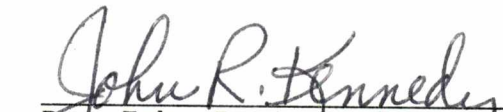


To the Graduate Council:

I am submitting herewith a thesis written by Sharon A. Trently entitled "The Biological Effects of Silicon Carbide Whiskers." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

  
Dr. Gerald L. Vaughan, Major Professor

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Associate Vice Chancellor  
and Dean of The Graduate School

**THE BIOLOGICAL EFFECTS OF SILICON CARBIDE WHISKERS**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Sharon A. Trently

December 1991

## **DEDICATION**

This thesis is dedicated to the Chimielewski family

and to

Brianna, within whom the future lies.

**DZIENKUJE!**

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## ABSTRACT

Occupational exposure to mineral fibers, such as asbestos, has been associated with increased risk of pulmonary fibrosis, malignant mesothelioma and lung carcinoma. It has been proposed that fibers with diameters  $< 0.25$  and lengths  $> 8$   $\mu\text{m}$ , possess the greatest capacity for cytotoxicity and cell transformation. (Stanton, 1977). The present study was prompted by the limited data on the health effects of silicon carbide whiskers (SiCW), a recently marketed man-made mineral fibers. This study was designed to compare two SiCW of differing dimensions as well as a non-fibrous form.

In order to examine the immediate biological effects of SiC, cultured explants of tracheal epithelium were exposed to a range of fiber concentrations ( $5 \text{ ug/cm}^2$  -  $100 \text{ ug/cm}^2$ ). There were no dramatic effects on ciliary function as measured by optical spectrum analysis which provided discrete ciliary frequencies. Particles were swept by ciliary activity into non-ciliated regions where foci of extensive cell damage and death were observed with whiskers penetrating epithelial layers into the underlying connective tissues. Similiar necrotic foci were observed in tracheas from rats exposed by

intratracheal instillation to silicon carbide whisker (SiCW) *in vivo*.

Investigation into the intermediate and long term pulmonary response began with intratracheal administration of SiCW-1 (0.8  $\mu$ m diameter), SiCW-2 (1.5  $\mu$ m diameter), SiC platelets (SiCP), boron carbide fiber (BC) and crocidolite asbestos. By bronchopulmonary lavage, phagocytic cells (macrophages, monocytes, lymphocytes) were harvested for examination of cell population changes. Lavage analysis revealed a significant ( $p < 0.05$ ) increase in macrophages still in evidence 28 days after SiCW exposure, an early sign of chronic inflammatory processes.

Lungs removed from fiber-exposed animals exhibited tissue alterations characterized by severe fibrosis and granulomatous tumors at 18 months post exposure. The highest frequencies of occurrence, 88% and 91%, were noted in SiCW-1 treatment groups. Additional pathological abnormalities included hyperplasia, metaplasia and interstitial pneumonia. Interperitoneal injections of SiCW similarly resulted in inflammatory fibrosis and benign peritoneal tumors.

SiCW-1 was shown to produced the greatest cellular damage. The single whisker form of SiC may pose a serious

health threat without precautionary handling measures.

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## **PART 1: GENERAL INTRODUCTION AND OVERVIEW**

## I. LITERATURE REVIEW

### HISTORY:

Occupational exposure to natural mineral fibers has been implicated in varying degrees of pulmonary disease ranging from chronic fibrosis to terminal neoplasia (Mossman and Gee, 1989). The commercially used mineral fiber, asbestos, has been the focus of great controversy and research in the past decade (Mossman et al., 1990). Prior to the passage and enforcement of the Occupational Safety and Health Act (1970), millions of workers were regularly exposed to high concentrations of these fibers which frequently resulted in an elevated incidence of pulmonary diseases.

Asbestos is a commercial term for a family of hydrated silicates which tends to crystallize in a fibrous form. Each of the two broad families, serpentines (e.g. chrysotile) and amphiboles (e.g. crocidolite), varies in chemical and dimensional geometry creating a variety of industrial uses. Industrial attractiveness relates to asbestos' unique chemical and physical properties, including heat resistance and tensile strength as well as low cost due to natural availability. Each family of asbestos differs in biological activity,

apparently stemming from differences in particle geometry. Studies have shown the long, thin amphibole fibers have a far greater potential for the induction of fibrotic and neoplastic disease than serpentines (Finkelstein et al., 1984 / Churg et al., 1988).

#### GEOMETRIC CONSIDERATIONS:

A material which has been tested as a dust may possess an entirely different biological effect when tested as a fibrous form. Following intrapleural administration of mineral fibers into animals, several investigators noted the development of malignant lesions, particularly mesothelioma of the pleura (Stanton and Wrench, 1972 / Wagner et al., 1973). Stanton and Wrench suspected particle geometry was crucial in determining a mineral fiber's carcinogenic potential. Stanton further examined 72 fibrous and nonfibrous respirable minerals, with the best correlation for cell transformation and cytotoxicity being with fibers of  $< 0.25 \text{ } \mu\text{m}$  in diameter and  $> 8.0 \text{ } \mu\text{m}$  in length. Deleterious effects in fibers with diameters up to  $1.5 \text{ } \mu\text{m}$  and lengths down to  $4 \text{ } \mu\text{m}$  were still observed (Stanton et al., 1977 / Stanton and Layard, 1978 / Stanton et al., 1981). This "Stanton Hypothesis" suggests that dimension and

durability are the major determinants of malignant cell transformation. Glass, for example, is thought to possess little or no cytotoxic or carcinogenic characteristics as a dust or coarse fiber. However, glass, as small fibers is an effective cell transforming agent *in vitro* with results analogous to those caused by asbestos (Chamberlian and Brown, 1977 / Lechner et al., 1983 / Oshimura et al., 1984 / Sincock, 1977). Further milling or chemical leaching of the later fibers abolishes this effect. Additional *in vivo* studies involving intratracheal, interperitoneal and intrapleural exposures have further substantiated the "Stanton Hypothesis" (Wagner et al., 1973 / Pott et al., 1974 / Pott, 1980).

#### DEPOSITION AND CLEARANCE:

In order for fibers to elicit pulmonary disease, they must be respirable and must be capable of prolonged tissue retention. Airborne particles are deposited in the respiratory tract by a combination of mechanisms - impaction, sedimentation, interception and diffusional displacement (Asgharian and Chu, 1988). The fate of these respirable fibers is dependent on the deposition site and localized clearance mechanisms. Particle clearance is accomplished by

two major events, initially by the mucociliary escalator and subsequently by phagocytic cells. Within hours of exposure, the protective epithelium of the upper respiratory airways transports some fibers upward by ciliary activity on a mucus layer. Upon reaching the laryngeal region, these fibers are either exhaled or swallowed. This mechanism is rapid and nonselective. Of the remaining fibers, a portion will be retained as cilia sweep them into non-ciliated areas where necrotic foci may develop. Some fibers may then penetrate the epithelium and progress into the underlying connective tissue (Vaughan et al., 1991b).

The majority of deep-penetrating fibers are selectively removed by cellular phagocytes, specifically alveolar macrophages. Fiber-burdened macrophages migrate to terminal bronchioles to be removed by ciliary activity. A macrophage's ability to competently remove fibers appears limited by fiber geometry. Short fibers,  $x < 10 \mu\text{m}$ , are effectively removed while longer ones are incompletely engulfed, consequences of which is cell membrane damage. These "injured" macrophages release chemical mediators that stimulate inflammation leading to fibrosis (Mossman and Craighead, 1981).

The remaining fibers rely on a slower and less effective

clearance pathway - disintegration. Disintegration of a fiber may be the result of a number of processes that alter external dimensions allowing for clearance by previously mentioned methods. These processes may include subdivision of fibers to shorter segments, dissolution of the chemical matrix or surface etching. Not all fibers are susceptible to these processes as durability differs with each fiber group. For example, chrysotile asbestos undergoes chemical leaching and fragmentation in whole animal studies (Roggli and Brody, 1984 / Roggli et al., 1987). In contrast, crocidolite asbestos and many man-made mineral fibers, eg. silicon carbide, show limited disintegration (Lippmann, 1990).

Respiratory clearance appears to favor the elimination of short, thick fibers. It seems likely that the remaining longer, non-removable fibers will interact with various pulmonary cells and dictate future pathogenic events.

#### FIBROGENESIS:

Despite decades of scientific investigation, little is understood concerning cellular events in fibrosis. Pulmonary fibrosis results from progressive collagen production and accumulation. The lung subsequently stiffens leading to

impairment of gas exchange. This link between collagen deposition and inflammatory fibrosis primarily stems from the interaction of macrophages and foreign particulates (Miller, 1978 / Harrington, et al., 1975 / Brody and Bonner, 1991).

Following fiber deposition, initial lesions involved a massive influx of alveolar macrophages as patterns of fiber localization correlate with sites of tissue injury. During chronic exposure, fibers are found in large numbers within AMs, the alveolar epithelium and pulmonary interstitium (Warheit et al., 1984 / Chang et al., 1988 / Skinner et al., 1988). While smaller fibers are sufficiently removed by AMs, incomplete phagocytosis of longer fibers triggers the "injured" macrophage to release a mixture of chemical mediators - chemotactic agents, growth factors and active oxygen species (AOS) (Pinkerton et al., 1984 / Warheit et al., 1984). Recruitment of additional macrophages and other inflammatory cell populations (neutrophils and lymphocytes) is accomplished through chemotactic agents such as leukotrienes, interleukins, cytokines, prostaglandins and, in some situations, complement (C5b) (Bissonnette et al., 1990 / Warheit et al., 1985).

Several growth factors (PDGF, FGF, AMDGF), released by

alveolar and interstitial macrophages, appear to signal fibroblast proliferation in both cell culture and animal studies. Platelet-derived growth factor (PDGF) has been identified as being mitogenic to mesenchymal cells of the lungs, fibroblast and smooth muscle cell culture (Kumar et al., 1988 / Laveck et al., 1988 / Van der Meeren, 1989 / Adamson and Bowden, 1990 / Bauman et al., 1990 / Brody and Bonner, 1991 / Brody, 1991). PDGF binds to specific membrane receptors on fibroblasts, initiating DNA synthesis and fibroblast replication, yielding exorbitant collagen production (Shimokado et al., 1985 / Osornio-Vargas et al., 1990). Pulmonary lavage of asbestos-treated animals found increased production of fibroblast growth factor (FGF) by AMs coinciding with the development of asbestos-induced fibrosis (Lemaire et al., 1986). Recently, an alveolar-macrophage derived growth factor (AMDGF) has been isolated from asbestosis patients however much of its function remains unclear (Rom et al., 1988).

Reactive metabolites of oxygen such as superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^\cdot$ ) are implicated in several harmful biological processes. These AOS are able to break DNA strands, to destroy proteins

and to initiate lipid peroxidation (Gulumian and Van Wyk, 1987 / Hansen and Mossman, 1987 / Holian and Roney, 1989 / Petruska et al., 1990 / Urano et al., 1991).

In clinical studies, the production of AOS is being used as early indicator of interstitial fibrosis (Clement et al., 1987) Cell cultures treated with antioxidants (eg. superoxide dismutase, catalase), scavengers (eg. dimethylthiourea) and iron chelators (eg. deferoxamine) have all shown dose-dependent reductions of asbestos-associated cell death but not a complete abolishment of toxicity (Janssen et al., 1990). These results suggest that cell damage and/or death is mediated by AOS (Mossman et al., 1986 / Mossman et al., 1990). In addition to cell damage, the presence of macrophage-liberated AOS increased fiber uptake by cells and increased fibroblast proliferation leading to enhanced collagen production (Hobson et al., 1990 / Klockars et al., 1990).

