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Sumas Mountain Chrysotile Induces Greater Lung Fibrosis in Fischer 344 Rats Than Libby Amphibole, El Dorado Tremolite, and Ontario Ferroactinolite

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The physical properties of different types of asbestos may strongly affect health outcomes in exposed individuals. This study was designed to provide understanding of the comparative toxicity of naturally occurring asbestos (NOA) fibers including Libby amphibole (LA), Sumas Mountain chrysotile (SM), El Dorado tremolite (ED), and Ontario ferroactinolite (ON) cleavage fragments. Rat-respirable fractions ($PM_{2.5}$) were prepared by water elutriation. Surface area was greater for SM (64.1 m^2/g) than all other samples (range: 14.1–16.2 m^2/g), whereas mean lengths and aspect ratios (ARs) for LA and SM were comparable and greater than ED and ON. Samples were delivered via a single intratracheal (IT) instillation at doses of 0.5 and 1.5 mg/rat. One day post-IT instillation, low-dose NOA exposure resulted in a 3- to 4-fold increase in bronchoalveolar lavage fluid (BALF) cellularity compared with dispersion media (DM) controls, whereas high-dose exposure had a more severe effect on lung inflammation which varied by source. Although inducing less neutrophilic inflammation than ON and ED, exposure to either LA or SM resulted in a greater degree of acute lung injury. Three months post-IT instillation, most BALF parameters had returned to control levels, whereas the development of fibrosis persisted and was greatest in SM-exposed rats (SM > LA > ON > ED). These data demonstrate that fiber length and higher AR are directly correlated with the severity of fibrosis and that, in the rat, exposure to SM is more fibrogenic than LA which suggests that there may be cause for concern for people at risk of being exposed to NOA from the Sumas Mountain landslide.

Key Words: **Libby amphibole; El Dorado; Sumas Mountain; cleavage fragments; fibrosis.**

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Naturally occurring asbestos (NOA) is the general name given to asbestos minerals found in their natural state. This term is typically used in areas where asbestiform minerals are found, either in such low quantity that mining for commercial uses is not feasible or as contaminants of other minable materials. Asbestiform minerals tend to occur in metamorphic, igneous, and ultramafic rock terrains, which encompass ~30–40% of the contiguous United States (Lee *et al.*, 2008). Additionally, the Agency for Toxic Substances and Disease Registry (ATSDR, 2007) has reported overlap between the fastest growing counties of the United States and areas of known NOA deposits. Development of these areas may lead to potential airborne asbestos and result in environmental health hazards.

The potential public health issues related to exposure to NOA have gained the regulatory and media spotlight in recent years. Arguably the most well-known example is Libby, MT, the site of the largest vermiculite ore mine in the United States, which was operational from 1920 to 1990. The ore mined from this site contains a significant amount of amphibole asbestos, termed "Libby Amphibole" (LA). Occupational and environmental exposure to LA has been associated with a number of asbestos-related diseases including asbestosis, pleural disease, lung cancer, and mesothelioma in workers and residents of the immediate town and surrounding communities (ATSDR, 2002, 2003; McDonald *et al.*, 2004; Sullivan, 2007). Whereas a significant body of research has demonstrated the relative toxicity of LA, both epidemiologically (Larson *et al.*, 2010a,b, 2012a,b; Vinikoor *et al.*, 2010; Weill *et al.*, 2011; Whitehouse *et al.*, 2008) and in animal models (Cyphert *et al.*, 2012; Padilla-Carlin *et al.*, 2011; Putnam *et al.*, 2008; Shannahan *et al.*, 2012a,b; Smartt *et al.*, 2010), little is known about other sites of NOA within the United States that are not associated with mining

operations, such as El Dorado Hills, California and Whatcom County (Swift Creek/Sumas Mountain), Washington.

Although activity-based sampling studies by the U.S. Environmental Protection Agency (U.S. EPA) Regions 9 and 10 have provided valuable characterizations of the NOA present and the personal exposures that may occur in these areas, the relative toxicity of these NOA fibers is still poorly understood (Ecology and Environment, Inc., 2007; Ladd and Ecology and Environment, Inc., 2005; Wroble, 2010). This study was designed to provide understanding of the acute toxicological effects of El Dorado tremolite (ED) and Swift Creek/Sumas Mountain chrysotile (SM) in rats after a single intratracheal (IT) exposure of elutriated $PM_{2.5}$ fractions of site-specific NOA. LA was included as a positive control, as animal models of LA exposure have demonstrated acute inflammation and lung injury, as well as the development of fibrosis (Padilla-Carlin *et al*., 2011; Smartt *et al*., 2010). Although studies in our lab have shown LA to be less pathogenic than the control fiber RTI amosite (which has comparably longer fibers) (Cyphert *et al*., 2012; Padilla-Carlin *et al*., 2011), we have conducted additional studies that have shown that IT exposure to LA in rats results in similar toxicological outcomes (i.e., lung inflammation and fibrosis) as following exposure to the same mass dose of the control fiber UICC amosite (which has comparably equal fiber length) (unpublished data). We also tested the potential toxicity of ferroactinolite cleavage fragments from Ontario, Canada which are thought to be less biologically active (Gamble and Gibbs, 2008; Ilgren, 2004; Mossman, 2008). In addition to fiber characteristics, we evaluated markers of lung inflammation and injury, as well as histopathological evaluation of lung tissue inflammation and fibrosis 1 day and 3 months following a single IT exposure of site-specific NOA. We hypothesized that the fiber characteristics and toxicity of the two amphibole samples (LA and ED) would be similar, whereas the serpentine chrysotile (SM) would be more fibrogenic than the amphiboles, as has been previously suggested (Davis *et al*., 1985; Hirano *et al*., 1988).

METHODS

Asbestos. LA was collected from the Rainy Creek Complex located near Libby, Montana in 2007. Ontario ferroactinolite (ON) cleavage fragments were collected in 1983 from the Marmora Mine in Marmora, Ontario, 90 m north of Rochester, New York. Swift Creek/SM was collected in 2007 from the banks of Swift Creek just below the toe of the Sumas Mountain landslide due east of Everson, Washington. ED was collected in 2004 from the south shore of Flagstaff Hill along Folsom Lake near El Dorado Hills, California. All rock samples were sent to the U.S. Geological Survey (USGS, Denver, CO) for further processing into fine-grained materials by several methods including crushing with a pneumatic press, horizontal grinder, ball mill grinder, and/or mortar and pestle.

