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Separation and Characterization of Respirable Amphibole Fibers from Libby, Montana

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The vermiculite mine in Libby, Montana, was in operation for over 70 yr and was contaminated with asbestos-like amphibole fibers. The mining, processing, and shipping of this vermiculite led to significant fiber inhalation exposure throughout the community, and residents of Libby have developed numerous pulmonary diseases such as lung cancer and mesothelioma. The present study describes the separation of Libby 6-mix into respirable and nonrespirable size fractions by means of aqueous elutriation. The elutriator, designed to separate fibers with aerodynamic diameters smaller than 2.5 μm (respirable) from larger fibers, used an upward flow rate of 3.4×10^{-4} cm s^{-1}. The resultant respirable fraction constituted only 13% of the raw Libby 6-mix mass, and less than 2% of the fibers in the elutriated fraction had aerodynamic diameters exceeding 2.5 μm. Surface area of the elutriated fibers was 5.3 m^{-2} g^{-1}, compared to 0.53 m^{-2} g^{-1} for the raw fibers. There were no detectable differences in chemical composition between the larger and smaller fibers. Such harvesting of respirable fractions will allow toxicological studies to be conducted within a controlled laboratory setting, utilizing fiber sizes that may more accurately simulate historical exposure of Libby residents' lungs. Importantly, this work describes a method that allows the use of material enriched in more uniform respirable material than raw Libby 6-mix, making comparisons with other known fiber preparations more valid on a mass basis.

Vermiculite historically extracted from Zonolite Mountain near Libby, Montana, was contaminated with a toxic form of naturally occurring fibrous and asbestiform amphibole, occurring in veins throughout the deposit (Pardee & Larsen, 1929). As a result of nearly 70 yr of mining and processing activities, the Libby area was contaminated with a complex mixture of dust including these amphibole fibers, and many of the homes within Libby are likewise contaminated (Wright et al., 2002). High concentrations (>100 million fibers/cm^2) of these amphibole fibers have even been found in the bark of Libby trees (Ward et al., 2006). Asbestos exposures have led to considerable health problems in the community, including reduced pulmonary function, elevated autoimmune responses, and increased mortality from lung cancer, malignant mesothelioma, and fibrosis (McDonald et al., 2004; Pfau et al., 2005; Whitehouse, 2004).

The form of asbestos in Libby's contaminated vermiculite has been characterized as belonging to the amphibole (double-chain silicate) mineral group, including both regulated (tremolite) and unregulated (primarily winchite and richterite) fibers (Meeker et al., 2003). These various fibers within Libby amphibole asbestos differ in their relative proportions of cations (Mg, Ca, Fe, Na, K). On the basis of their diverse chemical composition, Libby amphibole fibers are distinct from the more typical and well-characterized and -studied amphibole fibers.

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According to Meeker et al. (2003), most of the Libby particles display characteristics that are "intermediate between cleavage fragments and long flexible fibers." Nonetheless, this assemblage has been shown to induce oxidative stress as well as apoptosis in macrophages (Blake et al., 2007; Blake et al., 2008).

Fiber dimension is widely considered to be the most important determinant of fiber pathogenicity in terms of cancer development (Oberdorster, 2000). Early work suggested that fibers longer than 8 μm were more potent inducers of mesothelioma than were shorter fibers (Stanton et al., 1981). The greater pathogenicity of longer fibers, relative to shorter ones, has been determined in vivo (Mossman et al., 1989). However, more recent evidence supports the premise that asbestos fibers of all lengths, not simply long fibers, induce pathological responses (Dodson et al., 2003; Suzuki et al., 2005). Indeed, fibers of differing lengths are thought to induce separate types of pathological outcomes such as asbestosis, mesothelioma, and lung cancer (Lippman, 1990).

Aerodynamic diameter of a fiber is a crucial parameter that dictates whether a fiber is respirable. Inhaled fibers with a large aerodynamic diameter have sufficient inertia to be deposited in the upper respiratory tract, where they are cleared from the lung by the mucociliary escalator, which may then transport them to extrapulmonary sites. As a result, these fibers are less likely contribute to the pulmonary diseases caused by asbestos (Shusterman, 2003). In contrast, fibers that are small enough to be able to bypass the upper respiratory tract and penetrate into alveolar spaces are termed *respirable*. The largest alveolar deposition of fibers in the lung occurs when fibers are between 1 and 2 microns in aerodynamic diameter (Dai & Yu, 1998). Alveolar macrophages attempt to clear fibers of this size (Mossman & Churg, 1998) and may contribute to inflammatory processes. These respirable fibers produce the pathologies associated with humans exposed to asbestos.