It seems likely that one or more of the above mentioned mediators can cause proliferation or functional impairment of neighboring epithelial cells and fibroblasts following fiber exposure. **Figure 1** presents a hypothetical scheme of the cellular events in fibrosis. Deposited fibers activate

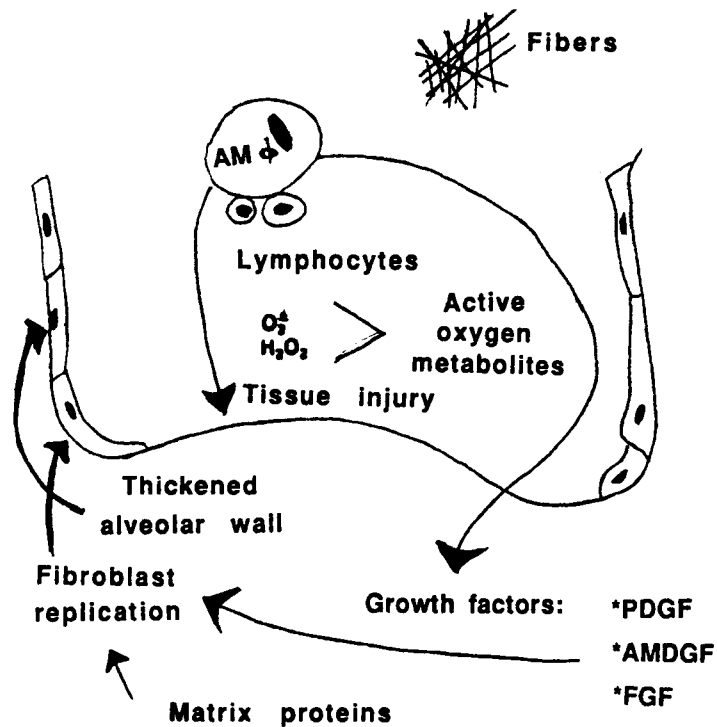


**Figure 1** Hypothesized Cellular Mechanism of Fiber-Generated Fibrosis in Alveoli

macrophages which in turn release chemical mediators for enhancing the inflammatory response. Oxidants trigger cell injury or death while growth factors stimulate increased DNA synthesis for enhanced connective tissue protein production resulting in fibrotic lung tissues (Rom et al., 1991).

## CARCINOGENESIS:

Asbestos-related cancers, lung and mesothelial, occur in an environment of chronic fibrotic lesions. Studies of asbestos workers appear to substantiate the correlation between fibrosis and carcinogenesis (Warnock et al., 1986 / Kipen et al., 1987). The inflammatory processes induced by asbestos fibers may damage DNA by either altering the regulation of protooncogenes or inactivating tumor suppressor genes (anti-oncogenes) (Gerwin, et al., 1987). The presence of active oxygen species, particularly the hydroxyl radical (OH<sup>•</sup>), has been shown to trigger DNA damage. Treatment with oxidant scavengers and iron chelators prevents DNA damage (Doelman, et al., 1990).

The incidence of lung cancer in humans apparently increases from synergistic events between asbestos and other carcinogens, particularly tobacco smoke (Selikoff et al., 1968 / Hammond et al., 1979 / Brown et al., 1990). Several mechanisms by which fibers act as cocarcinogens have been documented (Mossman et al., 1984 / Mossman et al., 1985). Fibers may absorb chemicals, such as oils or solvents, and transport them across the plasma membrane where they act *in situ* (Lackowicz and Hylden, 1978 / Mac Fadden et al., 1986 /

Churg and Wright, 1989). Recently, fiber-cell interactions resulting in altered intercellular communications have been examined as yet another possible mechanism (Marsh and Mossman, 1988 / Brown et al., 1989).

Asbestos has the capacity to bind directly to nucleic acids causing DNA strand breaks (Libbus et al., 1989). Mammalian cell cultures demonstrated dose dependent chromosomal aberration, anomalies and cellular transformation after asbestos exposure (Sincock and Seabright, 1975 / Lavappa et al., 1975 / Rom et al., 1983 / Paterour et al., 1985). By gaining access to the perinuclear region, asbestos fibers may cause transformation by binding to microtubules and other cytoskeletal proteins that are important during mitosis resulting in aneuploidy and polyploidy (Wang et al., 1987 / Palekar et al., 1988). Recent studies have documented SiCW as having close nuclear association accompanied by increased DNA and chromosome contents. (Jordon, 1991 / Vaughan et. al., 1991) This common association of fibers and nuclear structures appears to suggest the initiation of carcinogenesis.

## 2. RATIONALE

In recent years, a variety of man-made mineral fibers have entered the industrial market in the form of silicates and monocrystalline whiskers. Whiskers are very fine fibers with minimal dimensional variation. When added to composites, whiskers offer smoothness, precision and hardness to the product. The major drawback to these new forms is their geometric similarity to the known carcinogen, asbestos.

Silicon carbide is one such whisker presently being manufactured for use in ceramic composites. SiCW fall within the threshold range associated with cell toxicity and transformation. Aside from one previous study (ARCO, 1985), little data was available on the health impact of this material. This lack of data prompted a broad study into its health hazards.

This study examines the immediate cellular response to SiCW by investigation of ciliary function, *in vivo* and *in vitro*, and alveolar macrophage activity by bronchopulmonary lavage. Animal studies and pathological tissue examinations extended the investigation into the long term consequences of

SiCW exposure. Test materials included three sizes of SiCW plus a non-fibrous analog SiC platelets. Crocidolite asbestos served as the positive control for each experiment.

**PART 2: IMMEDIATE EFFECTS OF SILICON CARBIDE WHISKERS  
UPON CILIATED TRACHEAL EPITHELIUM**

## 1. INTRODUCTION

It well known that many materials encountered in the environment, both "natural" and manufactured, are harmful to human health. Exposure to some of these in the workplace has been judged unavoidable and acceptable within limits. In many cases, however, the guidelines that have been established are unrealistic for materials which are being produced in novel forms or applied in novel manners. A material which has been tested as a milled dust may have entirely different biological effects when tested in fiber or crystalline whisker form (Stanton et al., 1981) Glass, for instance, has little or no cytotoxic or cell transforming potential in powder or coarse fiber form, but small glass fibers may, *in vitro*, be as effective as some forms of asbestos in producing chromosomal aberrations and transforming cells (Sincock, 1977 / Oshimura et al., 1984 / Jaurand et al., 1986). Milling those same fibers to dust abolishes the effect.

Silicon carbide may show similar characteristics. It is classed as a nuisance dust and is known, in high concentrations, to cause irritation of the respiratory tract. With long-term exposure it may produce symptoms of pulmonary

fibrosis (Begin et al., 1981 / Brody et al., 1986 / Mossman and Gee, 1989 / Mossman, 1990 ). Currently, it is being produced as single crystal whiskers for use as reinforcing reagents in the manufacture of high-strength ceramics and other composites. The total emergent biological effects of these new forms are not certain. Considering the general hypothesis (Stanton et al., 1981) relating particle size and geometry to toxicity, we have undertaken a broad study of the effects of silicon carbide whiskers (SiCW) on biological systems. This report concerns the initial responses of mammalian tracheal cells and tissues to SiCW in both the intact animal and *in vitro*.

## 2. MATERIALS AND METHODS

### FIBER CHARACTERIZATION:

The three SiCW samples used in this study were SiCW-1, manufactured by Tateho, Japan, SiCW-2, provided by American Matrix, Inc., Knoxville, Tennessee, and SiCW-3, also from American Matrix, Inc.. Measurements of fiber size (length and width) were made from scanning electron micrographs which included images of latex bead size standards (Dow, Midland, MI; 2.68  $\mu$ m) for comparison. **Table I** shows size distribution for these materials. Test materials were weighed, autoclaved and then suspended in 1 mg/ml in the appropriate sterile solution. For *in vitro* use, fibers were suspended in Waymouth's MB 752/1 medium (Gibco Laboratories, Grand Island, NY ) whereas for intratracheal instillation, phosphate buffered saline (PBS) [grams per liter: NaCl, 8.0; KCl, 0.20;  $\text{KH}_2\text{PO}_4$ , 0.20;  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 2.16; pH=7.4] was used. Since SiCW is a dense, insoluble material which in aqueous suspension settles rapidly to the bottom of a vessel, it is necessary to calculate delivered dose rates *in vitro* in terms of mass per surface area.

TABLE I - DIMENSIONAL CHARACTERIZATION

| MATERIAL              | DIAMETER (uM) | LENGTH (uM) | ASPECT RATIO (L/D) |
|-----------------------|---------------|-------------|--------------------|
| SiCW-1                | 0.8 (0.3)     | 18.1 (14.3) | 23.3 (18.7)        |
| SiCW-2                | 1.5 (0.6)     | 19.0 (11.0) | 15.3 (11.2)        |
| SiCW-3                | 1.6 (0.5)     | 22.6 (2.7)  | 14.6 (5.2)         |
| -----                 |               |             |                    |
| () standard deviation |               |             |                    |

#### TRACHEAL EXPLANTS:

Dog tracheal tissues were obtained from animals euthanized for other reasons. Ciliated epithelium from fresh tracheas were prepared free of cartilage and maintained as previously described (Kennedy and Ranyard, 1983) in Waymouth's MB 752/1 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% Nu Serum (Collaborative Research, Bedford, MA) in a water saturated-atmosphere of 5% CO<sub>2</sub> at 37°C.

#### CILIARY FREQUENCY MEASUREMENTS:

Once outgrowth of ciliated cells from explanted tissue was established (5-7 days), cultures were used to test the sensitivity of ciliary activity to SiCW. Ciliary frequency was measured optically from videotaped experimental and control tissue using an Oriel photomultiplier detection system (Model 7070) and a Hewlett-Packard spectrum analyzer (Model 358A) to provide a spectral characterization through a Fast Fourier transform (FFT) conversion (Kennedy and Duckett, 1981). This method allows the major ciliary frequency for a cell to be measured with a precision of 0.1 hertz. Control levels of ciliary activity were determined just before addition of medium containing one of the test materials at

concentrations ranging from 5.0 to 100.0 ug/cm<sup>2</sup>. Successive determinations were made after 24, 48 and 72 hours of exposure. Data were collected from ten randomly selected cells within a tracheal sample from each of three different donor tissues at every time point.

#### ELECTRON MICROSCOPY:

At various points in the procedure, additional cultures were prepared for observation by scanning electron microscopy. Samples were fixed in gluteraldehyde (3.0%) followed by osmium tetroxide (2.0%), both in 0.025 M sodium cocadylate buffer (pH 7.4), each for one hour and dehydrated with acetone or alcohol. Finally they were dried with Peldri II (Ted Pella, Inc. Redding, CA) or by the critical point method, coated with gold palladium wire (60:40) and examined using an ETEC Autoscan scanning electron microscope.

Other samples prepared for electron microscopy had been taken from tracheal tissues removed from Sprague Dawley rats exposed via intratracheal instillation to SiCW at a dose of 5.0 mg per 100 ml of minute respiratory volume. For these animals, samples were taken 24, 48 or 72 hours after SiCW instillation. Histological preparations of tracheal samples

for light microscopy were made by Specialized Assays, Inc., 210 12 Ave. S., Nashville, TN 37204. Animals used in this study were maintained and used with the supervision and approval of an institutional animal care committee in an AALAC-approved facility.

### 3. RESULTS

#### CILIARY ACTIVITY:

Cilia from normal outgrowth cells derived from dog tracheal epithelium have a frequency of ciliary beat in the range of 7 - 13 Hz. The ciliated outgrowths used for this study fell within that range. SiCW whiskers, even at the highest dose studied, 100 ug/cm<sup>2</sup>, had no measurable effect upon ciliary motility (**Figure 1**), even after 72 hours of exposure. Considering the importance of the mucociliary elevator as a mechanism for clearance of foreign materials from the respiratory system, this absence of cilia toxicity is an encouraging finding from the standpoint of health effects.