Water elutriation. Water elutriation was utilized to separate asbestos fibers with aerodynamic diameters smaller than 2.5 µm from larger fibers according to a settling velocity based upon the particle density and radius ($PM_{2.5}$) (Webber *et al*., 2008). Briefly, each sample was sonicated in deionized water for a total of 3 min (30-s bursts followed by 30-s rest intervals), and placed into the elutriation system. This system consists of a glass separatory funnel (500 ml; where the sample is placed), piston pump (Cole-Parmer Instrument Co., Vernon Hills, IL), and Tygon tubing (Saint-Gobain Performance Plastics Co., Valley Forge, PA). Deionized water was pumped through the system at a flow rate to produce an upward settling velocity of 3.4×10^{-4} cm/s, which captures respirable fibers (*i.e.,* aerodynamic diameter ≤ 2.5 µm) into a collection bottle. $PM_{2.5}$ fibers were collected by filtration onto a 0.22-µm pore-size nylon filter, dried for 48 h within a high-efficiency particulate air-filtered hood, peeled off the filter, and stored at room temperature in a sterile collection vial until time of study.

The SM sample was elutriated as described above with the exception that the elutriation was carried out in 0.1% aerosol OT surfactant (dioctyl sulfosuccinate, Sigma-Aldrich) in deionized water due to the hydrophobic nature of the chrysotile fibers. The collected fibers were washed thoroughly with filtered deionized water to remove the surfactant before the drying stage.

Characterization of elutriated asbestos preparations. Scanning electron microscopy (SEM) images were acquired for each elutriated sample (Fig. 1). Particle size distributions (length, width, and aspect ratio [AR]) were counted for at least 500 particles for each sample using transmission electron microscopy (TEM). A full description of each fiber is detailed in Supplemental Data Set 1. Briefly, the chemical and physical properties of all particles with a minimum length of 0.2 µm were analyzed. Mineral identification was obtained by a combination of energy dispersive x-ray spectroscopy and x-ray diffraction analysis using standard reference samples for comparison (EMSL Analytical, Inc., Libby, MT). Total surface area (TSA) of all particles was measured in duplicate by krypton gas absorption using Brunauer-Emmett-Teller (BET) theory (Micromeritics Analytical Services, Norcross, GA). Table 1 summarizes the characteristics of total particles in the sample. Table 2 summarizes the characteristics of those particles that are defined as a fiber, having an AR > 5:1.

Animals and experimental design. All procedures were approved by the Institutional Animal Care and Use Committee (U.S. EPA) and were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology. All animals used in this study were housed in an AAALAC-accredited, specific pathogen-free, facility (21 ± 1°C, 50 ± 5% relative humidity, and 12/12 h light/dark cycle). Healthy male Fischer 344 rats (Charles River Laboratories, Raleigh, NC), 6–8 weeks of age, were double housed in polycarbonate cages with beta-chip bedding and acclimatized for at least 2 weeks prior to use in this investigation (total $n = 144$ rats). All animals received standard Purina rat chow (Brentwood, MO) and water *ad libitum*. After acclimatization, the rats were randomized by body weight, and the weight at IT instillation was 251 ± 25 g (mean ± SD). The animals ($n = 8$ per group) were placed into nine groups: dispersion media (DM = 0.0 mg/rat), LA (0.5 mg/rat; 1.5 mg/rat), ON (0.5 mg/rat; 1.5 mg/rat), SM (0.5 mg/rat; 1.5 mg/rat), or ED (0.5 mg/rat; 1.5 mg/rat) for each necropsy time point (1 day and 3 months after instillation). The doses of asbestos were chosen based on preliminary experiments showing that LA from 0.15 to 5 mg/rat can induce dose-dependent lung injury and fibrosis similar to standard UICC amosite (unpublished data), as well as data presented by Hirano *et al*. (1988) using instilled chrysotile showing that a single instillation of 1.6 mg/rat is sufficient to induce both obstructive pulmonary disease and fibrosis.

IT instillation. To ensure more uniform distribution of asbestos throughout the lung, asbestos suspensions were prepared daily in DM as previously described (Porter *et al*., 2008). DM consisted of 5.5mM D-glucose (Sigma-Aldrich, St Louis, MO), 0.6 mg/ml rat serum albumin (Sigma-Aldrich), and 0.01 mg/ml 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Sigma-Aldrich) in Ca^{2+}/Mg^{2+}-free PBS (Sigma-Aldrich). Samples were prepared daily and sonicated for 5 min, and then shaken by hand prior to each IT instillation. Animals were anesthetized with isoflurane (IsoFlo, Abbott Laboratories, Abbott Park, IL) prior to IT instillation. The IT procedure was conducted as previously described (Driscoll *et al*., 2000). The dosing was performed in a biological safety cabinet with laminar flow. Animals received an IT instillation of either DM (control; 0.0 mg/rat), LA (0.5 mg/rat; 1.5 mg/rat), ON (0.5 mg/rat; 1.5 mg/