Since Libby amphibole comprises a diversity of fiber sizes and types, the isolation, characterization, and utilization of respirable fibers will be an important step in mimicking real-life asbestos exposures that have historically occurred (and may be still occurring, even though mining was stopped in 1990) throughout the Libby area, as well as wherever Libby's vermiculite was shipped, processed, and used. To date, many of the health studies conducted on Libby amphibole have utilized a sample provided by the U.S. Geological Survey, a sample that includes a combination of asbestos samples from six separate collection areas. However, this composite sample, called "Libby 6-mix," includes a complex mixture of fiber sizes and nonfibrous material in addition to the respirable size fraction, and may not accurately reflect historical inhalation exposures that have occurred in Libby. Importantly, it is very difficult to make comparisons of the complex Libby 6-mix with other more uniform preparations of asbestos such as crocidolite through in vitro and animal studies where comparisons are based on dosage masses.

To that end, this article describes the isolation and characterization of respirable Libby amphibole fibers. Respirable Libby 6-mix fibers were fractionated through aqueous elutriation, which separated the fibers according to aerodynamic diameters. Through this process, larger particles were discarded, leaving a more uniform preparation of respirable particles, most of which were fibers. These elutriated fibers were individually characterized to ensure that the desired fiber sizes were recovered during elutriation. Elutriated fibers were also chemically characterized to determine if elemental composition varied among different size fractions. Nonfibrous particles that were undoubtedly in the Libby 6-mix were not characterized.

MATERIAL AND METHODS

Determination of Size of Respirable Fibers

Elutriation was based on the U.S. Environmental Protection Agency (EPA) designation of fine particles, as possessing a diameter of 2.5 μm or less. These particles are potentially harmful in that they can circumvent the upper respiratory gauntlet and reach the alveoli (Green & Armstrong, 2003). For the purpose of this article, *respirable* refers to particles with aerodynamic diameters of 2.5 μm or less. For fibers, the term *aerodynamic diameter* takes into account density and length.

Aerodynamic diameter increases with density (Lastow & Podgorski, 1997):

$$D_{ae} = D_p\left(\frac{\rho_p}{\rho_0}\right)^{0.5} \quad [1]$$

where D_{ae} is the aerodynamic diameter, D_p is the particle diameter, ρ_p is the particle density, and ρ_0 is 1.0 g/cm^3. Solving for D_p, we find that a 1.4-μm-diameter sphere with a density of 3.0 (density of amphiboles) is equivalent in aerodynamic diameter to a 2.5-μm sphere with a density of 1.0.

An elongate shape affects a fiber's settling velocity. The equivalent diameter (D_{eq}) is calculated (Timbrell, 1965) by:

$$D_{eq} = 66D_f\left(\frac{\beta}{2+4\beta}\right)^{2.2} \quad [2]$$

where D_f is the fiber diameter and β is the fiber aspect ratio (length divided by diameter). Solving this equation for a fiber with a 5:1 aspect ratio (the minimum aspect ratio for asbestos in this study as discussed later) and an equivalent diameter of 1.4 μm (determined earlier) yields a fiber diameter of 0.55 μm (and hence a length of $5 \times 0.55 = 2.8$ μm). Utilizing the same equation, we find that the largest respirable 10:1 aspect-ratio fiber would have a diameter of 0.50 μm, the largest respirable 50:1 aspect-ratio fiber would have a diameter of 0.47 μm, and the largest respirable 100:1 aspect-ratio fiber would have a diameter of 0.45 μm.

Elutriator Flow Determination

The settling velocity (V_t) of a particle is a function of water's viscosity and the particle's density and morphology

(Hanna et al., 1982):

$$V_t = \left(\frac{2r^2 g \rho_p}{9\mu}\right) \quad [3]$$

where ρ_p is particle density, μ is the dynamic viscosity of water (0.01002 g s^{-1} cm^{-1}), r is the particle radius (cm), and g is the gravitational constant (980 cm s^{-1}). Solving for a respirable sphere (r = 1.25 μm, ρ_p = 1.0 g/cm^3) yields a settling velocity of 3.4 × 10^{-4} cm s^{-1}. An upward flow at this velocity would theoretically buoy respirable particles, while allowing larger particles to sink.

