are similar to those observed in the present study (Figure 3). The cell types elicited in the chronic response to silica exposure in this study (Figure 5c) are also comparable to those found by other investigators. Sykes et al (1983b) reported large numbers of both PMN and macrophages in the lavage fluid of rats at 50 and 100 days post-exposure. Adamson and Bowden (1984) noted a similar response in mice at ten weeks post-exposure that continued to increase for the remaining ten weeks of the study. Elevations in cell-free protein are also observed in the chronic response to silica exposure. Sykes et al (1983b) demonstrated elevated protein in the lavage fluid at 50 and 100 days post-exposure. In contrast to the protein levels observed in the present study (Figure 8), Callis et al (1985) reported a decrease in cell-free protein levels at four weeks post-exposure. However, the protein levels increased in weeks 4 through 12 to levels that were greater than those seen at four weeks. Callis et al (1985) also noted elevated lung indices at four weeks post-exposure as did Sykes et al (1983b) at 50 and 100 days. These results are in contrast

with those found in the present experiment (Figure 11). Elevations in lung tissue hydroxyproline levels are usually attributed to an increase collagen deposition. This occurrence has been observed by several investigators (Callis et al, 1985; Sykes et al, 1983b; Adamson and Bowden, 1984 and Dauber et al 1980) but was not evident in this investigation (Figure 12). Indeed, Callis et al noted an average of 330 micrograms of hydroxyproline/lungs four weeks post-silica exposure whereas in this study, silica induced 260 micrograms of hydroxyproline/ lungs five weeks after exposure.

The majority of previous studies report that silica and asbestos are more cytotoxic to pulmonary tissue than glass fibers (Beck, Holt and Manojlovic, 1972; Haugen et al, 1982; Pickrell et al, 1983). It has been suggested by several investigators (Hunninghake et al, 1984; Bowden and Adamson, 1984; Sykes, et al, 1983b; Lugano, Dauber and Daniele, 1982) that the development of pulmonary inflammation and damage leading to fibrosis after particle exposure may be due to the infiltration of large numbers of inflammatory cells. In this investigation, glass fibers appear to have a pathogenicity that is similar to that of silica and greater than that of asbestos (Figures 2, 3, 7, 8, 9, 10, 11 and 12). This may be due to the presence of large numbers of inflammatory cells and their potential mediators in the lungs. A proposed scheme of glass fiber-inflammatory cell interaction is represented by Figure 13. The marked infiltration of eosinophils (Figures 4a and 5a) in glass fiber exposed animals may also contribute to the pulmonary damage seen in these animals. This eosinophilic