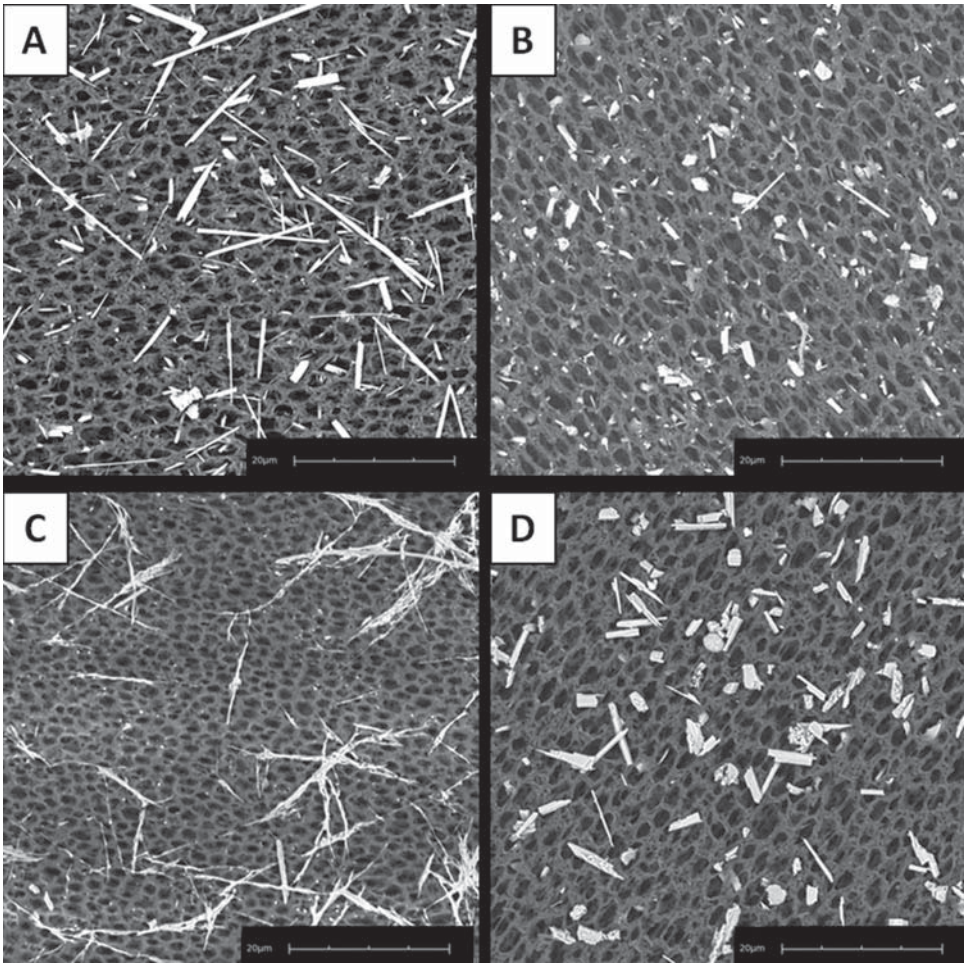

FIG. 1. SEM images of NOA preparations following size fractionation by water elutriation. (A) LA, (B) ON cleavage fragments, (C) SM, and (D) ED. Scale bars are 20 µm; magnification was ×4050.

rat), SM (0.5 mg/rat; 1.5 mg/rat), or ED (0.5 mg/rat; 1.5 mg/rat) in a total volume of 250 µl.

Bronchoalveolar lavage collection. Bronchoalveolar lavage fluid (BALF) was collected as described previously (Cyphert *et al*., 2012). Briefly, the trachea was cannulated, the left lung was ligated, and the right lung was lavaged using a volume representing 60% of total lung capacity (35 ml/kg). Three washes were conducted using the same buffer aliquot.

BALF analysis. Total cell counts from BALF were determined using a Z1 Coulter Counter (Coulter, Inc., Miami, FL). An aliquot was centrifuged at 600 rpm for 3 min (Shandon 3 Cytospin, Shandon, Pittsburg, PA) to prepare cell differential slides. Slides were dried at room temperature, stained with Leukostat (Thermo Fisher Scientific Co., Waltham, MA) and examined under light microscopy to determine numbers of alveolar macrophages, neutrophils, eosinophils, and lymphocytes. At least 300 cells were counted from each animal to determine percentage of cell types. The remaining BALF was centrifuged at 1500 × g for 10 min at 4°C, and the supernatant fluid was analyzed for markers of lung injury, including protein content (Coomassie plus Protein Assay Kit, Pierce, Rockford, IL), albumin (DiaSorin, Stillwater, MN), lactate dehydrogenase (LDH) activity levels (Thermo Trace Ltd., Melbourne, Australia), N-acetyl glucosaminidase (NAG) activity (Roche Diagnostics, Indianapolis, IN), and γ-glutamyl transferase (GGT) activity (Thermo Trace Ltd.). All assays were run using commercial kits with only slight modifications for use on the Konelab Arena 30 clinical analyzer (Thermo Chemical Lab Systems, Espoo, Finland).

Histopathology. The left lung was removed and inflation-fixed by IT instillation of 10% formalin (Z-Fix, Anatech Ltd., Battle Creek, MI) and embedded in paraffin. Longitudinal slices, 5 µm thick, were cut (cranial and caudal to the hilus of the left lung) and stained with hematoxylin and eosin or Masson's trichrome for collagen accumulation. All lung sections were examined by a certified veterinary pathologist by light microscopy. The European Society of Toxicologic Pathology International Harmonization of Nomenclature and Diagnostic (IN-HAND) criteria classification method was used to evaluate the histopathological lesions (www.goreni.org). Semiquantitative scoring was conducted by evaluating a variety of pathological indices using severity scores (0 = not present, 1 = minimal [< 10% of examined area], 2 = mild [11–40%], 3 = moderate [41–80%]; and 4 = marked [81–100%]).

Statistical analysis. Statistical analysis was performed using Prism 4 (GraphPad Software). Comparisons of the mean were made by Student's *t*-test or ANOVA followed by Tukey-Kramer's honestly significant difference or Dunnett's *post hoc* test as necessary. Data are shown as mean ± SE. Differences with $p < 0.05$ were considered statistically significant.

TABLE 1

Parameters of Elutriated ($PM_{2.5}$) LA, ON, SM, and ED Particles Were Determined Using TEM Analysis. TSA Was Measured by Krypton Gas Adsorption Using BET Theory

Property	$LA_{2.5}$	$ON_{2.5}$	$SM_{2.5}$	$ED_{2.5}$
Total objects counted (*n*)	532	516	502	507
Total particles/mg ($\times 10^7$)	86	463	19	1331
TSA (m^2/g) ± SD	14.11 ± 1.0	16.23 ± 0.02	64.08 ± 0.06	15.53 ± 0.01
Length (μm)				
Mean ± SD	1.9 ± 3.0	1.1 ± 0.9	2.0 ± 2.4	0.9 ± 0.9
Median	0.8	0.8	1.2	0.6
Range	0.2–27.3	0.2–9.4	0.2–17.5	0.2–6.4
Width (μm)				
Mean ± SD	0.39 ± 0.3	0.40 ± 0.3	0.31 ± 0.4	0.42 ± 0.4
Median	0.30	0.30	0.20	0.26
Range	0.07–3.0	0.08–2.7	0.05–3.5	0.06–2.5
AR (μm)				
Mean ± SD	6.4 ± 11.7	3.1 ± 2.7	12.1 ± 16.4	2.6 ± 2.2
Median	2.0	2.2	7.1	1.9
Range	1.0–109.2	1.0–20.5	1.0–157.5	1.0–22.2