Amphibole Fiber

Libby 6-mix was obtained from the U.S. Geological Survey. These amphibole fibers, which have been chemically and physically characterized in detail, contain six amphibole fiber types, including winchite, richterite, and tremolite (Gunter et al., 2003; Meeker et al., 2003; Wylie & Verkouteren, 2000). Prior to elutriation, fibers were dispersed in sterile water by cup-horn sonication (Misonix, Framingdale, NY). Crocidolite asbestos was provided by the Research Triangle Institute (RTI, Research Triangle Park, NC) but was not elutriated.

Elutriation Procedure

The polypropylene elutriation funnel (Cole-Parmer, Vernon Hills, IL) had a maximum diameter of 6 cm (Figure 1). A hole was drilled into the screw-top cap to receive a plastic tube through which the overflow drained. Because the cross-sectional area of the funnel at the maximum diameter was 28.3 cm^2, a flow rate of 0.58 cm^3 min^{-1} was needed to produce an upward settling velocity of 3.4 × 10^{-4} cm s^{-1}. To allow for the possibility of equilibrium between flow and settling where the 2.5-μm-diameter particle remains suspended just below the maximum diameter, the target flow rate was increased to 0.6 cm^3 min^{-1} to ensure capture of all respirable particles.

One end of a length of nylon tubing was attached to the bottom of the elutriation funnel, with the other end in a reservoir of filtered sterile water. The tubing was run through a peristaltic pump (Fisher Scientific, Pittsburgh, PA) which operated at 4 rpm, to deliver water up through the elutriator at a rate of 0.6 ml min^{-1}. The outflow was directed to a sterile 1000-ml screw-top polypropylene bottle.

Prior to elutriation, the water was brought up level to roughly 10 cm from the top of the funnel. Immediately before the elutriation was begun, the 10-ml suspension of Libby asbestos was dispersed in sterile water by cup-horn sonication. The pump was turned on just before the introduction of the asbestos suspension into the top of the elutriation funnel. The pump was run for 24 h to ensure collection of all narrow fibers. The receiving bottle was capped following elutriation and placed in an ultrasonic bath for 15 min, after which a 50-ml aliquot was withdrawn and filtered through a tared 0.1-μm pore polycarbonate (PC) filter, for determination of the actual mass of the suspended particles. The total mass of the elutriated residue was calculated by division of the net mass on the filter by 50 (ml) and multiplication of this by the total volume (864 ml) of elutriated water. For determination of the percent recovery, the total mass of elutriated residue was divided by the original mass of Libby 6-mix placed in the elutriator at the beginning of the experiment.

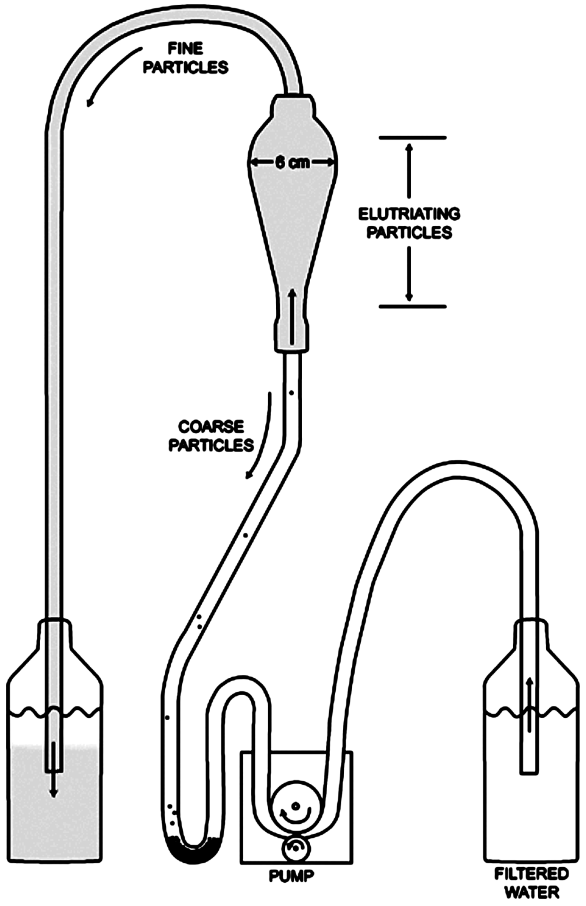

FIG. 1. Elutriation setup. Arrows indicate direction of flow. Elutriated fibers were harvested from the bottle on the left.

