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Long-Term Toxicity of Naturally Occurring Asbestos in Male Fischer 344 Rats

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ABSTRACT

Naturally occurring asbestos (NOA) fibers are found in geologic deposits that may be disturbed by mining, earthworks, or natural processes, resulting in adverse health risks to exposed individuals. The toxicities of Libby amphibole and NOA samples including Sumas Mountain chrysotile (SM), El Dorado tremolite (ED), and Ontario ferroactinolite cleavage fragments (ON) were compared in male Fischer 344 (F344) rats 15 mo after exposure. Rat-respirable fractions of LA and SM displayed greater mean lengths and aspect ratios than ED and ON. After a single intratracheal (IT) instillation (0.5 or 1.5 mg/rat), persistent changes in ventilatory parameters and a significant increase in lung resistance at baseline and after methacholine aerosol dosing were found only in rats exposed to 1.5 mg SM. High-dose ED significantly elevated bronchoalveolar lavage lactate dehydrogenase (LDH) activity and protein levels, while high-dose SM increased γ -glutamyl transferase and LDH activities. A moderate degree of lung interstitial fibrosis after exposure to 1.5 mg SM persisted 15 mo after exposure, unchanged from previous findings at 3 mo. LA induced mild fibrosis, while ED and ON produced minimal and no apparent fibrosis, respectively. Bronchioloalveolar carcinoma was observed 15 mo after exposure to LA or ED. Data demonstrated that SM, given by bolus IT dosing on an equivalent mass basis, induced greater pulmonary function deficits, airway hyperresponsiveness, and interstitial fibrosis than other NOA, although unlike LA and ED, no apparent evidence for carcinogenicity was found. All NOA samples except ON cleavage fragments produced some degree of long-term toxicity.

ARTICLE HISTORY

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Exposure to airborne asbestos fibers is associated with increased rates of asbestosis, lung cancer, and mesothelioma (Baumann et al. 2015; McDonald, 1985; O'Reilly et al., 2007). Naturally occurring asbestos (NOA) refers to asbestos found in its natural state in rocks and soil, and may also include fibrous minerals that do not fit the regulatory definition of asbestos (Harper, 2008; Wylie and Candela, 2015). Disturbance of NOA in geological formations by erosion or human activities such as construction, farming, or recreational activities increases airborne fiber concentrations and risk of adverse health effects (Case et al., 2011; Wylie and Candela, 2015). Accordingly, the relative toxicity of NOA fibers is of great concern to potentially impacted communities. In Libby, MT, the site of the largest vermiculite mine in

the United States, high proportions of fibrous amphiboles were present in the mined ore (Meeker et al., 2003). A comprehensive review of this Libby amphibole (LA) in support of risk assessment documented several noncancer and cancer health effects of LA, including lung parenchymal and pleural changes, reduced lung function, asbestosis, lung cancer, and mesothelioma (U.S. Environmental Protection Agency [EPA], 2014). Recent *in vivo* toxicology studies assessed the health effects of LA relative to other forms of asbestos (Cyphert et al., 2012b, 2015; Padilla-Carlin et al., 2011; Kodavanti et al., 2014).

An ongoing landslide in the upper watershed of Swift Creek on Sumas Mountain in Whatcom County, Washington may deposit up to 150,000 cubic yards of sediment annually into Swift Creek

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and Sumas River (Whatcom County Public Works [WCPW], 2012). Analyses of creek and river bank sediments and upland soils from flooded areas showed high levels of chrysotile asbestos (2–27%; U.S. EPA, 2009). Activity-based sampling (such as moving deposits, shoveling, or light recreation) indicated that some activities generated enough fibers at certain locations to exceed the U.S. EPA acceptable level for excess lifetime cancer risk (U.S. EPA, 2011), suggesting that the Sumas Mountain NOA poses potential adverse health risks for residents in the area. However, age-adjusted rates of lung and bronchial cancer, mesothelioma, and asbestosis in Whatcom County and the Swift Creek/Sumas River drainage area were not higher than for the state of Washington as a whole (Vander Kelen and Patrick, 2013). Insufficient exposures of area residents to SM chrysotile material, the relatively small populations in the Swift Creek/Sumas River drainage area, or long disease latency period after exposure may all contribute to the lack of reported adverse health effects to date.