TABLE 2

Parameters of a Subset of Elutriated ($PM_{2.5}$) LA, ON, SM, and ED Particles Defined as Fibers (AR ≥ 5:1) Were Determined Using TEM Analysis

Property	$LA_{2.5}$	$ON_{2.5}$	$SM_{2.5}$	$ED_{2.5}$
Total objects counted (*n*)	138	71	302	48
Percentage of sample defined as "fibers"	26	14	60	9
Length (μm)				
Mean ± SD	5.0 ± 4.5	1.9 ± 1.5	2.7 ± 2.8	1.9 ± 1.4
Median	3.6	1.5	1.6	1.4
Range	0.5–27.3	0.5–9.4	0.5–17.5	0.4–6.4
Width (μm)				
Mean ± SD	0.29 ± 0.19	0.24 ± 0.3	0.18 ± 0.5	0.26 ± 0.4
Median	0.23	0.24	0.10	0.22
Range	0.07–1.15	0.2–2.7	0.1–3.5	0.2–2.5
AR (μm)				
Mean ± SD	19.5 ± 17.3	8.4 ± 2.7	18.8 ± 16.3	7.8 ± 2.2
Median	14.3	7.5	12.5	6.8
Range	5.0–109.2	5.0–20.5	5.0–157.5	5.0–22.2

RESULTS

Characterization of Size-Fractionated NOA Samples

Prior to characterization, samples were size-fractionated by water elutriation to yield a sample representative of "rat respirable" ($PM_{2.5}$) particles as described previously (Duncan *et al.*, 2010; Padilla-Carlin *et al.*, 2011). SEM images of elutriated samples (Fig. 1) show a visible difference in particle size distribution between NOA samples, specifically showing longer, thinner fibers in the LA sample (Fig. 1A) compared with the other amphibole samples (Figs. 1B and D) and the serpentine nature of the chrysotile sample (Fig. 1C). Table 1 summarizes the length, width, and AR values for total particles in each size-fractionated sample as determined by TEM analysis, in addition to the TSA of each sample measured by gas absorption and BET analysis, whereas Table 2 describes the subset of total particles that were defined as fibers (i.e., particles having an AR > 5:1); additionally, a detailed description of each individual particle analysis is available in Supplementary Data Set 1. Examination of the distribution data shows comparable length, width, AR, and TSA between the ON cleavage fragments and ED samples, suggesting that the ED sample consists of primarily short, thick particles. Conversely, the LA and SM samples consist on average of longer particles and thus greater ARs. Although the mean lengths of all LA and SM particles are similar, LA fibers were on average longer than SM fibers, whereas SM particles and fibers were comparably thinner, resulting in similar mean AR between the two samples. In addition, the TSA of the LA sample was equivalent to the ON and ED samples, whereas the surface area of the SM was ~4-fold greater than all other samples. Finally, using the data acquired from TEM analysis, the total number of particles per milligram of sample was determined to be vastly different between samples (Table 1). The total number of particles per milligram of samples appears to decrease with increasing mean length and AR, with ~1331×10^7, 463×10^7, 86×10^7 and 19×10^7 particles/mg for ED, ON, LA, and SM, respectively.

Acute Inflammatory Response

The acute inflammatory response to NOA was assessed in BALF 1 day and 3 months following instillation (Fig. 2, left panel and right panel, respectively). One day after IT instillation, total BALF cell numbers were significantly elevated in all rats exposed to either low- or high-dose NOA (data not shown). Low-dose NOA exposure resulted in a 3- to 4-fold increase in BALF cellularity compared with DM controls regardless of the sample type, whereas high-dose exposure had a more severe effect on lung inflammation which varied by source. Surprisingly, exposure to high-dose cleavage fragment-like ON and ED resulted in the greatest number of total cells, 9-fold greater than DM, whereas high-dose LA and SM resulted in only 4-fold and 7-fold increases, respectively. Generally, inflammation consisted mostly of neutrophils (Fig. 2B) with some macrophages (Fig. 2A) and very few eosinophils and lymphocytes; however, instillation of SM resulted in significantly increased macrophage, eosinophil, and lymphocyte recruitment compared with other NOA, although these represented only 26, 3, and 1%, respectively, of total cells (eosinophil and lymphocyte data not shown). Three months post-IT instillation, total cell numbers were reduced to control levels in most groups, but remained slightly, yet significantly elevated (1.5-fold) in the high-dose LA, ON, and ED groups. This elevation in total cells was due to persistent elevation of neutrophils in the BALF (Fig. 2B) compared with controls,