Fiber Characterization

A suspension of elutriated Libby particles was filtered through 0.1-μm PC filters, carbon-coated, and transferred to transmission electron microscope (TEM) grids. These grids were examined in a Hitachi 7100 scanning transmission electron microscope (STEM) equipped with a fixed high-angle x-ray detector (ultrathin window PGT Omega) and an SIA-7C 3.2 megapixel CCD camera above the viewing screen. Grid openings were selected from a computer-generated randomized list (Webber, 1987) to prevent analyst bias. Mineral fibers that had aspect ratios greater than 5 were measured (length directly from the TEM phosphor screen and width from CCD images) at a calibrated 16,000× magnification. Selected-area electron diffraction (SAED) was used to measure on-screen row spacings, using an objective-aperture-eclipsing technique (Webber, 1997); these

SAED images were collected by the CCD camera. Finally, the electron beam was focused in the STEM mode, with a spot size smaller than 0.1 μm, on fibers showing amphibole SAED patterns, and a 30-s (live time) energy-dispersive x-ray (EDX) spectrum was collected. Full-width half-maximum counts for each element were derived by a process that used single-smoothing and background subtraction software. K-factors to account for system bias at different x-ray energies were then applied from calibrations with NIST SRM 2063a (O, Mg, Si, Ca, and Fe) and crushed kaersutite (Na, Al, K).

Nonelutriated Libby 6-mix fibers and crocidolite fibers were analyzed separately at a commercial laboratory (DataChem, Cincinnati, OH) on a Philips CM10 TEM equipped with an EDAX detector.

Masses of individual fibers were calculated by multiplying their volume (length $\times$ (width^2)) by mineral density (Deer et al., 1992), 3.3 pg/μm^3 for crocidolite, 3.2 pg/μm^3 for Libby 6-mix, and 3.0 pg/μm^3 for wollastonite. Surface area was similarly calculated by 4 $\times$ (length $\times$ width).

RESULTS AND DISCUSSION

Physical Characteristics of Elutriated Libby Asbestos Fibers

Elutriated particles constituted only 13% of the total mass of the original 6-mix material placed into the elutriator. Even before TEM characterization was performed, a marked difference between the two fiber populations was apparent by light microscopy. Figure 2A reveals that the nonelutriated thick fibers left in the funnel are absent from the elutriated fraction (Figure 2B). Figure 2 also corroborates the finding of Meeker et al. (2003: Figure 11.b) that fibers outnumber nonfibers in the Libby 6-mix. Calculations from individual dimensions of fibers used in an earlier study of apoptosis in macrophages (Blake et al., 2008) had indicated a respirable mass fraction of only 1.2%. However, those calculations were based on a limited population of 31 fibers.

In our study, in total, 101 elutriated fibers were characterized by TEM from a total of 4 grid openings on three grids. The vast majority were dispersed individually on the carbon film. The sole cluster (Figure 3) was composed of typical elutriated fibers. Mean fiber dimensions were 2.7 μm = length, 0.19 μm = width, and 16 = aspect ratio. When fiber diameters equivalent to 2.5 μm aerodynamic diameter (0.45 to 0.55 μm from Equation 2) are plotted (the line of Xs in Figure 4, A and B) against fiber length, it is apparent that many of the fibers in the raw 6-mix (Figure 4A) are larger than respirable. This abundance of thicker fibers is consistent with previous results (see the almost identical Figure 11 in Meeker, 2003). In contrast, 98% of the elutriated fibers were at or below the respirable diameters (Figure 4B). Interestingly, the largest amphibole fiber internalized by the murine macrophages (Figure 1D in Blake, 2007) had an aerodynamic diameter of less than 1 μm, even though the cells had been exposed to raw 6-mix.