#### ELECTRON MICROSCOPY:

Not so encouraging, however, are the effects of whiskers on tracheal cell structure both *in vitro* and *in vivo* as demonstrated with scanning electron microscopy. Dog tracheal tissue in culture is composed of two cell types when viewed from the surface with scanning electron microscopy. The majority of cells have long slender cilia projecting from their apical surfaces with non-ciliated cells scattered among the ciliated cells. (**Figure 2**) As the cells grow from the

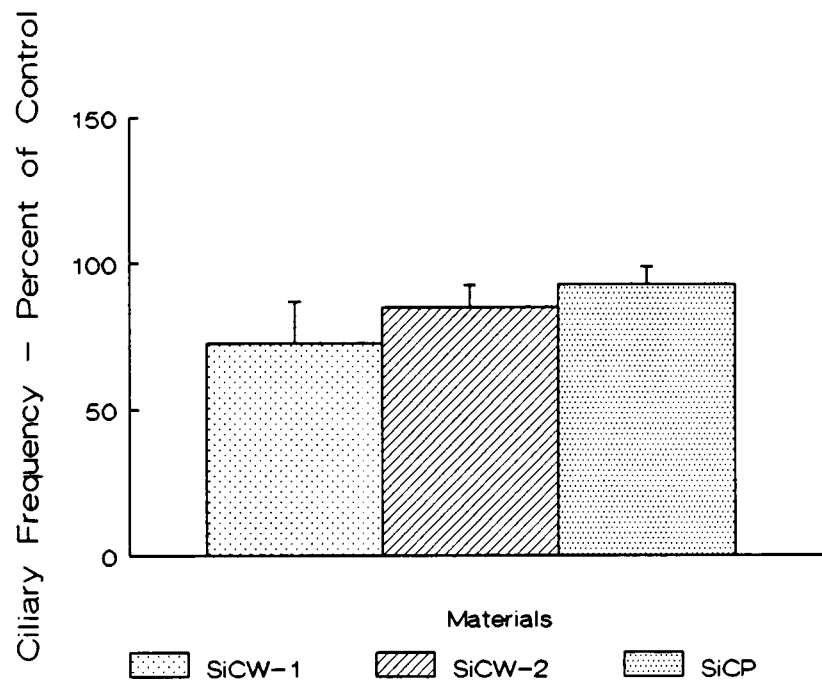


**Figure 1** Ciliary frequency as a function of duration of exposure to SiC at a concentration of 100 ug/cm<sup>2</sup>. Ciliary frequency is expressed as a percent of representative control values. Vertical bars show the standard error about the mean.

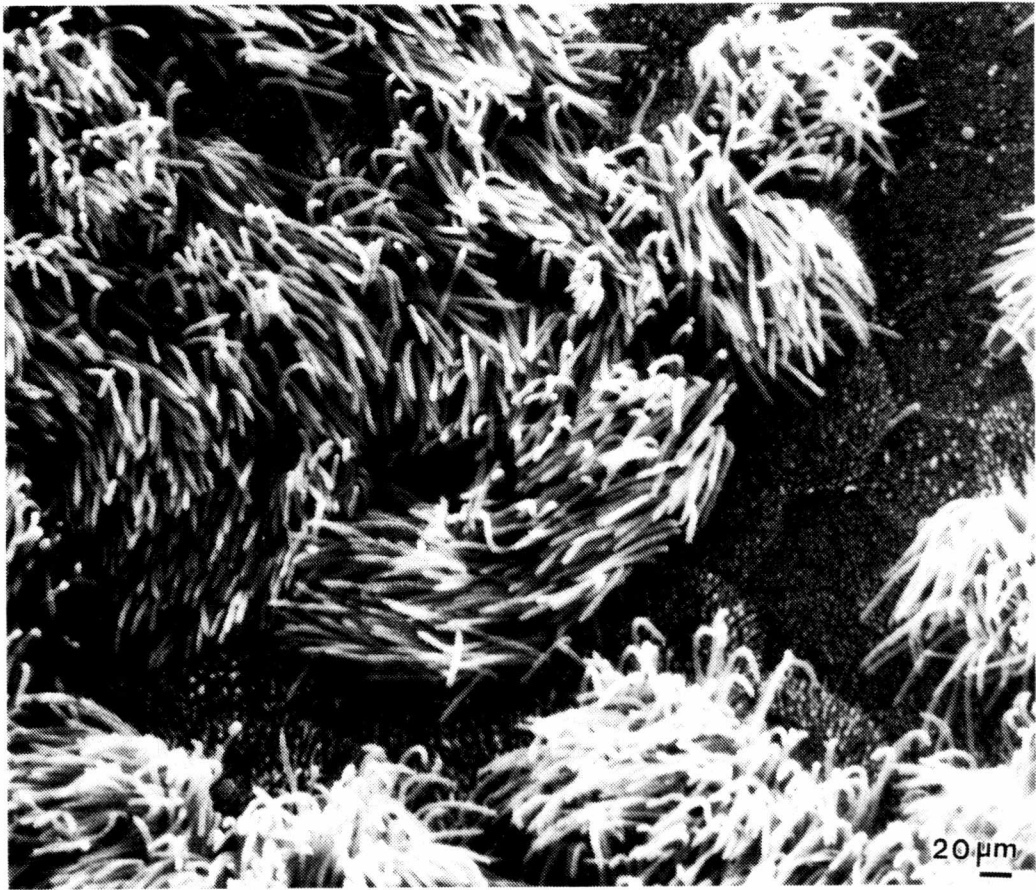


**Figure 2** A field of ciliated and non-ciliated regions in normal outgrowth of dog tracheal cells. Ciliary frequencies shown in Figure 1 were extracted from similar tufts of coherently beating cilia. Bar = 20  $\mu\text{m}$ .

tracheal explant, the ciliated cells become cuboidal to squamous in shape. Cilia are generally retained in a central location on the cell surface but as the cell surface increases, the ciliated regions in the outgrowth separate, increasing the non-ciliated surface area. Ciliary action in SiCW-treated cultures sweeps whickers to non-ciliated areas of the culture where they collect (**Figure 3**). In these areas whiskers penetrate and kill cells producing necrotic foci with fibers extending into the underlying connective tissue (**Figure 4**). Close examination of these foci indicates that both ciliated and non-ciliated cells are pierced by SiCW whiskers (**Figure 5A**). In some cases it appears that whiskers have been phagocytosed. Cells which seem to have phagocytosed whiskers often appear to have ruptures in their cell membranes (**Figure 5B**).

SiCW doses of 1.0 - 5.0 mg/100 ml of minute respiratory volume, when delivered to intact rats by intratracheal instillation in a total saline volume of 0.1 - 0.2 ml, elicited an immediate injurious effect. The whiskers produced lesions in the trachea that were very similar to those observed in the cultured ciliated outgrowths described above. Three days after intratracheal instillation of SiCW-1,



**Figure 3** Ciliated outgrowth from dog tracheal tissue exposed *in vitro* to  $10 \text{ ug/cm}^2$  of SiCW-1 for three days. Whiskers are swept by cilia into non-ciliated regions where necrotic foci develop. Bar = 50  $\mu\text{m}$ .

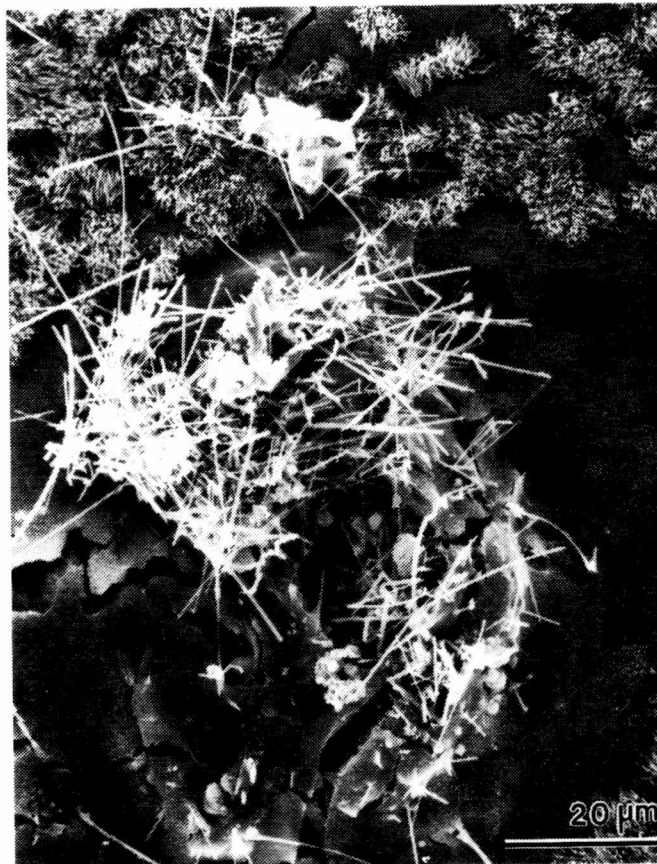


**Figure 4** Ciliated outgrowth from dog tracheal tissue exposed *in vitro* to 10 ug/cm<sup>2</sup> of SiCW-1 for three days. In a necrotic focus, whiskers penetrate the epithelium into the underlying connective tissue. Bar = 20 um

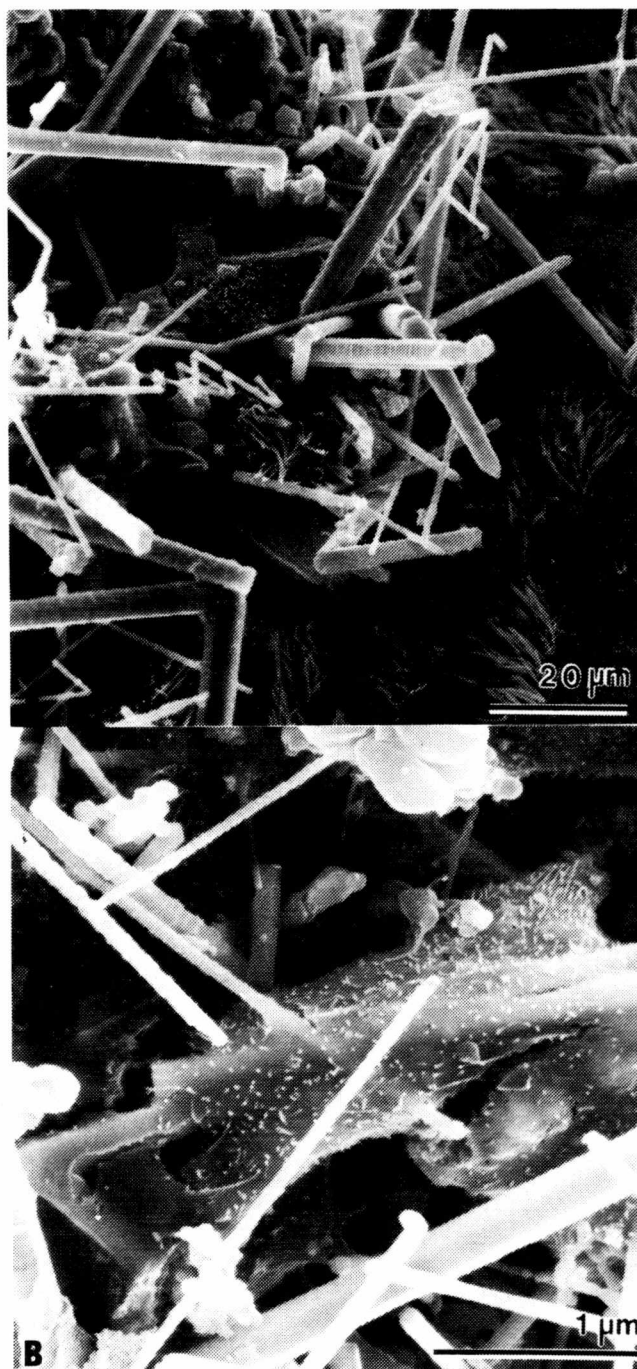


**Figure 5** Ciliated outgrowth from dog tracheal tissue exposed *in vitro* to 5 ug/cm<sup>2</sup> of SiCw-3 for three days. A. Whiskers deeply penetrate the epithelial layers. Bar = 20 um  
 B. Whiskers, free of membranes, transfix individual cells. A cell in the center of the field, which may have phagocytosed several fibers, has holes in its membrane. Bar = 1 um.

multiple necrotic foci were observed in relatively non-ciliated regions (**Figure 6**) with large numbers of whiskers piercing the epithelium. As in the cultured tracheal outgrowths, whiskers were observed to penetrate beneath the epithelium or to be engulfed by epithelial cells. One such cell containing a bent whisker can be seen in **Figure 7**.

Light microscopic examination of histological sections from tracheas of animal exposed to SiCW revealed one further important fact. Whiskers penetrated through the tracheal lining to the underlying lamina propria just superior to the supporting cartilagenous rings. Such SiCW whiskers remained in these tissues, apparently unchanged, for at least 1.5 years post exposure. Necroptic examinations of similar animals in a study running parallel to this one have variously presented pulmonary edema, hemorrhage, interstitial pneumonia, collapsed bronchioles and alveolar atelectasis.