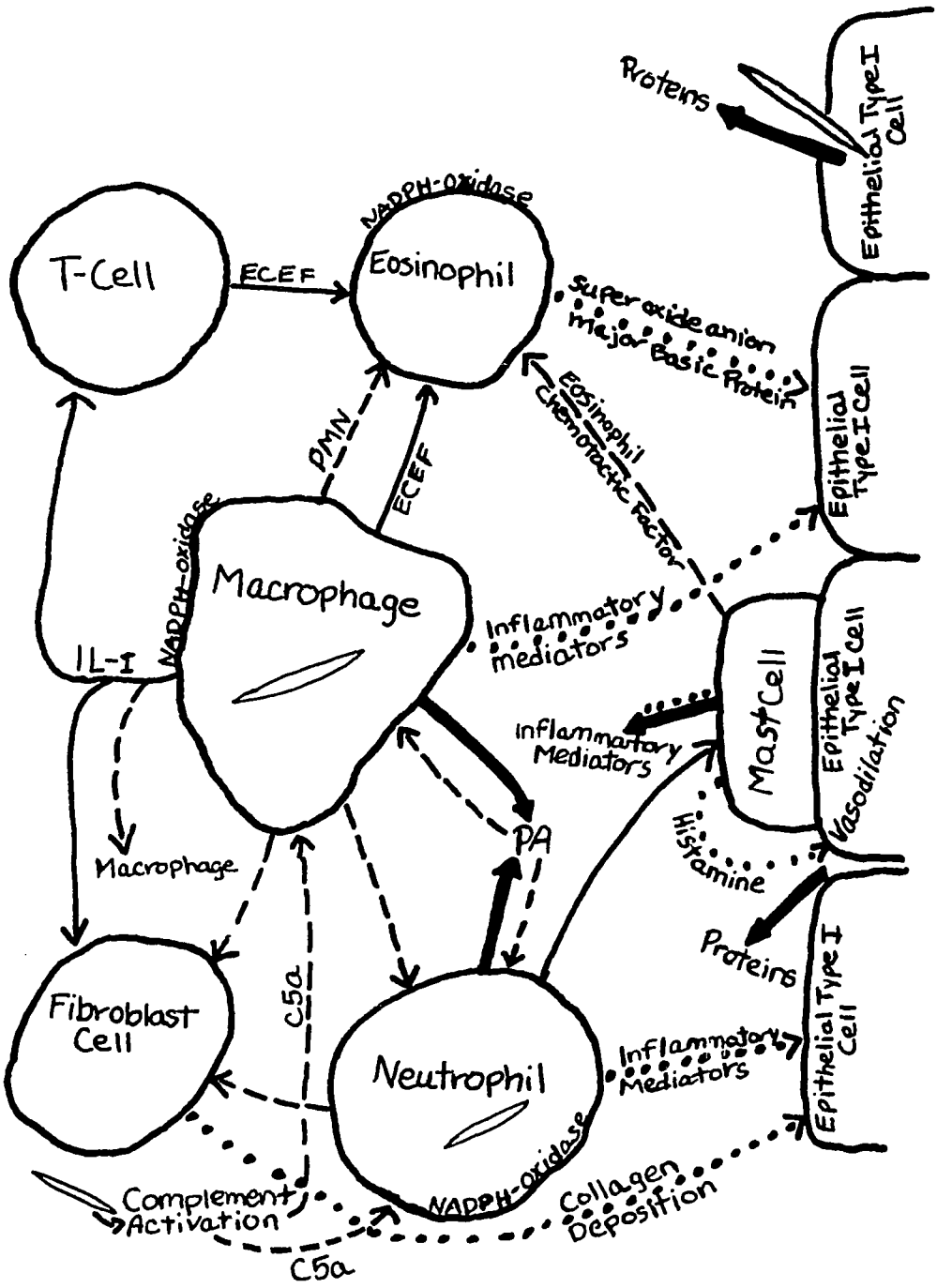

Figure 13. Proposed Scheme of Glass Fiber Induced Pulmonary Damage in the Alveolus. ECEF = eosinophil cytotoxicity enhancing factor; IL - I= interleukin I; PA= plasminogen activator; —— = activation; - - - = chemotactin; • • • = damaging; ➡ = release; ⬭ = glass fiber.

response appears to be related to the shape of the particle, as asbestos fibers also induced the influx of many of these cells (Figures 4b and 5b) whereas non-fibrous particles of silica did not (Figures 4c and 5c). Eosinophils have demonstrated cytotoxicity to lung tissue (Davis et al, 1984; Frigas, Loegering and Gleich, 1980; Frigas et al, 1981) in addition to inhibiting ciliary clearance (Hastie et al, 1987). This decrease in the clearance of fibers from the lungs increases the potential for the fibers to interact with and damage the pulmonary tissue. A decrease in clearance would also result in the elevated protein levels seen at both five days and five weeks post-exposure (Figures 7 and 8) as would the presence of inflammatory mediators such as plasminogen activator (Figures 9 and 10) and chemotactins released by inflammatory cells. Prolonged interaction between particles and inflammatory cells may also be responsible for the development of fibrosis. Hunninghake et al (1984) and Lugano et al (1984) reported that activated macrophages release chemotactic factors that attract fibroblasts to the area of inflammation and stimulate the production of collagen. In addition, Adamson and Bowden (1984) and Sykes et al (1983b) associated increased collagen synthesis (as indicated by elevated hydroxyproline levels) with chronically elevated levels of PMN and macrophages. In this experiment, elevated numbers of inflammatory cells in glass fiber exposed animals were associated with elevated tissue hydroxyproline levels (Figures 2, 3 and 12). Low levels of tissue hydroxyproline in silica exposed animals at five weeks post-exposure (Figure 12) has also been previously reported. Risendal et al

(1987) noted that at five weeks post-exposure, glass fiber exposed animals demonstrated significantly elevated hydroxyproline levels whereas silica exposed animals demonstrated only slightly elevated levels at this time. However, Risendal also reported that by fifteen weeks post-exposure, hydroxyproline levels in glass fiber exposed animals had decreased to saline control levels whereas in silica exposed animals, the hydroxyproline content of the lungs had increased significantly. Similar observations were made by Adamson and Bowden (1984). They noted that although levels of hydroxyproline in silica exposed animals were low at four weeks post-exposure, these levels increased steadily for the duration of the study. These results demonstrate the need for the evaluation of the pulmonary inflammatory and fibrotic responses at later time points as the pulmonary responses generated by glass fibers appears to be delayed in comparison to silica (Risendal et al, 1987). The relatively weak response induced by asbestos (Figures 2, 3, 5, 6, 7, 8, 9 and 10) is somewhat surprising given its reputation for provoking more intense responses than either glass fibers or silica. However, the size of the asbestos fibers may have been such that adequate numbers of pathogenic fibers were unable to reach the alveolar spaces and elicit a full inflammatory and fibrotic response.

Conclusions and Implications for Future Investigations

Exposures to glass fibers induced acute and chronic cellular inflammatory responses. These responses were not potentiated by repeated exposures. However, exposures to glass fibers provoked a marked infiltration of eosinophils that was enhanced by repeated exposures. The acute and chronic non-cellular inflammatory responses to glass fiber exposures were, on the other hand, potentiated by repeated exposures. Glass fibers also induced fibrosis at five weeks post-exposure that like the cellular inflammatory response, was not enhanced by repeated exposures. Additionally, the pulmonary inflammatory responses to glass fibers closely resembled those of silica and were more severe than those of asbestos whereas the fibrotic responses induced by glass fibers were greater than those induced by either asbestos or silica.

These results suggest that exposures to glass fibers induce changes in the pulmonary integrity that may compromise respiratory function and lead to additional problems that are deleterious to the health of the host. Thus, the need for further investigation on the toxicity of glass fibers, especially of repeated exposures, is essential.

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