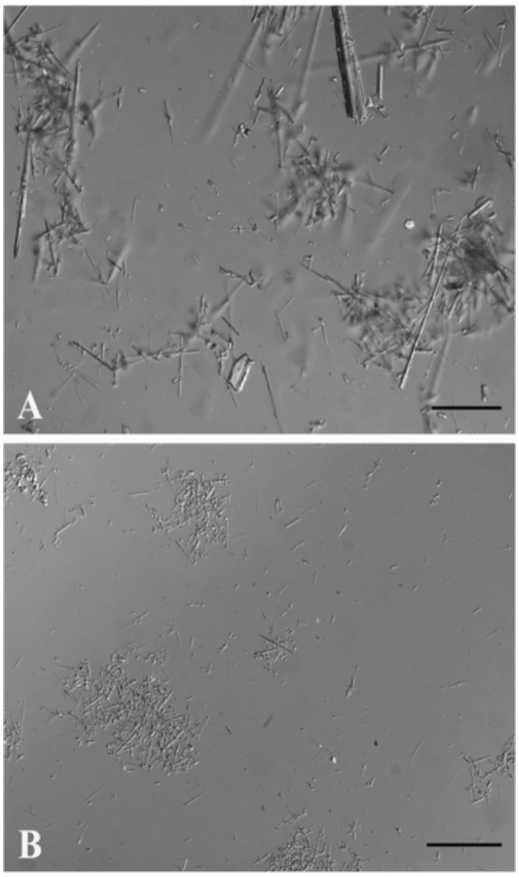

FIG. 2. Light micrographs of the two postelutriation fiber fractions. (A) Libby amphibole fibers taken from the funnel after elutriation. (B) Elutriated (respirable) fibers. Scale bar equals 50 μm.

The difference between the raw 6-mix and the elutriated portion is also large when we considered surface area of fibers exposed to cells during toxicity studies. Fibers in the raw 6-mix presented a surface area of 0.53 m^{-2} g^{-1}, compared to a surface area of 5.3 m^{-2} g^{-1} for the elutriated fraction (Table 1). Hence, the elutriated fraction more closely approximates asbestiform materials (surface area of 8.4 m^{-2} g^{-1} for crocidolite in this study, and 18 to 21 m^{-2} g^{-1} for ultrasonicated chrysotile; Seshan, 1983; Turci et al., 2007).

TABLE 1
Surface Areas of Fiber Populations (m^{-2} g^{-1}) Determined From Individual Fiber Measurements by TEM

Raw Libby 6-mix	Elutriated respirable fraction	Crocidolite
0.53	5.3	8.1

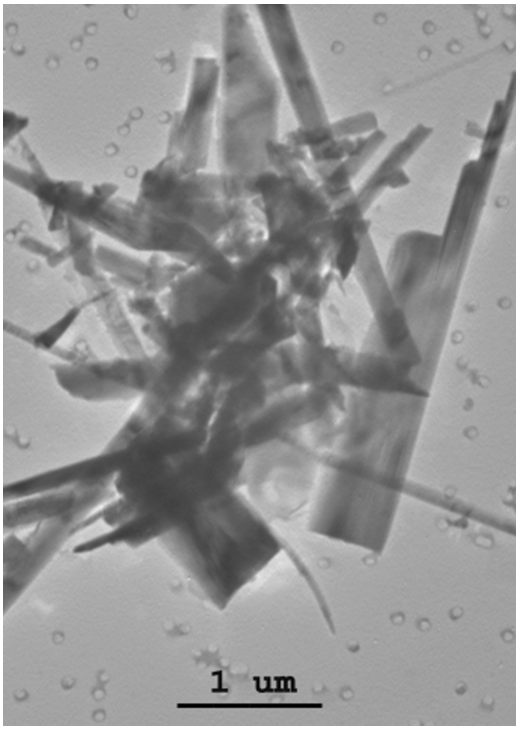

FIG. 3. Transmission electron micrograph of a cluster of elutriated 6-mix fibers.

TABLE 2
K-Factor-Corrected Elemental Compositions (Normalized to Si) for Respirable Libby Amphibole Fibers

	O	Na	Mg	Si	K	Ca	Fe
Mean	1.93	0.11	0.46	1.00	0.024	0.20	0.21
SD	0.24	0.05	0.05	0.00	0.019	0.06	0.08
RSD	0.12	0.45	0.10	0.00	0.79	0.32	0.38

TABLE 3
Correlation Matrix of Significant Elements (K-Factor-Corrected and Normalized to Si)

	O	Na	Mg	K	Ca	Fe
O	1					
Na	0.160	1				
Mg	0.019	–0.058	1			
K	–0.206	0.365*	–0.126	1		
Ca	–0.062	–0.465*	0.581*	–0.292*	1	
Fe	0.254	0.573*	–0.405*	0.040	–0.630*	1

Note. Absolute values larger than 0.26 are significant at $p < .01$ and are indicated by asterisks.