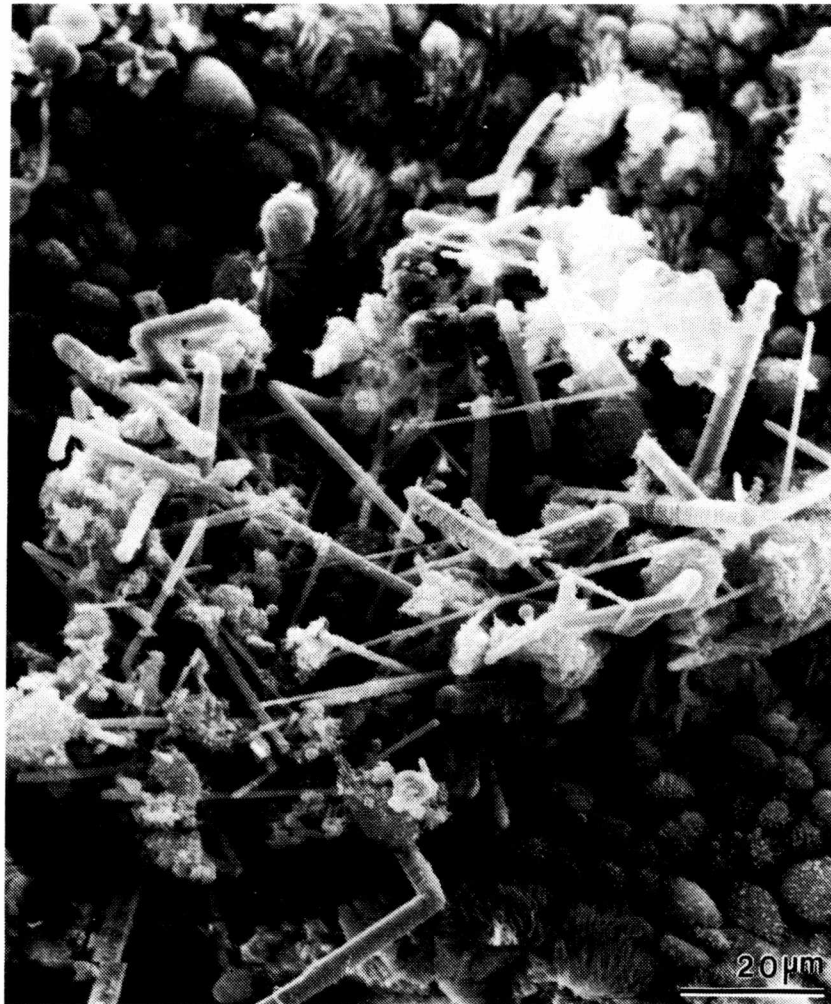


**Figure 6** Rat tracheal tissue two days after instillation of SiCW-3. Necrotic foci localized in non-ciliated regions of the tissue are similar to those observed *in vitro*. Bar = 20 um.



**Figure 7** Rat tracheal tissue exposed *in vivo* to SiCW-3. At the arrow, a cell is shown which has engulfed a bent whisker. Bar = 20  $\mu\text{m}$ .

#### 4. DISCUSSION

Zeolites, asbestos and other naturally occurring materials with a whisker or fiberform shape are known to be the etiologic agents of a variety of serious disease states (Brody et al., 1986 / Mossman and Begin, 1989 / Mossman and Gee, 1989). These range from simple eye, skin and pulmonary irritation to life-threatening pulmonary fibrosis, pneumoconiosis and mesothelioma. The primary relationship demonstrated between durability, geometry and size of whisker/fiberform materials and their toxicity relegates chemical identity to a secondary role (Stanton et al., 1981). Silicon carbide, the hardest material known, with the exception of diamond, is being produced as very strong, dense, single crystalline whiskers for use as reinforcing elements in the production of advanced ceramic, metal and polymer composites. Most SiCW being produced and researched for this use, including the samples described in this study, fall within the same range of size and aspect ratio (Table I) as the asbestos particles most often found to be associated with pulmonary disease in humans (Timbrell, 1982). All of this has stimulated an intense interest in the impacts of SiCW and

similar materials on biological systems. We have, as a consequence, undertaken a broad-based study of those impacts using *in vitro* systems, as well as both short and long-term animal studies. This report describes results from the first phase of that study.

The major locus of damage to the intact animal by airborne particulates is the pulmonary system which, necessarily, incorporates several mechanisms to protect itself from such exposure. Ciliated epithelium, in conjunction with mucus producing cells, provides one clearance mechanism. While there may have been a slight inhibition of ciliary function, *in vitro*, within the first 24 hours of exposure to SiCW, by 48 hours motility was back to normal. By that time, of course, most of the whiskers were swept and settled in areas sparsely populated with ciliated cells. Within those areas whiskers disrupted membranes, piercing and killing cells. It is reasonable that, no other force being apparent, penetration of cells results in part from attempted phagocytosis of whiskers which, too long to be entirely engulfed, compromise membrane integrity. Even more interesting is the fact that once cells on the surface of the tracheal epithelium are pierced, the SiCW particles continue to

penetrate tissues through to the region of the supporting cartilage. The mechanism for this penetration is not apparent.

Pulmonary fibrosis developing after extensive exposure to asbestos and similar materials is thought to be an indirect consequence of cell death, particularly that of macrophages (Aalto and Heppleston, 1984 / Brody, 1985 / Lemaire et al., 1986 / Cantin et al., 1988). Enzymes, peroxides and growth factors released during cell lysis damage other cells and stimulate the synthesis of collagen by fibroblasts leading to scar tissue formation. Mechanically and chemically durable materials which are not easily cleared, remaining in place to stimulate additional macrophage response and further damage may be particularly effective in pulmonary fibrogenesis. SiCW, in light of the results presented here, has the potential to produce extensive immediate damage to the pulmonary system, and, further, remains in the tissue for extended periods of time. We suggest care and adequate safeguards should be provided to those handling this material to protect them from pulmonary exposure.

PART 3: INTERMEDIATE AND LONG TERM *IN VIVO* PULMONARY  
RESPONSE TO SILICON CARBIDE WHISKERS

## 1. INTRODUCTION

Exposure to natural and man-made mineral fibers in occupational and environmental settings has been associated with health conditions ranging from eye and skin irritation to life threatening fibrosis, lung cancer, pneumoconiosis and pleural neoplasia (Suzuki and Kohyama, 1984 / Mossman and Gee, 1989). Of these conditions, fibrosis is perhaps the most debilitating as the resultant collagen buildup leads to lung stiffness and impaired gas exchange. When fibrous materials, including asbestos, glass, and silicon carbide (SiC), of varying dimensions were examined, fiber length and diameter appeared to be the most critical factors in determining cytotoxicity. It has been proposed that both natural and man-made mineral fibers with aspect ratios  $> 5:1$ , lengths  $> 8.0 \mu\text{m}$  and diameters  $< 0.25 \mu\text{m}$ , possess the highest cell transforming potential *in vitro* (Brown et al, 1978 / Stanton and Layard, 1979 / Stanton et al., 1981). Durability, residence time and surface chemistry are secondary considerations in toxicity determination. Ground glass, for instance, exhibits little to no cytotoxicity but glass fibers are effective cell transforming agents (Sincock, 1977 / Stanton et al., 1977 /

Lechner et al., 1983). Reducing fiber diameter from 1.0  $\mu\text{m}$  to 0.1  $\mu\text{m}$  yielded an order of magnitude increase in frequency of chromosomal aberrations and anomalies (Oshimura et al., 1984).

In the lungs of intact animals, the situation is more complex, in that several factors determine particulate respirability. For a fiber to exert an effect in the lungs, it must first be capable of deep penetration into the alveolar regions. The most important property appears to be fiber diameter (Timbrell, 1983). As fibers align with airflow, smaller fibers have a greater chance of deep penetration. The limiting factor for determining respirability has been shown to be fiber dimensions. Fibers with diameters greater than 3  $\mu\text{m}$  and lengths less than 200  $\mu\text{m}$  are more likely to be internalized (Timbrell, 1965 / Timbrell, 1982 / Ashgarian and Chu, 1988). Deposition of these particles occurs through a variety of mechanisms: impaction, sedimentation, interception and diffusion (Lippmann, 1990). While diffusion occurs throughout the respiratory tract, interception and impaction predominate in the upper regions due to the higher flow velocities. Sedimentation is the primary mechanism in the lower respiratory tract.

Clearance of fibers is accomplished by a combination of

pulmonary protective mechanisms. Shorter fibers are cleared by the mucociliary escalator and phagocytic macrophages while the majority of long fibers, 10.0  $\mu\text{m}$ , are less likely to be removed. Long term interactions between these remaining inhaled particulates and pulmonary cell populations, particularly alveolar macrophages and fibroblasts, may dictate future pathogenic events. The primary interaction of macrophages against foreign particulates has been suggested as the link between collagen deposition and subsequent inflammatory fibrosis (Begin et al., 1983). By releasing active oxygen species and growth factors, macrophages stimulate fibroblast proliferation resulting in excessive collagen (Lemaire et al, 1986 / Bauman et al., 1990 / Bissonnette et al., 1990 / Hobson, 1990).

In recent years, man-made mineral fibers have entered the commercial market. Presently, silicon carbide ( $\text{SiC}$ ) is being constructed as fibers (whiskers) and crystalline plates used in the manufacturing of high strength ceramics and other composites. These new forms have not been sufficiently assessed for cellular toxicity. In addition, present guidelines for fiber-form materials may be unrealistic and inadequate. Considering the relationship between toxicity and

particle geometry, as exhibited by asbestos, we are investigating the *in vitro* and *in vivo* consequences of SiC exposure.

Previous *in vitro* work, Vaughan et al., 1991, examined a range of sizes of silicon carbide whiskers (SiCW) and platelets (SiP) in ciliated tracheal epithelium explants. After 24 hours of exposure, no significant effect on ciliary function was noted. However, SiCW were swept by ciliary activity into non-ciliated regions resulting in development of necrotic foci. Scanning electron microscopy further revealed extensive cell damage to underlying tissues. Similiar regions were observed following *in vivo* SiCW treatment. This paper presents data from continued investigation into the underlying *in vivo* cellular events leading to pulmonary fibrosis and necrosis. Information collected will aid in the establishment of preliminary guidelines for human exposure to SiCW.

## 2. MATERIALS AND METHODS

### FIBER CHARACTERIZATION:

Three SiCW samples were used in this study. SiCW-1 was manufactured by Tateho Inc., Japan, and SiCW-2, SiCW-3, SiC platelets (SiCP) and boron carbide (BC) were provided by American Matrix, Inc., Knoxville, Tennessee. Crocidolite asbestos (UICC) was supplied by Dr. L.W. Ortiz of Los Alamos National Laboratory, Los Alamos, New Mexico. Measurements of fiber size (length and width) were made from scanning electron micrographs with latex bead size standards (Dow, Midland, Michigan; 2.68  $\mu\text{m}$ ) used for comparison. Size distributions for these materials are reported in Table 1. Test materials were weighed, autoclaved and then suspended to 1mg/ml in the appropriate sterile solution. For *in vitro* use, fibers were suspended in Waymouth's MB 752/1 medium (Gibco Laboratories, Grand Island, New York ) whereas for intratracheal instillation, phosphate buffered saline (PBS) [grams per liter: NaCl, 8.0; KCl, 0.20;  $\text{KH}_2\text{PO}_4$ , 0.20;  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 2.16; pH=7.4] was used. Since the test materials are a dense, insoluble material which settles rapidly in aqueous suspension, it is necessary to calculate delivered dose rates

TABLE I - DIMENSIONAL CHARACTERIZATION

| MATERIAL    | DIAMETER ( $\mu\text{M}$ ) | LENGTH ( $\mu\text{M}$ ) | ASPECT RATIO (L/D) |
|-------------|----------------------------|--------------------------|--------------------|
| SiCW-1      | 0.8 (0.3)                  | 18.1 (14.3)              | 23.3 (18.7)        |
| SiCW-2      | 1.5 (0.6)                  | 19.0 (11.0)              | 15.3 (11.2)        |
| SiCW-3      | 1.6 (0.5)                  | 22.6 (2.7)               | 14.6 (5.2)         |
| SiCP        | 24.5(14.0)                 | 1.2 (0.4)*               | ---                |
| BC          | 6.1 (6.7)                  | 80.9 (48.1)              | 16.1 (11.9)        |
| Crocidolite | 0.4 (0.2)                  | 4.2 (3.6)                | 14.3 (3.8)         |

( ) standard deviation  
 (\*) thickness

in terms of mass per surface area. Animal respiratory rates were determined to be an average of 100 respirations/minute based on a sampling of 25 animals. From a rodent ventilation graph, minute respiratory volumes for each animal were calculated based on the weight and the average respirations/minute for each animal. (Harvard Equipment Inc., Boston, MA). The delivered low (1 mg/100 ml MRV) and high (5mg/100 ml MRV) doses are standards determined by the National Cancer Intstitute for respiratory carcinogens.