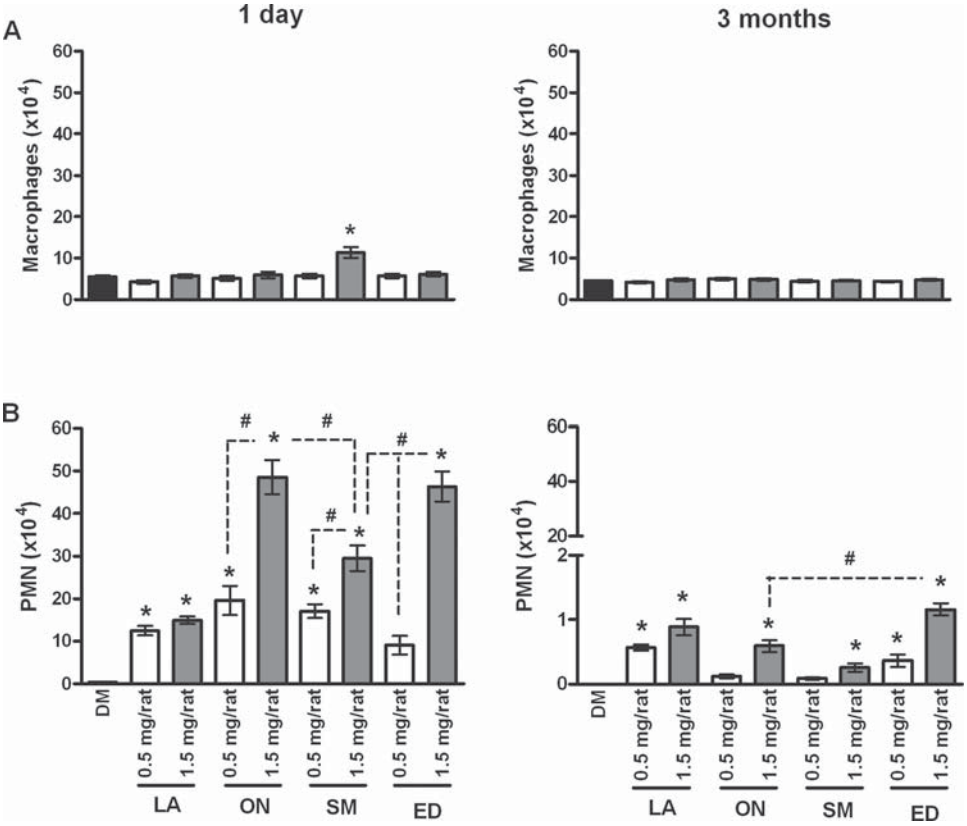

FIG. 2. Total macrophages (A) and neutrophils (B) were counted in BALF 1 day (left panel) and 3 months (right panel) after a single IT instillation of NOA (results show means ± SE of cells/ml BALF). At 1 day postexposure inflammation consisted mostly of neutrophils, while by 3 months the majority of cells were macrophages, although neutrophils numbers remained significantly increased compared with controls in most groups. $^{*}p < 0.05$ compared with DM; $^{\#}p < 0.05$ compared with indicated group(s); $n = 8$ rats/group.

as macrophages (Fig. 2A), eosinophils, and lymphocytes had returned to control levels in all groups by this time (some data not shown).

Different patterns of response to NOA were exhibited with respect to biomarkers of lung injury, which were also assessed in BALF at these time points (Fig. 3, left panel and right panel, respectively). At 1 day post-exposure, although markers of macrophage lysosomal activation (NAG, Fig. 3A), membrane damage (GGT, Fig. 3B), cellular toxicity (LDH, Fig. 3C), and epithelial permeability/lung damage (albumin and total protein, Figs. 3D and E) were elevated in almost all groups (excluding low-dose ED), these levels were greatest in rats exposed to LA and SM. Most notably, markers of epithelial permeability and lung damage in high-dose SM rats were increased by 21-fold compared with DM controls and by 4-fold compared with high-dose LA (Figs. 3D and E). With all other markers, the patterns of LA and SM responses were similar, whereas the patterns of ON and ED were lower and comparable to each other. By 3 months post-IT instillation, lung injury markers had returned to control levels in most groups, although they remained slightly elevated in rats exposed to both low- and high-dose LA (Figs. 3B–E) and in some cases in high-dose SM and ED groups (Figs. 3B, D, and E).

Histopathology

The histopathological response to NOA exposure was assessed in the left lung lobe 1 day and 3 months after a single IT exposure (Table 3). All lesions observed following asbestos exposure were localized to the centriacinar region of the lungs. One day after IT instillation (Supplemental fig. 1), no abnormalities were detected in DM control animals, as expected. However, exposure to low-dose LA, ON, and ED resulted in minimal (grade 1) intra-alveolar accumulation of mixed neutrophils and macrophages associated with minimal alveolar hyperplasia, whereas high-dose exposure resulted in mild (grade 2) intra-alveolar accumulation of mixed neutrophils and macrophages and alveolar hyperplasia. Both doses of SM resulted in mild (grade 2) intra-alveolar accumulation of neutrophils and macrophages accompanied with mild (grade 2) alveolar hyperplasia. Furthermore, high-dose SM resulted in moderate (grade 3) alveolar duct accumulation of macrophages and neutrophils. In addition to the above lesions, unstained fiber-like structures were noted within macrophages and occasionally present free within the alveolar space in LA, ON, and ED exposure groups. Conversely, SM exposure was associated with uniquely extracellular presence of elongated, relatively thick, amorphous foreign material possibly

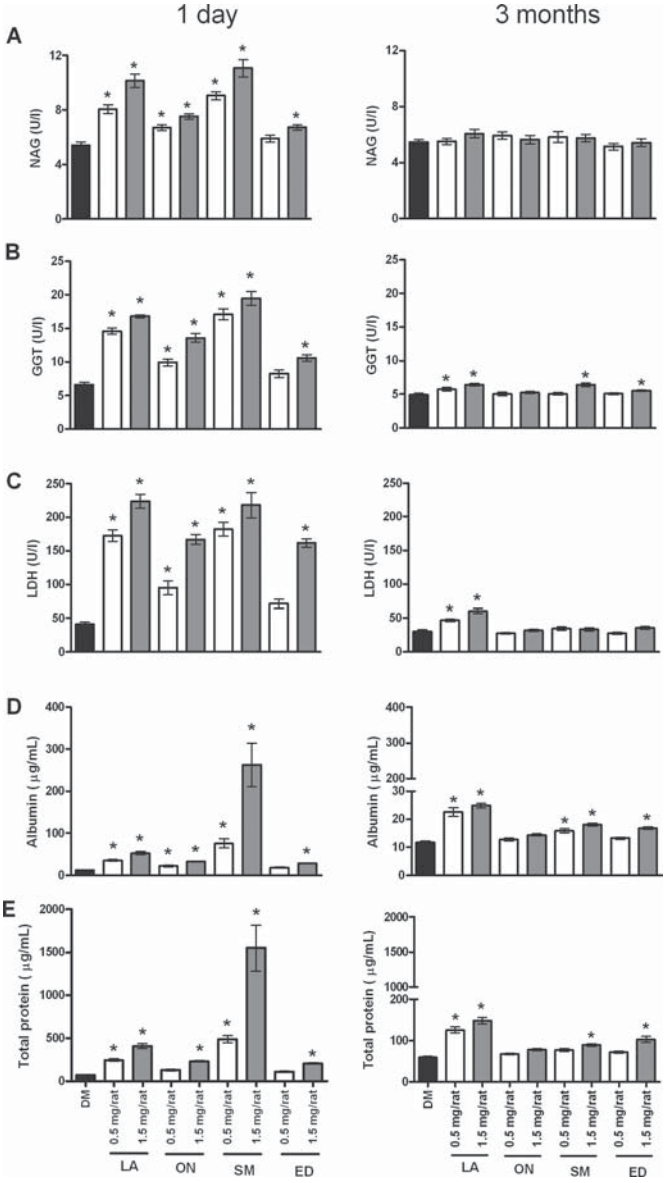

FIG. 3. Biomarkers of lung injury were measured in BALF 1 day (left panel) and 3 months (right panel) after a single IT instillation of NOA. Markers of macrophage lysosomal activation (NAG, A), membrane damage (GGT, B), cellular toxicity (LDH, C), and epithelial permeability/lung damage (total protein and albumin, D and E, respectively) were significantly increased in most groups 1 day post-IT instillation compared with DM controls and were approaching or at control levels by 3 months post-IT instillation. *$p < 0.05$ compared with DM; $n = 8$ rats/group.

representing agglomeration of chrysotile fibers. Staining with Masson's trichrome did not reveal any accumulation of collagen in any exposure group 1 day after IT instillation (data not shown).