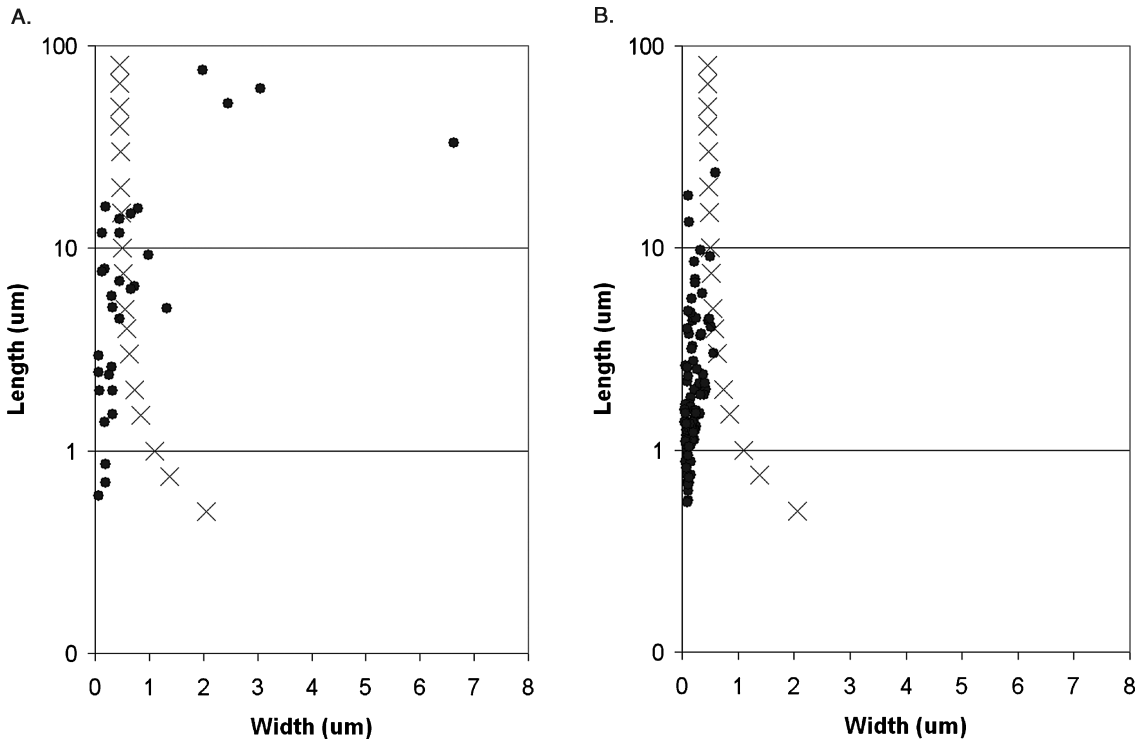

FIG. 4. Distribution of 6-mix amphibole fibers characterized by electron microscopy. Line of Xs marks the boundary between respirable and nonrespirable fibers. (A) Raw 6-mix fibers; (B) elutriated fibers.