NOA in the form of tremolite and actinolite fibers in western El Dorado County, California, has been a significant concern since asbestos deposits were uncovered during construction of homes and schools in the community of El Dorado Hills (Raloff, 2006). Activity-based air sampling including simulated activities such as cycling, running, and baseball in El Dorado Hills showed elevated levels of asbestos in breathing zone heights for children and adults (U.S. EPA, 2005). Concern over El Dorado County NOA led to new state regulations on proposed property development and evaluation of NOA at school sites (Van Gosen and Clinkenbeard, 2011). NOA may also include cleavage fragments, which are generated by patterned breakage during the crushing or grinding of nonfibrous amphibole material, generally have lower aspect ratios, and do not meet the definition of asbestiform fibers (Harper, 2008). Nevertheless, acicular (needle-like) or prismatic crystal structures of cleavage fragment may be of size and shape similar to asbestiform fibers, and their toxicity is disputed (Harper, 2008).

Given the potential risks of exposure, residents have expressed increasing concerns about NOA in their communities, and regional offices of the U.S. Environmental Protection Agency have addressed

these issues. To support risk assessment in U.S. EPA Regions, the comparative acute and long-term toxicological effects of LA and site-specific NOA have been investigated. Cyphert et al. (2012a) recently examined the toxicity of LA and several site-specific NOA following acute intratracheal (IT) exposures in rats. Lung inflammation and pathology were examined 1 d and 3 mo after exposure to samples of LA, Sumas Mountain chrysotile (SM), El Dorado tremolite (ED), and a ferroactinolite cleavage fragment sample from Ontario, Canada (ON). Data demonstrated that asbestos samples with high proportions of particles or shorter fibers (i.e., the ED and ON samples) contribute significantly to acute inflammation, while samples with longer fibers and greater aspect ratios (i.e., the LA and SM samples) were correlated with fibrosis 3 mo after exposure. Given the latency (>20 yr) period associated with development of asbestos-related diseases, assessment of longer term effects in the rat model may further inform site-specific risk assessment efforts. The purposes of this investigation were to evaluate toxicological effects up to 15 mo following a single IT exposure of the site-specific NOA used in our previous study, and to examine whether the observed short-term effects are predictive of chronic effects. Markers of lung inflammation and injury, lung and pleural tissue histopathology, incidences of tumor formation, and evaluation of pulmonary function were used to provide a comprehensive assessment of comparative toxicity.

Methods

Asbestos samples

Rock samples of Libby amphibole (LA, collected in 2007), Ontario ferroactinolite (ON, collected in 1983), Sumas Mountain chrysotile (SM, collected in 2007), and El Dorado tremolite (ED, collected in 2004) were processed by the U.S. Geological Survey (USGS, Denver, CO) into fine-grain materials. The samples were further processed at the U.S. EPA (Research Triangle Park, NC) using water elutriation (Webber et al., 2008) to obtain particles and fibers in the respirable range for rodents as determined by sedimentation settling velocity, equivalent to a median aerodynamic diameter of

Table 1. Key Physical Characteristics of NOA Samples.

Sample	All structures					Fibers (aspect ratio ≥ 5)		
	Mean length (μm)	Maximum length (μm)	Mean aspect ratio	Surface area (m^2/g)	Structures/mg ($\times 10^7$)	Mean length (μm)	Mean aspect ratio	Fibers/mg ($\times 10^7$)
LA	1.9	27.3	6.4	14.1	86	5.0	19.5	22
ON	1.1	9.4	3.1	16.2	463	1.9	8.4	64
SM	2.0	17.5	12.1	64.1	19	2.7	18.8	12
ED	0.9	6.4	2.6	15.5	1331	1.9	7.8	126

Note. Further details are found in Cyphert et al. (2012a).

2.5 μm or less. Details of the water elutriation technique were previously described (Cyphert et al., 2012a; Padilla-Carlin et al., 2011). At least 500 structures per sample were counted using transmission electron microscopy, and structures were identified using energy-dispersive x-ray spectroscopy and x-ray diffraction, and total surface area of all particles was measured by Kr gas absorption. Key physical dimensions of all structures and a subset of structures defined as fibers (aspect ratio ≥ 5) are shown in Table 1, and further details of physical and chemical properties of LA, SM, ON, and ED including bivariate size distributions were previously published (Cyphert et al., 2012a).