#### INTRATRACHEAL FIBER INSTILLATION:

Inhalation exposures were carried out using 9 week old female Fischer F344/NTacFBR rats (Taconic, Germantown, NY) for long-term exposure and mixed-sex Spague Dawley rats (Charles River, Wilmington, MA) for short-term exposure experments. Animals were anesthetized with 0.2 cc intramuscular injection of 60% ketamine - 100 mg/ml (Bristol Labs, Syracuse, NY) and 40% acepromazine - 10 mg/ml (Ayerst Labs, New York, NY). Deeply anesthetized animals did not survive the procedure, therefore animals were permitted to begin recovery from anesthesia prior to intratracheal instillation of test materials. Animals were restrained supine on an inclined

surface with intense external illumination provided to the neck region. A 16 guage, 5 1/4 inch angiocatheter (Deseret Medical Inc., Sandy, Utah) was inserted into the trachea. To insure entrance into the respiratory tract, airflow through the catheter was confirmed. Using an automatic pipettor, appropriate doses of fibers were removed from a stirred suspension and introduced as the animal inhaled. Two 5cc air injections followed. Animals recovered in a warmed environment before being returned to the colony.

#### BRONCHOPULMONARY LAVAGE:

Following exposures of 1, 7 and 28 days, animals were anesthetized with 0.5cc of 33% ketamine - 100 mg/ml, 40% acepromazine - 10 mg/ml and 27% xylazine - 20mg/ml (Miles Labs, Shawnee, Kansas) administered intramuscularly. The tracheae were exposed and cannulated with a 16 guage 2-inch angiocatheter (Deseret Medical Inc., Sandy, Utah), held in place by ligatures and clamped cranially with a hemostat. With continuous massage of the thorax, the accessible airways were lavaged 4 times with 5 ml aliquots of cold, sterile PBS. Washes were collected and pooled for each animal. Following lavage, animals were euthanized. Variations in recovered

lavage volumes were minimal.

Recovered bronchoalveolar cells were obtained by centrifugation at 250 X G at 4°C for 10 minutes. Following resuspension in PBS and immediate enumeration in a hemacytometer chamber, the cells were processed using Histopaque-1077 (Sigma, St. Louis, MO), a mononuclear cell isolation medium. Differential counts of lavaged cells were made from slides stained with Diff-Quik (American Scientific Products, Mc Graw Park, Il). A total of 200 cells in each sample including macrophages, lymphocytes and monocytes were counted and a total titer estimated from hemacytometer values. Additionally, macrophage diameters were determined from photomicrographs of the stained slides.

#### LONG TERM INTRATRACHEAL AND INTRAPERITONEAL ANIMAL STUDY:

Test materials were administered intratracheally to a minimum of 50 Fischer F344 rats for each material. Each treatment group involved 25 animals with a high dose, 5 mg/100 ml MRV and 25 animals at a low dose, 1mg/100ml MRV. Control animals were exposed to PBS under similar conditions.

For intraperitoneal administration, animals were lightly anesthetized with an intramuscular injection of 0.1 cc 60%

ketamine - 100 mg/ml and 40% acepromazine - 10 mg/ml. With a 20 gauge syringe, each animal received a 20 mg/ml interperitoneal injection of PBS or SiCW-2.

Each animal was maintained in an AALAC-approved facility for eighteen months. Body weight and general health were monitored biweekly during the first two months and monthly thereafter. At the end of the each test period, they were euthansized in a carbon dioxide chamber. The liver, spleen, heart, lungs, kidneys and incidental tumors were preserved in 10% neutral buffered formalin (NBF) [per 900 ml H<sub>2</sub>O: NaH<sub>2</sub>PO<sub>4</sub> 4.0 g; NaHPO<sub>4</sub> 6.5g; 37% formalin 100 ml]. Lungs and heart were excised *en bloc* with the lungs expanded with 10% NBF and ligated at the trachea. Histopathological evaluation was preformed under contract by Specialized Assays, 210 12th Ave., Nashville, TN, 37204.

#### STATISTICAL ANALYSIS:

Chi Squared analysis and Students two tailed/single group t-tests were employed for statistical comparison of treatment groups to their respective PBS controls. Because of the small sample size,  $N < 25$ , histological data were tested at  $P < 0.10$  to reduce the possibility of committing a Type II statistical

error.

### 3. RESULTS

#### INTERMEDIATE RESPONSE:

Pulmonary phagocytes were obtained in order to examine the cellular response to inhaled particulates. Following intratracheal instillation of SiCW-1, SiCW-2 or crocidolite at the appropriate intervals, phagocytes were harvested by bronchopulmonary lavage. Pulmonary lavage fluids from control animals yielded an average of  $0.4 \times 10^6$  (+/- 0.1) cells with a high alveolar macrophage concentration. Red blood cell contamination was minimal with the exception of (SiCW-1)-treated animals wherein high red cell counts resulted from acute pulmonary hemorrhage. Results of differential analysis of macrophage cell populations, shown in **Figure 1**, revealed no significant differences between crocidolite and PBS controls at the end of the first week. However, at 28 days post exposure a marked elevation in macrophages were observed in both SiCW-1 and SiCW-2 groups. At 7 days, macrophage populations in SiCW-1 groups had increased to  $0.86 \times 10^6$  cells, 600 % of the control values (**Figure 1A**). At 28 days, AMs remained significantly high ( $0.86 \times 10^6$ ) in both SiCW-1 and SiCW-2 groups, reflecting prolonged inflammatory response

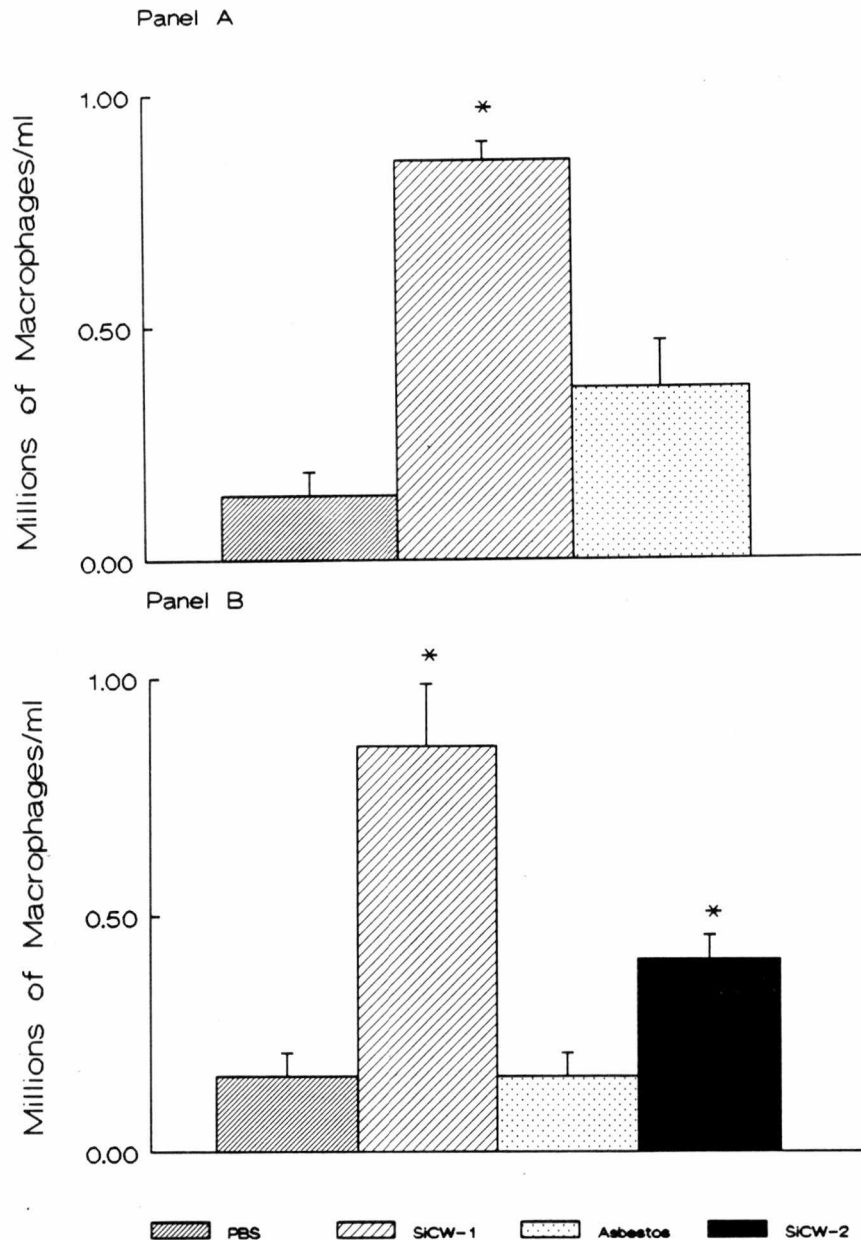


**Figure 1** Analysis of macrophage populations following intratracheal instillation of PBS, SiCW-1 , crocidolite and SiCW-2 for 7 (Panel A) and 28 (Panel B) days post exposure. Values represent means +/- standard error of at least five animals per group. \*  $P < 0.05$  with respect to PBS controls at identical time intervals.

in the respiratory tract (Figure 1B).

Macrophages demonstrated morphological changes characterized primarily by cell membrane damage (Figure 2). Membranes often appeared ruffled, fragmented and even torn especially following SiCW-1 treatment. Previous studies have documented macrophage size changes following asbestos exposure. We found no statistical variation in macrophage diameters.

Although lavage cell populations were mainly composed of macrophages, cellular analysis also revealed changes in both monocyte and lymphocyte cell populations following SiCW-1 instillation (Figures 3 and 4). After 24 hours, an initial decrease (15% of the control values) in the monocyte titer was followed by a five-fold increase at 7 days (Figure 3A). Since monocytes are precursors to macrophages, such an increase in the titer is expected to occur with rising macrophage populations within 7 days. Additionally, during the first 7 days lymphocytes increased reaching 4500% of the control value (Figure 4). Several possible explanations for this dramatic response include non-specific interactions of the immune system to fiber-activated macrophages or bacterial invasion