Three months after IT instillation (Supplemental fig. 2), DM instillation had no histopathological effect in the lung tissue. Instillation of LA resulted in intra-alveolar accumulation of macrophages associated with alveolar epithelial hyperplasia, interstitial granulomatous inflammation (consisting of mixtures of macrophages, lymphocytes, and multinucleated giant cells), and fibrosis that was minimal (grade 1) after low-dose exposure and mild (grade 2) after high-dose exposure. Few histopathological changes were seen 3 months following ON or ED instillation, mainly dose-dependent intra-alveolar accumulation of macrophages, although high-dose ON exposure was also associated with minimal (grade 1) fibrosis (Fig. 4E). Conversely, the greatest histopathological changes were noted following exposure to SM, which resulted in mild to moderate (grade 2–3) dose-dependent interstitial granulomatous inflammation and interstitial fibrosis (Supplemental figs. 2 and 4F and G). Similar to 1 day postexposure, unstained fiber-like structures were found within macrophages of rats exposed to LA, ON, and ED. Extracellular fiber-like structures were also noted after low-dose SM exposure, whereas in the high-dose group the extracellular structures appeared to be possible agglomerations of fibers. In addition, bronchus-associated lymphoid tissue (BALT) aggregation of macrophages was also noted after LA, ON, or ED exposure (Supplemental fig. 3), but not after SM exposure, indicating active transport of amphibole particles to lymphoid tissues following exposure to amphiboles and suggesting reduced clearance of chrysotile fibers from the lung.

DISCUSSION

It is well known that workers involved in the extraction and/or processing of asbestos or materials contaminated with asbestiform minerals are at serious risk of disease and that this risk is shared by their families, due to take-home contamination, as well as local residents, due to environmental exposure (Linton *et al.*, forthcoming). The town of Libby, Montana is a paradigm of this health risk: mining and processing of amphibole-contaminated vermiculite has resulted in asbestos-related disease and death from both occupational and para-occupational exposures (miners and their family members) as well as from nonoccupational exposures in residents of the surrounding communities (ATSDR, 2002, 2003; Hart *et al.*, 2009; Larson *et al.*, 2010a, 2012a,b; McDonald *et al.*, 2004; Whitehouse *et al.*, 2008). Despite the evidence of significant risk from exposure to "environmental asbestos," (i.e., pollution from mines affecting residents not associated with occupational exposure), it is still unclear if NOA in the United States presents the same risks of exposure, as it is difficult to separate out the potential for exposure and disease from the presence of asbestos in the soil and rocks in the absence of mining or milling processes. However, there are several countries that have reported elevated asbestos-related disease, such as pleural plaques, asbestosis, lung cancer, and mesothelioma in the absence of asbestos mining in or near the town (Baris *et al.*, 1988a,b; Harper, 2008; Luo *et al.*, 2003; McConnochie *et al.*, 1987; Metintas *et al.*, 1999a,b, forthcoming; Murai *et al.*, 1997). In many of these countries, town residents developed

TABLE 3

Incidence Table of Pathological Changes 1 Day or 3 Months After a Single IT Exposure to LA, ON Cleavage Fragments, SM, or ED. Values Represent the Number of Observations in Each Group With the Average Histopathological Score Within Parentheses (n = 8 Per Group)

	Time	Dose	A-PMNL	A-M	AD-PMNL	AD-M	AH	IG	BU	LGC	IF
DM	1 day	—	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	3 months	—	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	1 day	Low	8 (1.0)	8 (1.0)	0 (0.0)	0 (0.0)	8 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
LA		High	8 (2.0)	8 (2.0)	8 (2.0)	8 (2.0)	8 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	3 months	Low	0 (0.0)	8 (1.0)	0 (0.0)	0 (0.0)	8 (1.0)	8 (1.0)	0 (0.0)	7 (0.9)	8 (1.0)
		High	0 (0.0)	8 (2.0)	0 (0.0)	0 (0.0)	8 (1.0)	8 (1.0)	0 (0.0)	7 (0.9)	8 (1.4)
	1 day	Low	7 (0.9)	7 (0.9)	0 (0.0)	0 (0.0)	7 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ON		High	8 (2.0)	8 (2.0)	0 (0.0)	0 (0.0)	8 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	3 months	Low	0 (0.0)	8 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (0.8)	0 (0.0)
		High	0 (0.0)	7 (1.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (1.0)
	1 day	Low	8 (2.0)	8 (2.0)	8 (2.0)	8 (2.0)	8 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
SM		High	8 (2.0)	8 (2.0)	8 (3.0)	8 (3.0)	8 (2.0)	0 (0.0)	8 (2.0)	0 (0.0)	0 (0.0)
	3 months	Low	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (1.0)	0 (0.0)	0 (0.0)	8 (1.6)
		High	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (1.0)	8 (3.0)	0 (0.0)	0 (0.0)	8 (3.0)
	1 day	Low	8 (1.0)	8 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ED		High	8 (2.0)	8 (2.0)	0 (0.0)	0 (0.0)	8 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	3 months	Low	0 (0.0)	8 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
		High	0 (0.0)	7 (1.6)	0 (0.0)	0 (0.0)	7 (0.9)	7 (0.9)	0 (0.0)	4 (0.5)	0 (0.0)

Notes. A-PMNL = intra-alveolar polymorphonuclear leukocyte infiltration; A-M = intra-alveolar accumulation of macrophages; AD-PMNL= intra-alveolar duct PMNL infiltration; AD-M = intra-alveolar duct accumulation of macrophages; AH = alveolar epithelial hyperplasia; IG = interstitial granulomatous inflammation (*i.e.*, interstitial presence of mononuclear cells, macrophages and multinucleated giant cells); BU = bronchi-epithelial ulcer; LGC = presence of multinucleated giant cells in peribronchial lymphoid tissues; IF = interstitial fibrosis.

asbestos-related disease by simply being exposed to road dust that contained NOA or using contaminated soil/clay as building materials. These same, or similar, exposure scenarios could be occurring in counties within the United States where NOA is present.