Animals and experimental design

The Institutional Animal Care and Use Committee of the U.S. EPA approved all procedures in this study. Male Fischer 344 (F344) rats (Charles River Laboratories, Raleigh, NC), 6–8 wk old, were double housed in polycarbonate cages with beta-chip bedding, and acclimatized for 2 wk prior to the study in an AAALAC-accredited, specific-pathogen-free facility ($21 \pm 1^\circ\text{C}$, $50 \pm 5\%$ relative humidity, 12/12-h light/dark cycle). Animals received standard Purina rat chow (Brentwood, MO) and water ad libitum. After acclimatization, rats were randomized by body weight, and average group weight at IT instillation was 231 ± 3 g (mean \pm SEM). The animals ($n = 24$ per group) were placed into 9 groups: control dispersion media (DM = 0 mg/rat), LA (0.5 or 1.5 mg/rat), ON (0.5 or 1.5 mg/rat), SM (0.5 or 1.5 mg/rat), or ED (0.5 or 1.5 mg/rat). DM, used to provide uniform distribution of asbestos in suspensions and facilitate consistent distribution in the lung (Porter et al., 2008), consisted of 5.5 mM 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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) (Sigma-Aldrich). Rat-respirable asbestos suspensions were prepared using DM and delivered via IT instillation as previously described (Cyphert et al., 2012a). Animals were necropsied 15 mo post IT to avoid excess natural spontaneous early deaths due to mononuclear cell leukemia in F344 rats.

Lung function measurements

Noninvasive baseline pulmonary parameters were measured in unrestrained rats in a subset of each exposure group ($n = 8$) by barometric whole-body plethysmography (WBP) (Buxco Research Systems, Wilmington, NC) at 1 wk and 3, 9, and 15 mo postexposure. Rats were placed in whole-body chambers, and several respiratory parameters, including frequency, minute volume, and enhanced pause (P_{enh}), were measured continuously for 5 min. WBP measurements were taken on successive days at each time point and averaged for each rat, and group data represent the averaged values.

At 15 mo postexposure, invasive pulmonary mechanics parameters were assessed on the same animals ($n = 8/\text{group}$) used for WBP already described (Cohen and Larson, 2005). Rats were anesthetized with urethane (1 g/kg), tracheotomized, and mechanically ventilated at a rate of 150 breaths/min, tidal volume of 10 ml/kg, and positive end-expiratory pressure of 3 cm H_2O with a computer-controlled small-animal ventilator (Flexivent, Scireq, Montreal, Canada). Once ventilated, rats were paralyzed with 0.8 mg/kg pancuronium bromide and allowed to equilibrate on the ventilator for 5 min or until spontaneous breathing ceased. Lung resistance (R_L), elastance (E_L),

and dynamic compliance (C_{dyn}) were determined by transducing airway pressure and airflow using a precision-controlled piston during a single inspiration and expiration over a period of 1 s (“snapshot” maneuver). Three separate baseline snapshot maneuvers were taken, and then increasing doses of methacholine (MCh) in sterile saline (6.25, 12.5, and 25 mg/ml) were nebulized for 10 s into the airways, and snapshot maneuvers were performed (12 each) beginning 15 s after each nebulization. Acceptable values (goodness of fit >90% for snapshot values) were averaged for the baseline and each of the three MCh doses for each rat.

Bronchoalveolar lavage fluid (BALF) collection and analysis

Right lung lobes were lavaged three times with a single aliquot of Ca^{2+}/Mg^{2+} -free PBS, using a volume representing 60% of total lung capacity (35 ml/kg). Total cell counts were determined using a Z1 Coulter Counter (Coulter, Inc., Miami, FL). Slides of bronchoalveolar lavage fluid (BALF) cells were stained with Leukostat (Thermo Fisher Scientific Co., Waltham, MA) and differentiated for macrophages, neutrophils, lymphocytes, and eosinophils (300 cells/sample). Cell-free BALF supernatant was analyzed for markers of lung injury, including protein (Coomassie Plus Protein Assay Kit, Pierce, Rockford, IL), albumin (DiaSorin, Stillwater, MN), lactate dehydrogenase (LDH) activity (Thermo Trace Ltd., Melbourne Australia), *N*-acetyl glucosaminidase (NAG) activity (Roche Diagnostics, Indianapolis, IN), and γ -glutamyl transferase activity (GGT) (Thermo Trace Ltd.), as described previously (Cyphert et al., 2012a).