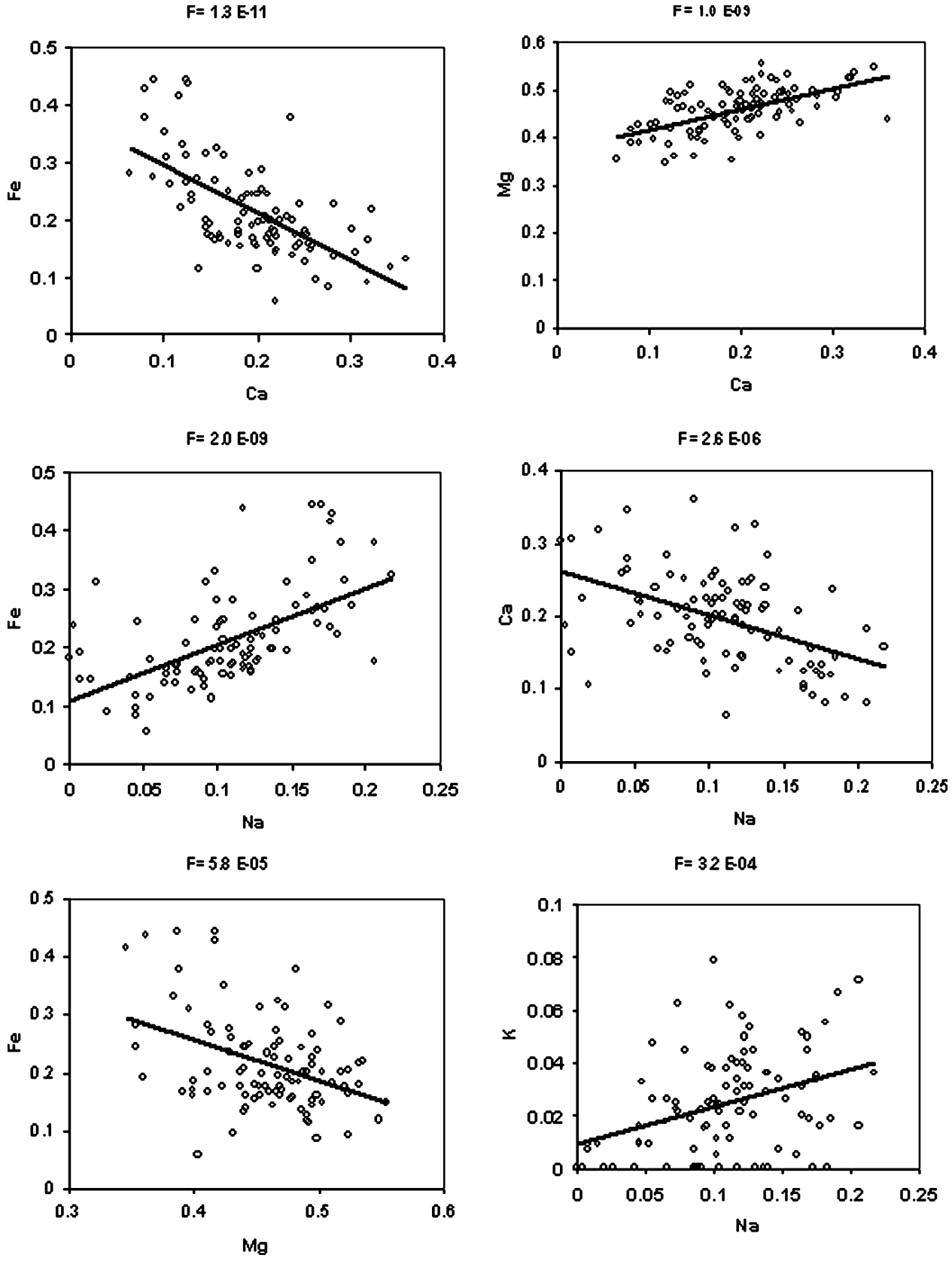

FIG. 5. Plots of significant correlations between major cations in elutriated Libby amphibole fibers. Significance of F is given at the top of each plot.

TABLE 4
K-Factor-Corrected Elemental Compositions (Normalized to Si) for Respirable Libby Amphibole Fibers Divided Into Two Width Categories

Width (μm)	O	Na	Mg	K	Ca	Fe
<0.16	2.05	0.11	0.44	0.021	0.19	0.23
>0.16	1.81	0.11	0.47	0.028	0.20	0.20
Ratio	1.13	0.98	0.94	0.73	0.92	1.15

Chemical Characteristics of Elutriated Libby Asbestos Fibers

Elemental data from the elutriated fibers were distributed through the continuum of compositions expected from the Libby amphiboles (Table 2). For this study, no attempt was made to identify specific amphibole mineral species (e.g., winchite, richterite, tremolite) by individual fiber; rather, any possible correlation of elemental composition with size was evaluated. Mg and O were the elements with the least variation (relative standard deviation (RSD) <15%), while K, Na, and Fe were the most variable (RSD >30%).