Although currently no epidemiological data has indicated asbestos-related disease in populations from El Dorado Hills, U.S. EPA Region 9 has conducted activity-based air sampling to assess exposures to NOA in response to concerns posed by the community (Ladd and Ecology and Environment, Inc., 2005). The EPA activity-based sampling tests simulated activities that could create airborne dust including outdoor sports, playground activities, running or biking on nature trails, and gardening. The study reported asbestos fibers in air samples collected during activity-based testing in greater quantity than levels present in ambient air samples, indicating personal exposure levels were significantly higher during these activities. However, a later assessment by RJ Lee Group, Inc. (2006a,b) reported that the materials identified by the EPA and its contract laboratories were not asbestos, but were amphibole cleavage fragments and therefore should not be considered a major health risk. In response to this, the USGS sampled multiple sites within El Dorado Hills and confirmed the presence of asbestos fibers (Meeker *et al*., 2006). Regardless of the ongoing debate concerning the nature of minerals in the area (RJ Lee Group, Inc., 2005; U.S. EPA, 2006), a health consultation recently released by ATSDR (2011) concluded that (1) breathing in NOA in the El Dorado Hills area, over a lifetime, has the potential to harm people's health and (2) reducing exposures to NOA will protect people's health and is warranted in El Dorado County based on estimates of past exposures.

In contrast to the El Dorado samples analyzed in the above studies, the average raw ED elongated particle was thicker which consequently resulted in many being removed during water elutriation. The resulting $PM_{2.5}$ ED sample, consisting of only 9% fibers by our standard, contained primarily particles and fibers less than 2 µm in length that had comparably thicker widths and smaller AR than the other samples. Although we had originally hypothesized that ED would be similar in toxicity to LA, ED exposure at both the low and high doses resulted in very few pathological changes, despite containing the greatest amount of particles/mg and causing significant acute inflammation. In fact, exposure to ED appeared to elicit less of a histopathological response than ON cleavage fragments. However, the lack of toxicity of the ED can most likely be contributed to the very low percentage of long, thin fibers in the sample. This is consistent with historical data showing that the toxicity and carcinogenicity of tremolite is increased with increasing fiber length (Davis *et al*., 1985; Wagner *et al*., 1982). The bulk ED sample collected for this study was recovered near Folsom Lake, ~3–5 mi from the center of the El Dorado Hills community (Meeker *et al*., 2006); therefore, this sample may not be representative of the amphiboles found within El Dorado Hills and more studies using multiple samples would be needed before a definitive conclusion could be reached.

Similar to the studies conducted in El Dorado Hills, U.S. EPA Region 10 has conducted several activity-based sampling assessments of personal exposure to NOA from sediment