Histopathology

The left lung was removed and inflation-fixed by IT instillation of 10% formalin (Z-Fix, Anatech Ltd., Battle Creek, MI). In addition, the heart, aorta, pericardium, thymus, diaphragm, and lower rib cage (including the parietal pleura) were removed and fixed in formalin, and all tissues were embedded in paraffin. The diaphragm and parietal pleura of the rib cage were examined in order to determine levels of fibrosis and evidence

for cancer or mesothelioma formation. Tissues were sectioned at 5 μ m thickness (lung tissues were cut longitudinally cranial and caudal to the hilus of the left lung), and stained with hematoxylin and eosin (H&E). Lung sections were also stained with Masson’s trichrome for analysis of collagen accumulation. All tissue sections, including those from rats that died before scheduled necropsies, were examined by a certified veterinary pathologist (AN) by light microscopy. Images were prepared by whole slide scanning of selected slides with representative lesions using an Aperio scanner (Vista, CA). Slides were scanned at 40 \times magnification, and from these scans, representative photos were taken. Severity of pathology was scored in a blinded manner using the following scale: 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 analysis of variance (ANOVA) followed by Tukey-Kramer’s HSD or Dunnett’s post hoc test as necessary. Data are shown as mean \pm SE. Differences with $p < .05$ were considered statistically significant.

Results

Respiratory parameters and airway responsiveness

Changes in respiratory parameters were measured in unanesthetized freely breathing rats at multiple times after IT dosing. At 1 wk post IT exposure, minute volume was significantly increased in the high-dose (1.5 mg) SM group compared with the DM control (Figure 1A). The significant rise in minute volume in the high-dose SM group persisted at 3, 9, and 15 mo postexposure. Elevated minute volume in the high-dose SM group was accompanied by increased inspiratory and expiratory flows (data not shown). After exposure to any of the other high dose NOA samples, no significant changes in minute volumes were observed. Plethysmography-derived respiratory parameters

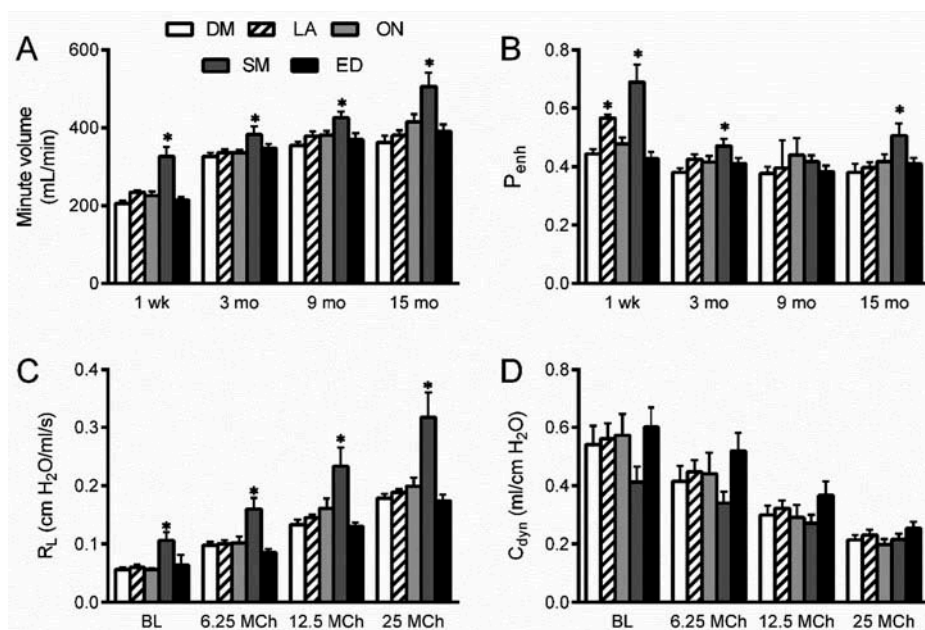


Figure 1. (A, B). Baseline lung function parameters determined by whole-body plethysmography at 1 wk and 3, 9, and 15 mo following exposure of rats to high-dose (1.5 mg/rat) NOA or vehicle (DM). (C, D). Airway responsiveness to MCh aerosol challenge evaluated in mechanically ventilated rats 15 mo after exposure to high-dose NOA or DM. Data show lung resistance (R_L) and dynamic compliance (C_{dyn}) at baseline and after aerosolization of 6.25, 12.5, and 25 mg/ml MCh. Significance: * $p < .05$ compared to DM; $n = 8$ per group.

in low-dose (0.5 mg) groups were not markedly different from DM controls at any time (data not shown). Enhanced pause (P_{enh}), a derived parameter often correlating with lung resistance (Hamelmann et al., 1997), was significantly elevated 1 wk after exposure to high-dose LA and SM (Figure 1B). No persistent elevation in P_{enh} was observed 3 mo or later after LA, but SM produced significant increases in this parameter at 3 and 15 mo postexposure.