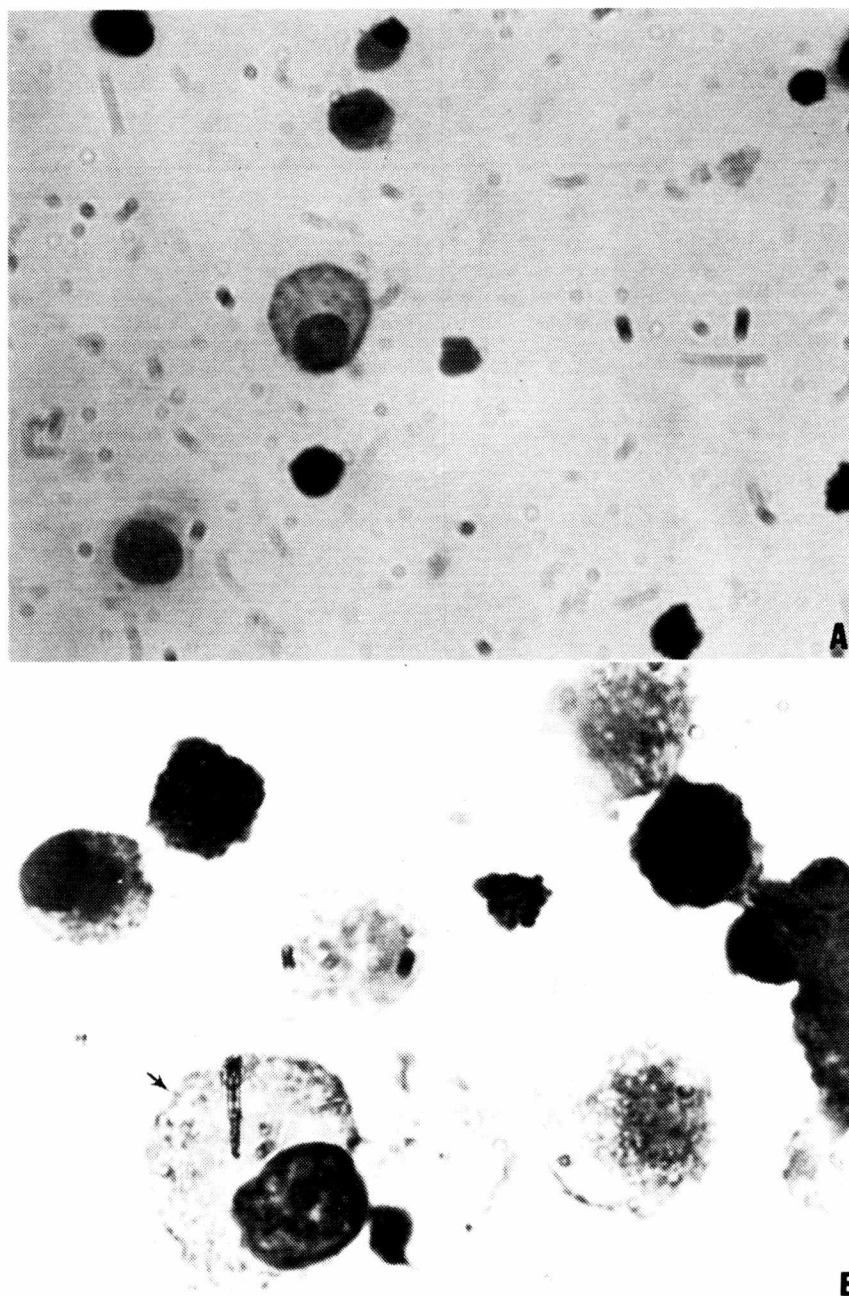


**Figure 2** Alveolar macrophages obtained from pulmonary lavage 28 days following PBS (Panel A) or SiCW-1 (Panel B) exposure. A fiber laden-macrophage demonstrates characteristic membrane fragmentation following fiber exposure. (Arrow) Magnification 990X.

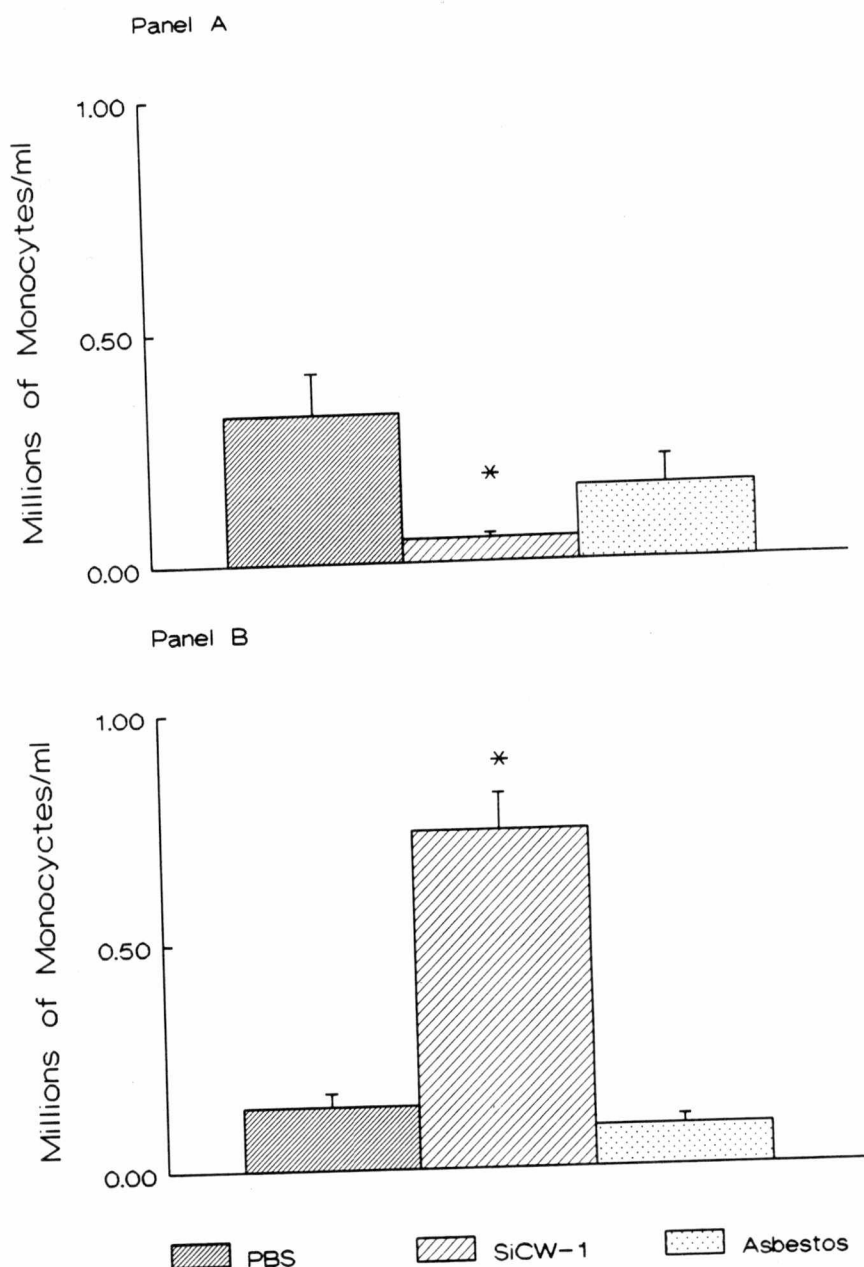


**Figure 3** Analysis of monocyte populations following intratracheal instillation of PBS, SiCW-1 and crocidolite for 24 hours (Panel A) and 7 days (Panel B) post exposure. Values represent means  $\pm$  standard error of at least five animals per group. \*  $P < 0.05$  with respect to PBS controls at identical time intervals.

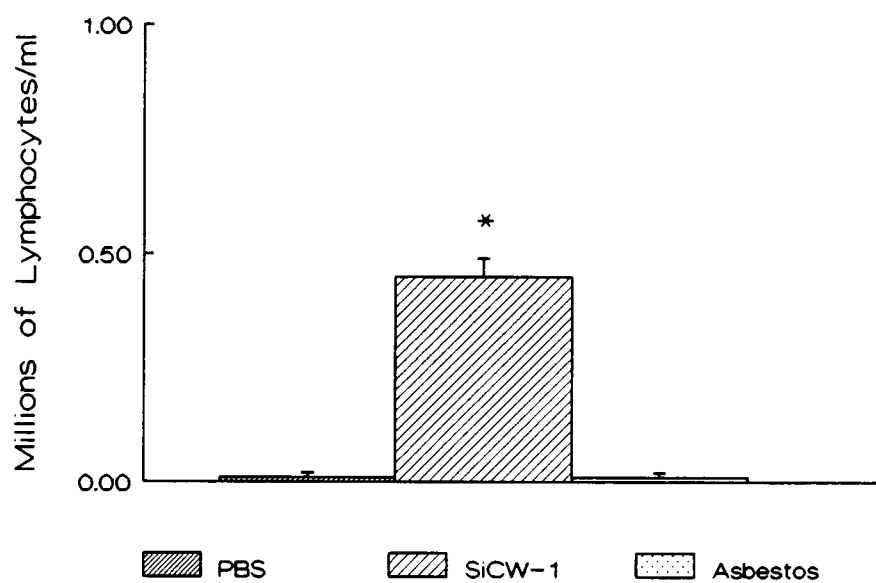


**Figure 4** Analysis of lymphocyte populations following intratracheal instillation of PBS, SiCW-1 and crocidolite for 7 days exposure. Values represent means  $\pm$  standard error of at least five animals per group. \*  $P < 0.05$  with respect to PBS controls at identical time intervals.

following such severe tissue damage.

LONG TERM RESPONSE:

Within the first 24 hours following fiber instillation, the SiCW-1 group exhibited a particularly high initial mortality with up to 50% of the animals dying within 24 hours of exposure. They exhibited severe pulmonary hemorrhage accompanied by pulmonary congestion, edema and interstitial pneumonia. An additional short term consequence of SiCW-1 exposure was body weight loss as great as 15% of the animal's initial weight within 14 days of treatment. After this initial period of weight loss, however, overall growth patterns were similar among all fiber treatment and control groups throughout the remainder of the 18 month study. Many of the animals, particularly those treated with SiCW-1, experienced chronic respiratory distress characterized by wheezing and diminished vocalization. Initial mortality and respiratory complications were rare in both boron carbide and silicon carbide platelet groups, an early indication of the relatively innocuous nature of these materials.

Histopathological examination after 18 months of exposure showed the greatest pulmonary damage in SiCW-1-exposed groups. (Table 2) Extensive pulmonary granuloma development

**Table 2** Histopathological findings in Fisher-344 rats 18 months after exposure. Treatment groups received LD=1.0 mg, HD=5.0 mg per 100ml MRV. Controls received volume equivalents of PBS. Frequencies tested by chi square at  $p < 0.10$ .

| CONDITION                  | TREATMENT   |      |        |      |        |      |
|----------------------------|-------------|------|--------|------|--------|------|
|                            | CROCIDOLITE |      | SiCW-1 |      | SiCW-2 |      |
|                            | HD          | LD   | HD     | LD   | HD     | LD   |
| N=(X)                      | (25)        | (25) | (23)   | (25) | (23)   | (23) |
| <b><u>LUNG:</u></b>        |             |      |        |      |        |      |
| Granulomas                 | 0.28        | --   | 0.91   | 0.88 | 0.30   | 0.30 |
| Hyperplasia                | --          | --   | --     | 0.31 | --     | --   |
| Fibrosis                   | 0.36        | --   | 0.31*  | --   | --     | --   |
| Giant Cells                | --          | --   | --     | 0.64 | --     | --   |
| Macrophages(f)             | 0.20        | --   | 0.57   | 0.40 | 0.30   | 0.21 |
| Macrophages(p)             | 0.64        | --   | --     | --   | --     | --   |
| Neutrophils                | --          | --   | 0.26   | 0.28 | --     | --   |
| Pneumonia                  | 0.32        | --   | --     | --   | --     | --   |
| Mucinosiis                 | --          | --   | --     | 0.24 | --     | --   |
| Atelectasis                | 0.28*       | --   | --     | --   | --     | --   |
| <b><u>TRACHEA:</u></b>     |             |      |        |      |        |      |
| Metaplasia                 | --          | --   | --     | 0.64 | --     | --   |
| Macrophages (f)            | --          | --   | 0.30   | --   | --     | --   |
| Macrophages (p)            | --          | --   | --     | 0.44 | --     | --   |
| Lymphoid                   |             |      |        |      |        |      |
| Infiltration               | --          | --   | --     | 0.56 | --     | --   |
| Neutrophils                | --          | --   | 0.28*  | --   | --     | --   |
| <b><u>LYMPH NODES:</u></b> |             |      |        |      |        |      |
| Macrophages (f)            | --          | --   | 0.23   | --   | --     | --   |
| <b><u>SPLEEN:</u></b>      |             |      |        |      |        |      |
| Splenomegaly               | 0.24        | --   | 0.22   | --   | --     | --   |
| <b><u>KIDNEY:</u></b>      |             |      |        |      |        |      |
| Monocytes                  | 0.25        | --   | --     | --   | --     | --   |
| <b><u>LIVER:</u></b>       |             |      |        |      |        |      |
| Lymphocytes                | --          | --   | --     | --   | --     | 0.48 |