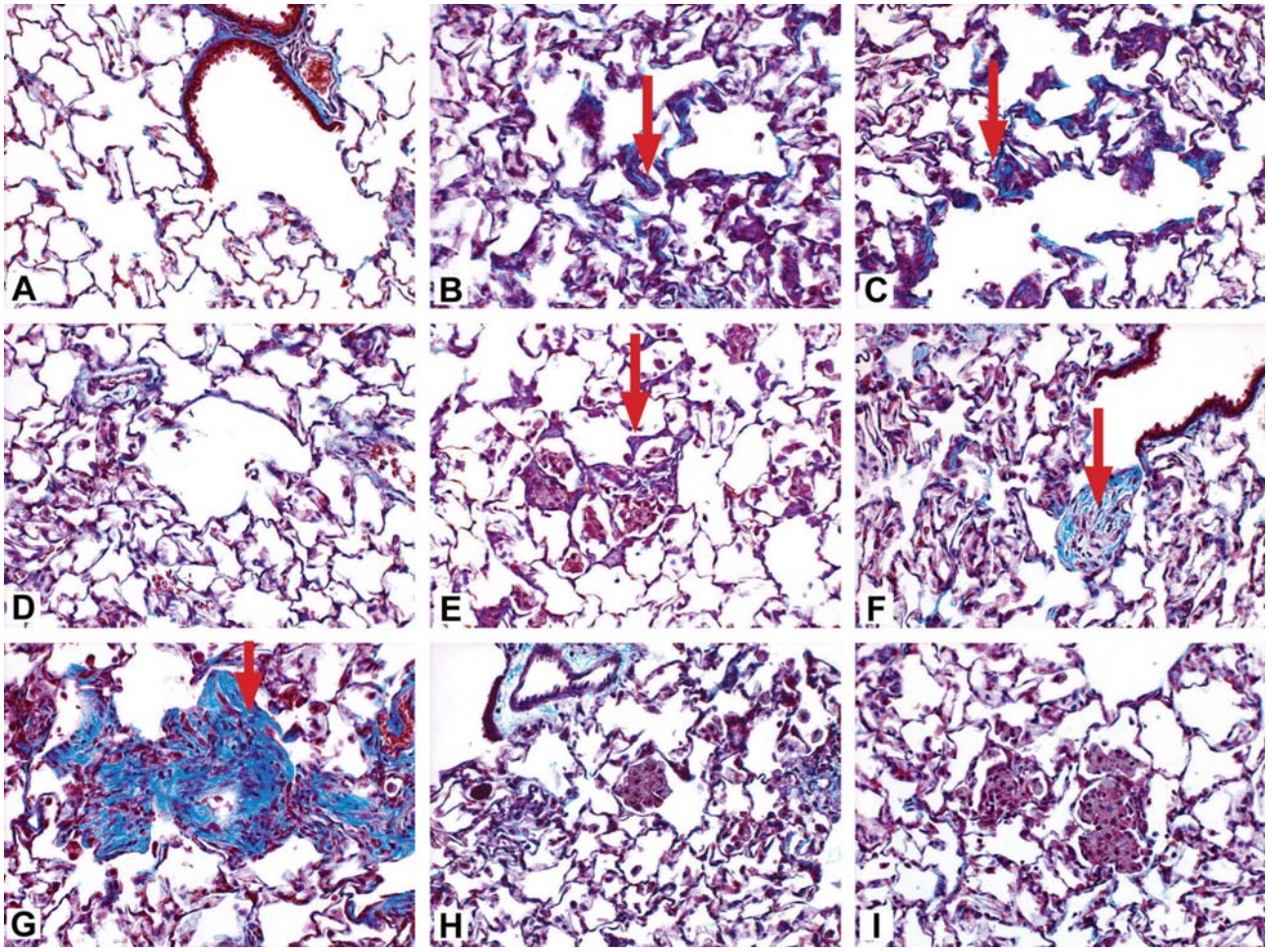

FIG. 4. Masson's trichrome staining of the left lung 3 months after a single IT exposure to DM (A), LA (0.5 and 1.5 mg/rat; B and C), ON cleavage fragments (0.5 and 1.5 mg/rat; D and E), SM (0.5 and 1.5 mg/rat; F and G), or ED (0.5 and 1.5 mg/rat; H and I). A dose-dependent increase in collagen accumulation was seen in all groups with the exception of rats exposed to ED, which did not develop fibrosis.

dredged from Swift Creek (deposits from the Sumas Mountain landslide), as well as from residential and agricultural properties in Whatcom County, Washington near the towns of Everson and Nooksack (Ecology and Environment, Inc., 2007; Wroble, 2010). Both studies showed that soil and sediment samples contained significant amounts of chrysotile asbestos and that disturbances of the materials could result in elevated airborne concentrations of fibers that could lead to elevated risk of exposure greater than what is considered acceptable under Federal guidelines. Additionally, a health consultation released by ATSDR (2008) concluded that (1) a public health hazard exists for people conducting activities regularly on dredge piles and (2) an indeterminate public health hazard exists for people who might be exposed at off-site locations; although they also determined that there was "no evidence of elevated asbestos related disease rates (i.e., mesothelioma and lung cancer) in the community near Swift Creek compared to Whatcom County or the state of Washington as a whole" at that time.

Although some chrysotile samples have been shown to be less biopersistent than amphiboles (Bernstein *et al*., 2010, 2011; Wagner *et al*., 1974), studies have shown chrysotile to be more fibrogenic than some amphiboles (i.e., amosite and crocidolite) (Davis *et al*., 1978; Hirano *et al*., 1988; Wagner *et al*., 1974). Davis *et al*. (1978) attributed this effect to the greater percentage of long fibers present in chrysotile compared with amphiboles. The same basic conclusion can be suggested from this study. Total particles in the elutriated SM sample were comparatively longer and thinner than the other amphibole samples, and consisted of ~60% fibers with an AR ≥5:1. Despite having more than 4-fold fewer particles/mg, exposure to SM resulted in a greater degree of pulmonary fibrosis than LA at the same mass dose. This effect might also be attributed to the high surface area of the serpentine asbestos compared with the amphibole samples; however, surface area of the amphibole samples was equivalent and does not explain the greater degree of lung injury and fibrosis in LA-exposed animals compared with those exposed to ED. Although the toxicity of SM is most likely

due to fiber length, it is also possible that despite the historic literature SM was more biopersistent than the amphiboles assessed in this study, as BALT aggregation of macrophages was only seen in lungs exposed to amphiboles. Although this suggests trafficking and removal of amphibole particles from the lungs, biopersistence was not directly assessed in this study. Additionally, possible agglomerations of chrysotile fibers were noted histologically (Supplemental fig. 1), which may have influenced the pathological outcome.

This is the first published animal study to describe the toxicity and pathogenicity of NOA collected from El Dorado Hills and the Sumas Mountain landslide. In rats, a single IT instillation of SM resulted in development of fibrosis that was greater than that induced by LA, whereas exposure to ED resulted in little to no biological effects 3 months after exposure and surprisingly appeared to be even less pathogenic than ON cleavage fragments (SM > LA > ON > ED). It has been proposed that the biological effects of asbestos are associated with production of reactive oxygen species and cellular damage that can lead to apoptosis, inflammatory cytokine release, and growth factor expression which result in inflammation and the development of fibrosis (Kamp and Weitzman, 1999; Sanchez *et al*., 2009). Although exposure to all asbestos samples induced elevation of acute lung injury biomarkers and resulted in significant inflammation compared with vehicle controls, the levels of these biomarkers did not necessarily predict the longer term severity of disease. Markers of lung injury were greatest after exposure to LA or SM compared with ON and ED; however, these responses were equal between LA- and SM-exposed rats at 1 day and greatest in LA-exposed rats at 3 months despite SM being more fibrogenic 3 months after exposure. In addition, total inflammation in BALF was actually greatest acutely after exposure to ON cleavage fragments and short, thick ED particles even though these particles resulted in minimal pathological effects 3 months after exposure. The average particle length and AR (Table 1) seemed to correlate well with the development of fibrosis as the rank order of all three was SM > LA > ON > ED. Although the average length of defined fibers was greater in the LA sample (Table 2), the SM sample contained 34% more fibers comparatively, which could account for its increased potency. These data support the long standing dogma that the proportion of long, thin fibers in an exposure is the primary determinant of adverse health outcomes (Stanton *et al*., 1981) and suggests that some early biomarkers might not be indicative of enduring toxicity or pathogenesis.

Although we can conclude based on the data presented here that our sample of ED is relatively inactive and will most likely not result in adverse health outcomes, we must stress that this study represents only one sample collected from a vast area of tremolite-contaminated soil and rock, and was obtained from an area 3–5 mi outside the town center. It is quite possible that other areas within El Dorado Hills contain a higher percentage of long, thin fibers that may be more pathogenic and examination of multiple samples throughout the community is warranted (Meeker *et al*., 2006). Conversely, chrysotile collected from the source of the contamination (the Sumas Mountain landslide) was shown to be potently fibrogenic. Soil and rock from the mountain landslide enter into Swift Creek and can be transported and deposited as sediments all the way from the source and continue into Canada via Swift Creek and the Sumas River, suggesting that there may be cause for concern for people at risk of being exposed to dust from the mountain as well as from river sediments. Collectively these studies show that environmental exposure to NOA in the absence of mining has the potential for adverse health effects and highlights the need for further study of more sites within the United States where NOA is present.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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