At the termination of the study, pulmonary mechanics assessment in anesthetized ventilated rats revealed a significant rise in lung resistance at baseline and following all doses of MCh aerosol challenge in rats exposed to 1.5 mg SM compared to the DM control (Figure 1C), while other groups displayed no marked change in R_L . Rats exposed to low doses of NOA demonstrated no significant changes in any pulmonary mechanics parameters (data not shown). The high-dose SM-exposed group showed a nonsignificant decrease in baseline dynamic compliance, and no marked differences in C_{dyn} were seen at any dose of MCh aerosol (Figure 1D). No significant differences were observed in lung elastance at baseline or after MCh challenge (data not shown).

Survival and assessment of lung inflammation and injury

Following exposure to LA, SM, ED, ON, or DM, health status was observed up to 15 mo after exposure. Body weights at final necropsy did not differ significantly among treatment groups (Table 2). Most treatment groups had some deaths prior to the scheduled 15-mo necropsy, ranging from 0 to 4 of 24 rats per group (83–100% survival), but analysis of survival days after exposure showed no relationship to dose or asbestos type (data not shown). Causes of early death included spontaneous mononuclear cell leukemia, a common cancer in F344 rats (Haseman et al., 1998).

Fifteen months after exposure to NOA, few changes were observed in total BALF cell numbers (Figure 2A). Exposure to 1.5 mg ED resulted in higher numbers of total BALF cells, macrophages, and neutrophils; however, these changes did not achieve statistical significance (Figures 2A–2C). Exposure to 0.5 mg ED or either dose of the other NOA samples produced cell numbers comparable to DM control. Few lymphocytes or eosinophils were found after NOA exposure (<3% of

Table 2. Body Weight at Necropsy, Survival to 15 mo, and Incidence Table of Pathological Changes 15 mo (or at Time of Early Death) After a Single IT Exposure to DM or 0.5 or 1.5 mg/rat LA, ON, SM, or ED.

	Dose (mg/rat)	Body weight (g)	Survival to 15 mo	A-M	AH	IG	IF	LGC	DF	Tumor
DM	0	479 ± 4	24/24	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0
LA	0.5	459 ± 7	23/24	23 (1.0)	23 (1.0)	2 (0.1)	14 (0.6)	3 (0.1)	0 (0.0)	1 (C) ^a
LA	1.5	457 ± 6	24/24	24 (1.0)	24 (1.1)	23 (1.0)	24 (2.0)	13 (0.8)	2 (0.1)	0
ON	0.5	484 ± 7	21/24	15 (0.7)	13 (0.6)	0 (0.0)	2 (0.1)	1 (0.0)	0 (0.0)	0
ON	1.5	457 ± 6	22/24	23 (1.4)	23 (1.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0
SM	0.5	479 ± 5	21/24	20 (0.9)	20 (0.9)	21 (0.9)	21 (1.7)	1 (0.0)	0 (0.0)	0
SM	1.5	469 ± 7	22/24	24 (1.5)	24 (1.1)	24 (2.0)	24 (3.0)	0 (0.0)	0 (0.0)	0
ED	0.5	477 ± 7	21/24	20 (0.9)	19 (0.8)	6 (0.3)	0 (0.0)	9 (0.4)	1 (0.0)	1 (C) ^b
ED	1.5	455 ± 9	20/24	22 (1.0)	22 (1.0)	22 (1.9)	22 (1.0)	22 (1.9)	1 (0.0)	0

Note. Body weight data represent means and SE at necropsy, 15 mo post instillation. Survival data represents number surviving to 15 mo/total *n* exposed. Values in pathological indices columns represent the number of animals with observed lesions in each group with the average histopathological score in parentheses for all animals. A-M = intra-alveolar accumulation of macrophages; AH = alveolar epithelial hyperplasia; IG = interstitial granulomatous inflammation (i.e., interstitial presence of mononuclear cells, macrophages and multinucleated giant cells); IF = interstitial fibrosis (lung); LGC = presence of multinucleated giant cells in peribronchial lymphoid tissues; DF = diaphragm fibrosis.