Correlations among the major cations were often significant, as indicated in Table 3. Linear regression analysis of cation pairs revealed extreme significance ($p < .0001$) for Fe/Ca, Mg/Ca, Fe/Na, Ca/Na, Fe/Mg, and K/Na. These correlations are plotted in Figure 5.

To determine whether fiber width and elemental composition were related, we divided fibers into equal halves at the median width of 0.16 μm (Table 4). O and K were the two elements that exhibited the strongest fiber-width bias. O's negative correlation with width was significant ($p = 3.1 \times 10^{-6}$) and was probably caused by self-absorption of O's weak (250 eV) x-rays with increasing width. Potassium's positive correlation with width was less significant ($p = .047$) and the K/width relationship had only a slight slope (0.033). Hence, there appears to be no correlation between mineral species and fiber width for respirable fibers.

CONCLUSIONS

Currently, Libby 6-mix is being used in toxicological studies investigating asbestos-related diseases associated with the Libby population, which include cancers, pulmonary fibrosis, and possibly connective tissue/autoimmune diseases. In order to study the autoimmune phenomena that we have reported in the Libby population (Pfau et al., 2005), we have shown that tremolite asbestos given to mice intratracheally leads to the production of autoantibodies (Pfau et al., 2008). However, when we repeated this study using 6-mix, the mice showed no increase in autoantibody production above control levels (personal communication, J. C. Pfau) and only limited lung fibrosis compared to either tremolite or crocidolite (Putnam et al., 2008). Because we perform toxicological comparisons based on equal mass of fibers given to the mice, we were concerned that the material we were using did not accurately represent what would actually deposit in the lung during inhalation exposure. We undertook the current study in order to collect and characterize fibers from the Libby 6-mix that would be considered respirable. Ideally, the material collected could fall within defined parameters as respirable, would more closely resemble other amphibole preparations used in toxicology studies, and yet would not differ from the original 6-mix in chemical composition.

In this study, we describe the fractionation and characterization of respirable fibers from a known asbestos source that has generated large-scale pathogenic effects in exposed humans. Importantly, our study reveals that only 13% of the mass of 6-mix stock is in the respirable size fraction, suggesting that the majority of the material being used in animal and cell biology studies of 6-mix would normally be eliminated from the body by mucociliary action and coughing. It is uncertain, and beyond the scope of this article, how much of the larger material caught in the upper airways or transported to extrapulmonary sites would contribute to pathology, and at what exposure. Nevertheless, their inclusion in toxicology studies where the comparison is made against equal-mass fibrous preparations that do not contain a significant portion of this nonrespirable material makes interpreting the results extremely difficult. Elutriation was demonstrated to be effective in separating respirable fibers from larger fibers in the stock Libby 6-mix. Respirable fibers were harvested with mean fiber dimensions of 2.7 μm = length, 0.19 μm = width, and 16 = aspect ratio. Based on aspect ratio and surface area, the elutriated fibers more closely resemble other asbestiform fibers such as crocidolite. The data revealed how dramatically surface area increases per unit of mass when large, nonrespirable particles are exclude by elutriation.

Finally, the respirable Libby amphibole fibers showed no size-selective difference in their elemental composition. This is critical information from both a toxicological and a geological perspective. We are unaware of other studies that specifically analyzed whether composition affects fiber dimensions, so that this information would be of interest to geological studies, as well as efforts to discover nontoxic materials with asbestos-like properties. For toxicology, the data suggest that this aqueous elutriation method will allow future segregation of targeted aerodynamic diameters for toxicity studies of any asbestos fibers. For example, a two-stage elutriation could be run to create a suspension of fibers in the thoracic range (~2.5 to 10 μm) to help explore the earlier mentioned possible extrapulmonary pathogenic role of larger fibers eliminated in the first step of our elutriation. In this way, various fractions can be selected from a given mass sample to study cellular interactions with fibers, specific cellular receptors involved in signaling cell death or activation, or membrane perturbations by specifically defined fiber sizes. Studies such as these could lead to explanations for the various pathologies seen with asbestos exposures that may be dependent on fiber dimensions rather than fiber composition.

The ability to harvest the respirable fraction from the Libby 6-mix will enable investigators to conduct in vitro and animal studies that will more accurately assess potential health consequences of historical (and current) inhalation asbestos exposures to the Libby population. Finally, elutriation can be used to separate respirable fractions from any types of dust where the focus is on interactions with lung cells.

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