**Table H-1 (continued)**

| CONDITION                  | TREATMENT  |            |            |            |
|----------------------------|------------|------------|------------|------------|
|                            | B4C        |            | SiCP       |            |
|                            | HD<br>(20) | LD<br>(24) | HD<br>(24) | LD<br>(24) |
| N=(X)                      |            |            |            |            |
| <b><u>LUNG:</u></b>        |            |            |            |            |
| Granulomas                 | --         | --         | --         | --         |
| Hyperplasia                | --         | --         | --         | --         |
| Fibrosis                   | --         | --         | --         | --         |
| Giant Cells                | --         | --         | --         | --         |
| Macrophages(f)             | --         | --         | --         | --         |
| Macrophages(p)             | --         | --         | 0.67       | --         |
| Neutrophils                | --         | --         | --         | --         |
| Pneumonia                  | --         | --         | --         | --         |
| Mucinosiis                 | --         | --         | --         | --         |
| Siderphages                | --         | --         | --         | 0.58       |
| <b><u>TRACHEA:</u></b>     |            |            |            |            |
| Metaplasia                 | --         | --         | --         | --         |
| Macrophages (f)            | --         | --         | --         | --         |
| Macrophages (p)            | --         | --         | --         | --         |
| Lymphoid                   |            |            |            |            |
| Infiltration               | --         | --         | --         | 0.58       |
| Neutrophils                | --         | --         | --         | --         |
| Siderphages                | --         | --         | --         | 0.63       |
| Mucinosiis                 | --         | --         | 0.25       | 0.38       |
| <b><u>LYMPH NODES:</u></b> |            |            |            |            |
| Macrophages (f)            | --         | --         | --         | --         |
| <b><u>SPLEEN:</u></b>      |            |            |            |            |
| Splenomegaly               | --         | --         | --         | --         |
| <b><u>KIDNEY:</u></b>      |            |            |            |            |
| Monocytes                  | --         | --         | --         | --         |
| Lymphoid                   |            |            |            |            |
| Infiltration               | --         | --         | --         | --         |
| <b><u>LIVER:</u></b>       |            |            |            |            |
| Lymphocytes                | --         | --         | --         | --         |
| Bile Duct                  |            |            |            |            |
| Hyperplasia                | --         | --         | --         | --         |

f - fibers

p - pigmented

\* - values not significant at  $p < 0.05$

was noted in more than 85% of SiCW-1 groups at both low and high doses. These tumors varied in size and number, frequently extending into bronchial lumens partially occluding airways (Figure 5A). Similar conditions were observed in SiCW-2 and asbestos treatment groups but with lower frequency, 28% and 30% respectively. No tumors were observed in boron carbide or silicon carbide platelet exposed animals. Additional pathological conditions, as further shown in Table 2 included severe fibrosis (Figure 5B), acute alveolar injury (Figure 6), and cuboidal metaplasia (Figure 7), developing predominately in SiCW and crocidolite exposure groups. Tracheal metaplastic changes occurred exclusively in SiCW-1 treated animals.

Strict localization of SiCW within the respiratory tissues did not exist. Fibers remained intact and unchanged after 18 months. The highest concentrations were found within granulomatous tumors (Figure 8) or under the epithelial layer, near the basement membrane (Figure 9). Regional lymph nodes containing fiber-laden macrophages indicated that fibers had been translocated from the lungs, most likely through macrophage involvement. Overall, the deleterious conditions were associated with SiCW and crocidolite in a ranking of SiCW-1 > SiCW-2 > crocidolite with respect to pulmonary

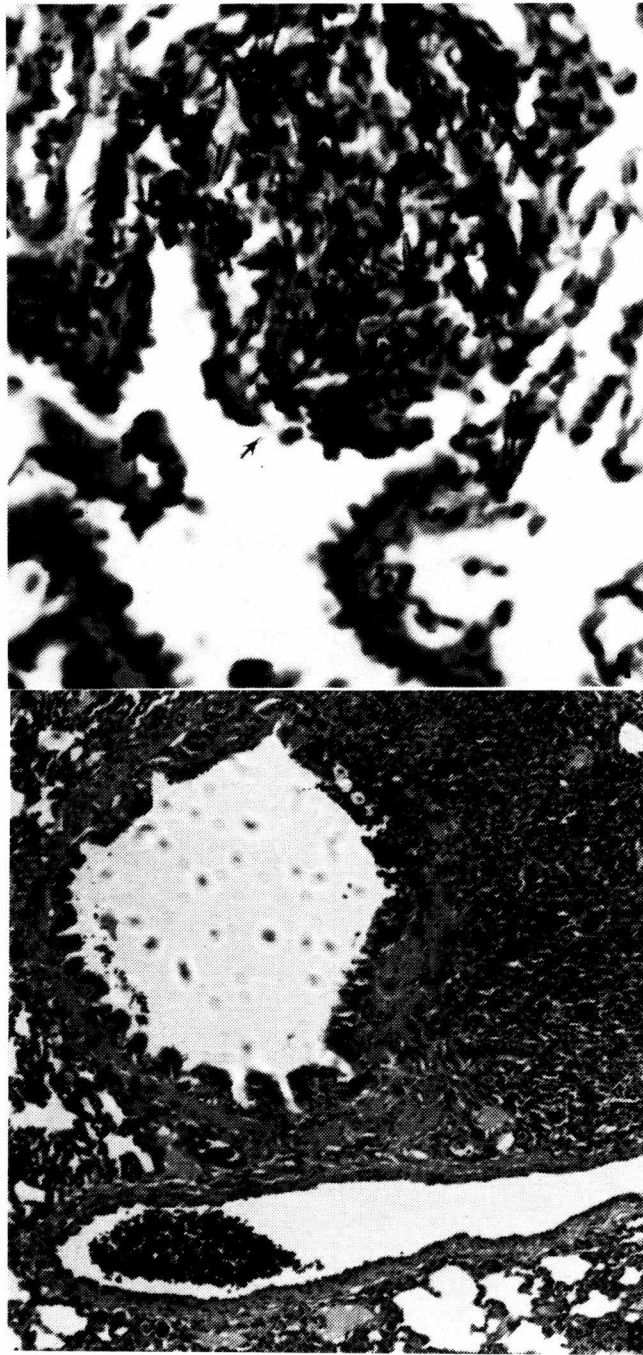


**Figure 5** Lung sections, 18 months post exposure, from rats treated with SiCW-2 at 1 mg/100ml MRV (Panel A) and 5 mg/100 ml MRV (Panel B). Peribronchiolar granulomas (arrows) protrude into the bronchial lumen partially occluding the airway. Irregular fibrosis surrounds the terminal bronchioles. Magnification 160 X.

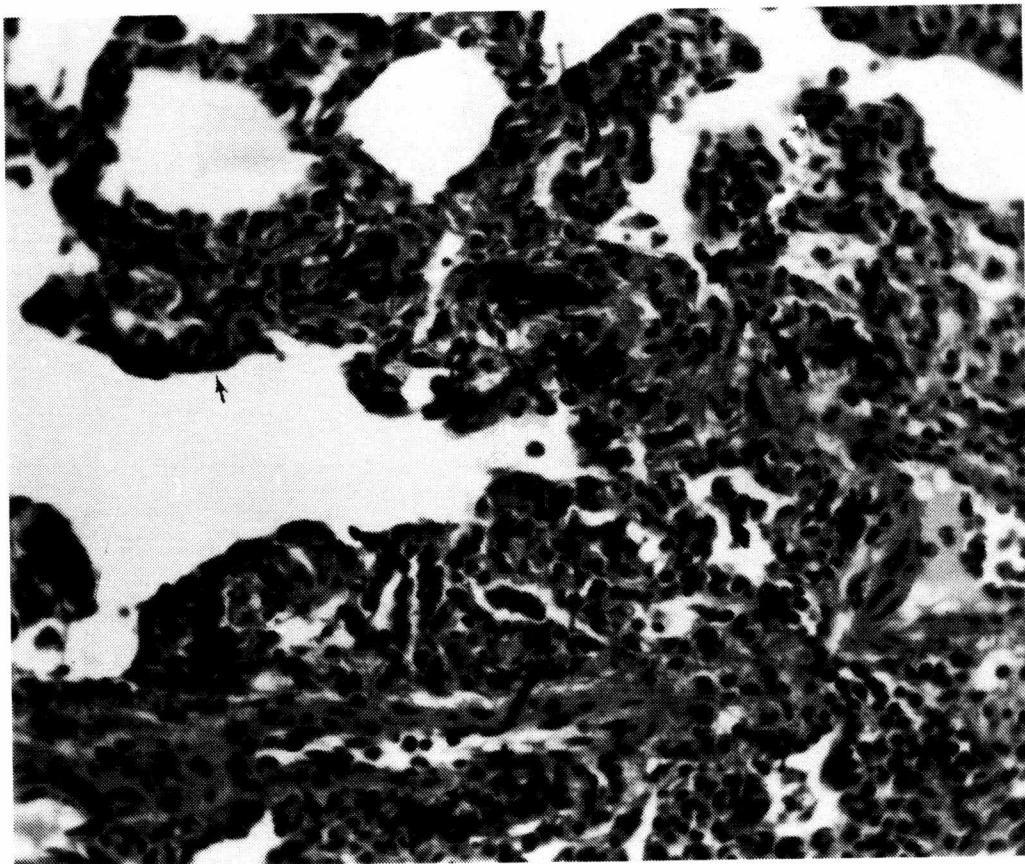


**Figure 6** Light micrograph showing the acute alveoli injury common to interstitial pneumonia with marked thickening of alveolar walls (arrow) following exposure to SiCW-1 at a dose of 5 mg/ 100 ml MRV. Magnification 990X.

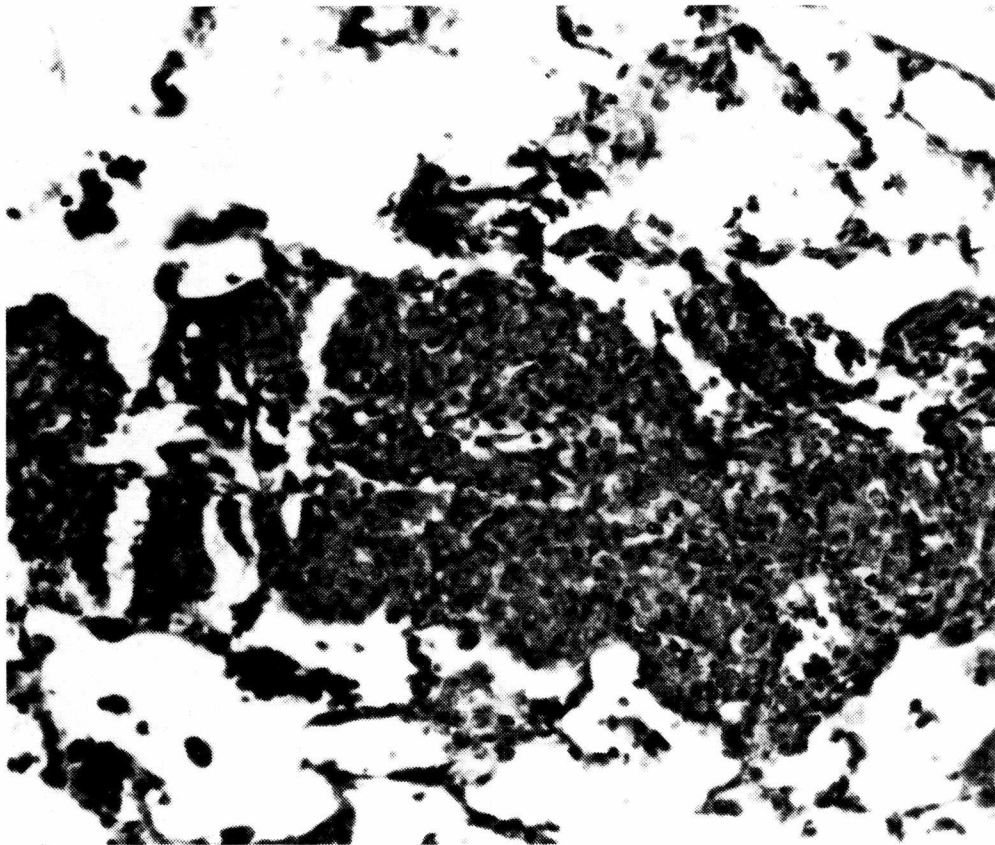


**Figure 7** Lung tissue removed from a SicW-2 treated animal after 1.5 years. This distorted airway demonstrated evidence of epithelial cell injury manifested by cuboidal metaplastic changes. Magnification 990X.