^aBronchioloalveolar carcinoma (C) observed which had metastasized to pericardium, diaphragm, and thymus.

^bBronchioloalveolar carcinoma observed without metastasis.

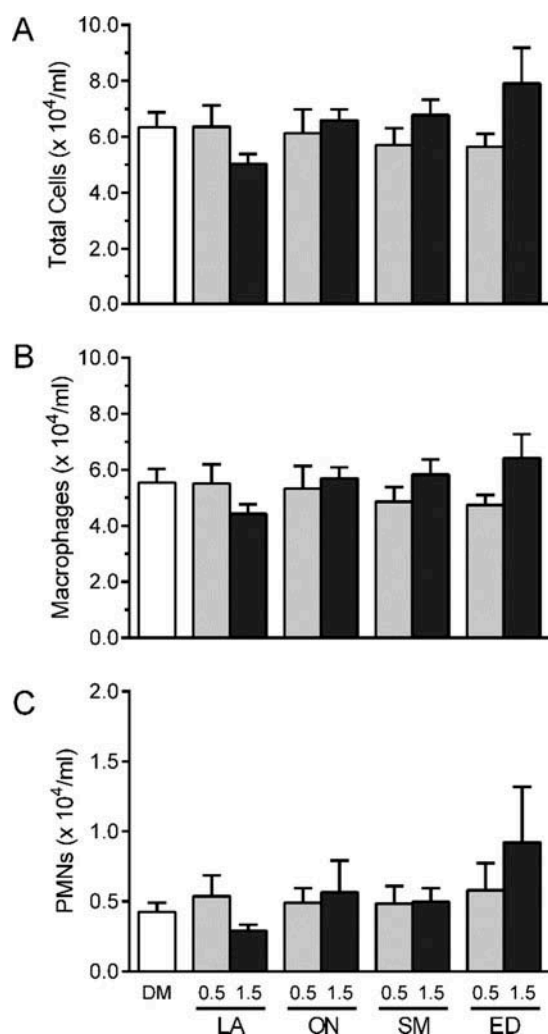


Figure 2. Total cells (A), macrophages (B), and neutrophils (C) in BAL 15 mo post IT instillation of DM or LA, ON, SM, or ED (0.5 or 1.5 mg/rat). At 15 mo post IT, BAL cellularity was not significantly different in exposed groups compared with control DM (*n* = 20–24 per group).

total cells), and these were not significantly different among groups (data not shown).

Biochemical indicators of acute lung injury were also examined in BALF supernatant 15 mo after exposure to the site-specific NOA. Activity levels of GGT and LDH (markers of membrane damage and cytotoxicity, respectively) were significantly elevated in the high-dose (1.5 mg) SM group (Figure 3). Markers of epithelial permeability (albumin [not shown] and total protein) and cellular toxicity (LDH) were significantly increased 15 mo after exposure to high dose ED compared to DM. NAG activity levels within the treatment groups were comparable to those for DM (data not shown).

Histopathology

Fifteen months after dosing of rats with NOA, various histopathological lesions were observed in lungs of animals (Table 2). Intra-alveolar macrophage accumulation and alveolar epithelial hyperplasia, common responses to many particle or fiber exposures, were comparable after exposure to all NOA, and the extent of reaction generally correlated with sample dose. These changes were located at the centriacinar regions (i.e., surrounding the terminal bronchioles), and asbestos-like fibers were observed within macrophages. In contrast, the interstitial fibrotic reaction and development of associated interstitial fibrotic granulomas varied markedly by sample type. Interstitial granulomatous inflammation was comparable between