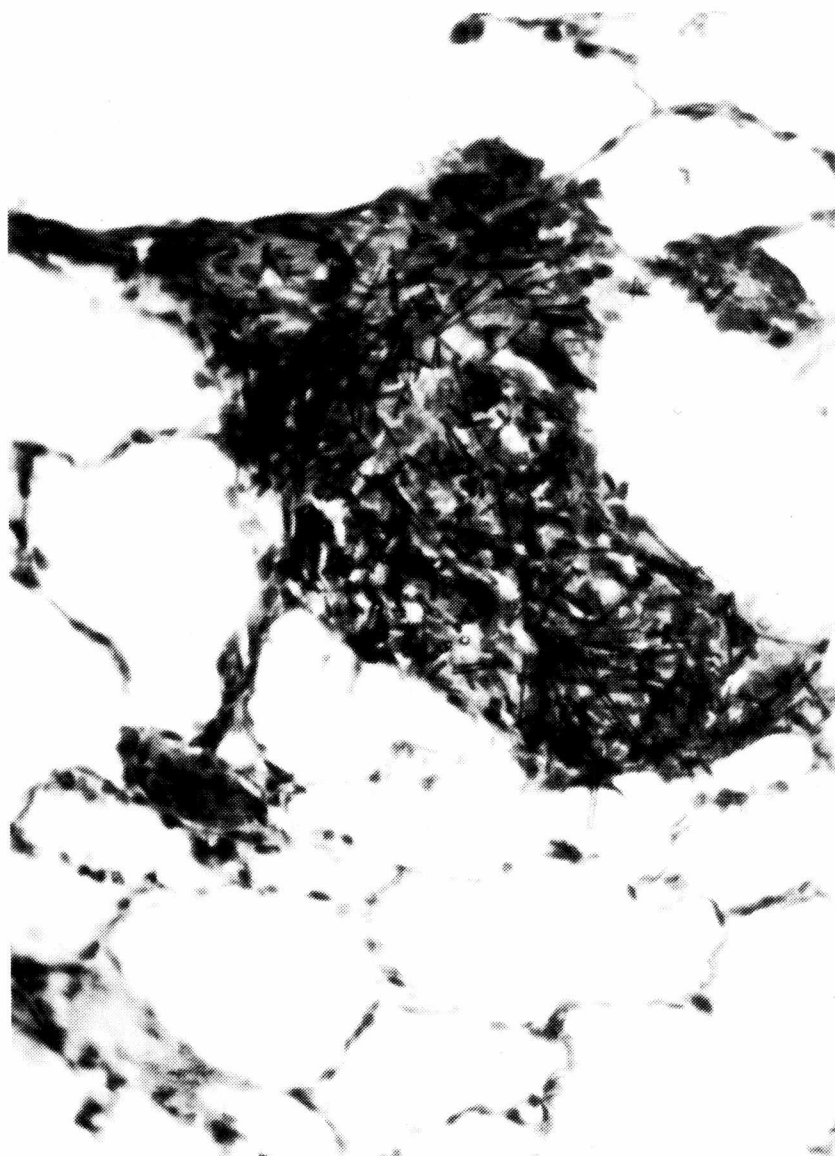


**Figure 8** Typical fiber-associated granulomatous tumor in the alveolar region of a rat exposed to 1 mg/100 ml MRV of SiCW-2. Magnification 950X.

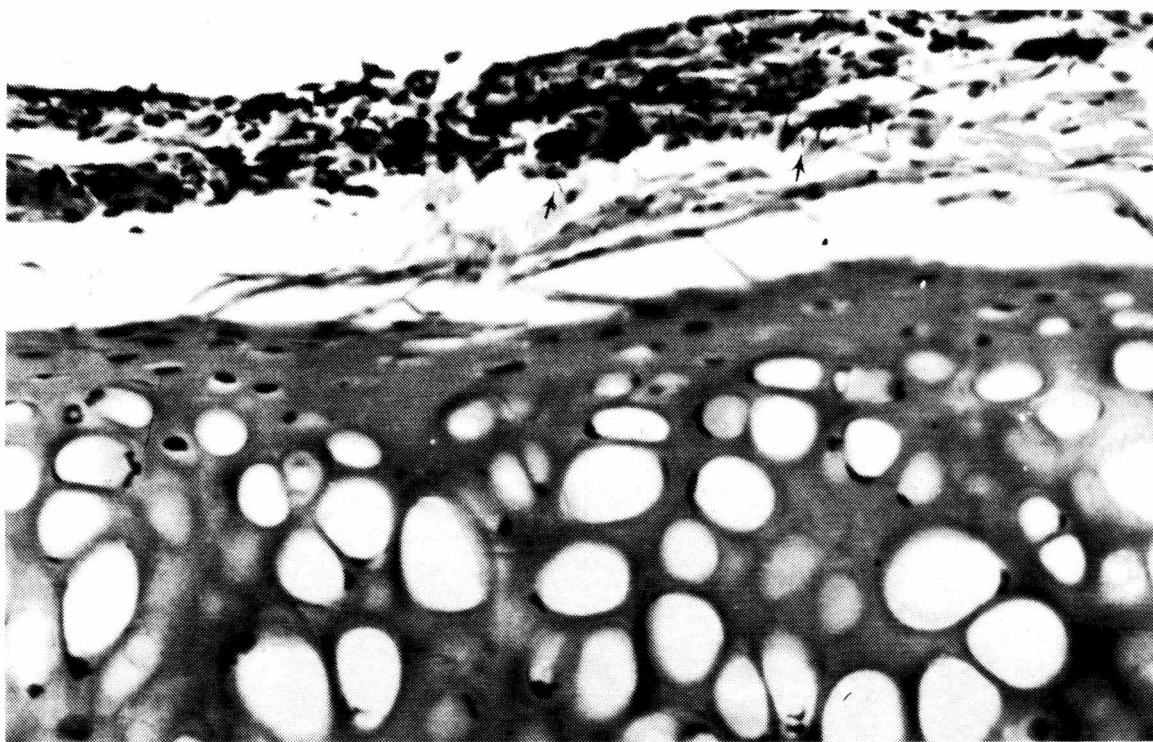


**Figure 9** Intact SiCW-1 fibers penetrate to the basement layer (arrows) 1.5 years after intratracheal instillation. Magnification 210 X.

damage. No changes of significance were observed in the tissues of boron carbide and silicon carbide platelets.

Fibers penetrating the lung may become lodged in the pleural membranes further triggering the development of malignant mesotheliomas. In order to examine this possibility, rats were given an intraperitoneal injection of PBS or SiCW-2 and maintained for 18 months. A few animals developed subcutaneous fibroadenoma and mononuclear leukemia, both of which are typically found in the aging Fischer rat. Although malignant mesotheliomas were absent, one incidence of fibrosarcoma and myxosarcoma were noted in the SiCW-2 group. The most frequently observed condition was severe peritoneal fibrosis, which occurred in 90% of the animals exposed to SiCW-2. Fibrotic conditions resulted in extensive abdominal organ adhesions, particularly between Glisson's capsule of the liver and the diaphragm. In two instances, fibrosis caused lethal intestinal obstruction. Often accompanying fibrosis were granulomatous tumors, as previously observed in intratracheally exposed animals. These tumors became localized at the capsules of the liver, spleen and pancreas as well as the diaphragm. One incidence of a tracheal tumor indicated that fibers can migrate or be transported to distal

tissues.

#### 4. DISCUSSION

Various man-made mineral fibers have recently entered the marketplace. Many of these fibers are fiber-form materials with particle geometry similar to the asbestos form linked to pulmonary disease. Utilization of new forms often precedes extensive testing for carcinogenicity and other biological effects. Silicon carbide fibers also fall within this category. Based on previous (AARCO, 1985) and current findings (Vaughan et al., 1991a), SiCW exhibit early signs of fibrotic and neoplastic disease.

The pulmonary response immediately following fiber instillation is characterized by an influx of neutrophils and macrophages. Alveolar macrophage titers remained constant for 28 days, an early indication of prolonged inflammation. The incomplete phagocytosis of fibers by AM is believed to initiate the cellular events leading to fibrogenesis and neoplasia (Lemaire et al., 1986 / Mossman and Begin, 1989). Chemical mediators, including AOS (eg.  $O_2^-$ ,  $OH^-$ ) and growth factors (FGF, AMDGF, PDGF) enhance the inflammatory process by the recruitment of additional macrophages and other pulmonary cells. Cultures of tracheal epithelium, lung fibroblast and

macrophages have all demonstrated release of active oxygen species following fiber exposure resulting in fibroblast proliferation, as well as cell injury and death (Hansen and Mossman, 1987 / Gulumian and Van Wyk, 1987 / Brody and Bonner, 1991 / Marsh and Mossman, 1991). Chronic accumulation of pulmonary inflammatory cells and fibroblasts leads to continual thickening and decreased elasticity of pulmonary tissues, i.e. fibrosis (Rom et al., 1991).

Fiber tumorigenicity of asbestos has been studied using both *in vivo* and *in vitro* systems (Pott et al., 1987 / Adachi et al., 1991). The general hypothesis states that a significant relationship between carcinogenicity and fiber morphology exists (Stanton, 1983). Man-made mineral fibers with particle geometry similar to asbestos were shown to generate neoplasia (Woodworth, et al., 1983 / Suzuki, 1984). Following intraperitoneal and intratracheal instillation, histopathology revealed benign granulomatous tumors associated with SiCW and crocidolite exposure. There was an overwhelming occurrence of tumors in SiCW-1 treatment group as compared to the SiCW-2 and asbestos groups. Non-fibrous SiCW analogs did not produce any forms of tumors.

The lack of malignancies in both intratracheal and

intrapleural studies is understandable. Development of malignancies in rodents approaches the 2-3 year life span of these animals, a period too brief for a slow carcinogen such as mineral fibers. Should circumstances had allowed for a longer incubation period, malignant tumors may have appeared.

Data present here suggest that the SiCW-1 is more cytotoxic than SiCW-2 or crocidolite. While both possess similar geometry, SiCW-2 is approximately twice the diameter of SiCW-1. This small difference in dimensions perhaps explains the levels of cellular damage observed. Secondly, it seems likely that cytotoxicity is a function of the number of fibers present. In this instance, there would be approximately four times as many SiCW-1 as SiCW-2 per weight of fibers. Furthermore, crocidolite which possesses the smallest dimensions of all tested material, would be expected to be the most cytotoxic based on the number of fibers present. The lack of such severe conditions following crocidolite exposure are possibly related to the fact that these fibers were small enough for the macrophage to successfully phagocytize. Other factors contributing factors may include differences in chemical composition and surface charge.

## 5. CONCLUSION

These data clearly demonstrate that SiCW have a far greater potential for fibrosis and neoplastic disease than crocidolite asbestos. Findings of this study are summarized as follows:

- \* cilia activity is not inhibited by fibers
- \* fibers are swept into non-ciliated regions of the epithelium where necrotic foci develop
- \* inflammation persists up to 28 days after fiber exposure
- \* benign granulomatous tumors develop in pulmonary tissue with frequent occlusions of conducting airways.
- \* fibers are retained in tissue 18 months following exposure

If MMMF continue to be manufactured in fibrous-form with dimensions similar to asbestos, they will remain a human health hazard.

Whenever possible, the size of SiCW should be selected so as not to fall in the respirable range determined to be toxic. Being completely innocuous, SiCP offer a far safer occupational environment therefore, making them a more desirable SiC form.

As long as SiC continues to be manufactured as whiskers, strict handling guidelines must be employed. Suggested

guidelines include:

- \* storage of fibers in airtight and unbreakable containers
- \* wearing of protective clothing and respirators with HEPA filters
- \* material transfers in a ventilated enclosure
- \* continual monitoring of airborne concentrations with maintenance below 0.2 fiber/cc/hour
- \* medical surveillance programs of all exposed employees

Through strict enforcement of these guidelines it will be possible to attain the lowest feasible airborne concentrations.

## LIST OF REFERENCES

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## VITA

Sharon Ann (Milewski) Trently was born in the antiquated town of Dupont, Pennsylvania on August 13, 1963. After attending Pittston Area Schools, for what seemed like forever, she graduated in June, 1981. Following graduation, she entered King's College, Wilkes-Barre, Pennsylvania and received a B.S. in Biology on August 24, 1984. Shortly after moving to east Tennessee, she entered the University of Tennessee to pursue a Master of Science degree in Zoology. The degree was conferred in December 1991.

Presently, Sharon resides with her husband, David and Burmese python, Radagast, in West Knoxville. She is employed by Carson Newman College and The University of Tennessee. In her spare time, she can be found roaming the southeastern United States in search of a folk dance.