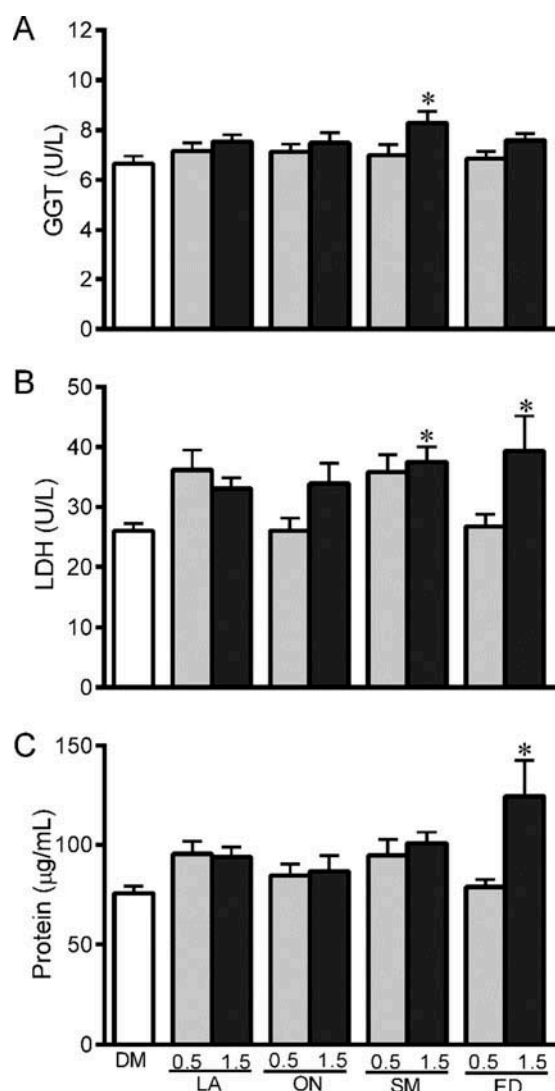


Figure 3. Biomarkers of lung injury measured in BAL 15 mo after a single IT of NOA. Markers of membrane damage (GGT, A), cellular toxicity (LDH, B), and epithelial permeability/lung damage (total protein, C) were significantly different than controls 15 mo after exposure to high-dose SM (GGT, LDH) or ON (LDH, protein). Significance: * $p < .05$ compared to DM control; $n = 20$ –24 per group.

high-dose SM and ED (grade 2), lower with LA (grade 1), and absent with ON. Exposure to SM resulted in the greatest degree of interstitial lung fibrosis at both doses (Figure 4F and 4G), with high-dose SM producing a moderate (grade 3) fibrotic response (Table 2). Reduced average responses were seen for LA (Figures 4B and 4C) and ED (Figures 4H and 4I), with average grades of 2 and 1, respectively, at the high dose, while ON cleavage fragment sample did not induce any apparent fibrotic reaction (Figures 4D and 4E). Development of dose-related aggregates of

multinucleated giant cells located within the peribronchial lymphoid tissue (BALT) occurred in LA and ED groups, but virtually no lymphoid giant cells were observed in ON or SM groups (Table 2).

A single bronchioloalveolar carcinoma in the peribronchial region of the lungs that had metastasized to the pericardium, diaphragm, and thymus was found after exposure to 0.5 mg LA (Figure 5A). Two cases of minimal focal fibrosis in the diaphragm were observed after exposure to 1.5 mg LA (Figure 5B and Table 2). Similarly, a single bronchioloalveolar carcinoma in the perivascular region of the lungs was detected after exposure to 0.5 mg ED (Figure 5C) along with focal fibrosis and subchronic inflammation in the diaphragm, and 1 case of focal fibrosis and subchronic inflammation in the diaphragm was observed after exposure to 1.5 mg ED (Figure 5D and Table 2). No apparent evidence for mesothelial proliferation was noted in any group. No lung tumors were detected in the DM control or after exposure to SM or ON. In the hearts of both control and treated animals, minimal to mild multifocal fibrotic lesions, without any suggested dose-related incidence, were observed. Such lesions are known to occur spontaneously in aged F344 rats. No other lesions were observed in the diaphragm, parietal pleura of the ribs, thymus, aorta, and pericardium.

Discussion

Decrements in lung function, asbestosis, mesothelioma, and lung cancer are established long-term health effects of asbestos (Miles et al., 2008; Mossman et al. 2011; O'Reilly et al., 2007; Weiss, 2000; Wilken et al., 2011). The most significant example of asbestos exposure from a public health perspective in the United States is from the former vermiculite mine in Libby, MT (i.e., LA), which is the subject of the greatest number of exposure and health effects studies. The noncancer health effects of LA include lung parenchymal changes (asbestosis), pleural changes (localized or diffuse pleural thickening), respiratory symptoms, inflammatory reactions, and reduced lung function (U.S. EPA, 2014; Kodavanti et al., 2014). Localized pleural thickening of the parietal pleura along the chest wall or diaphragm appears to be the most sensitive

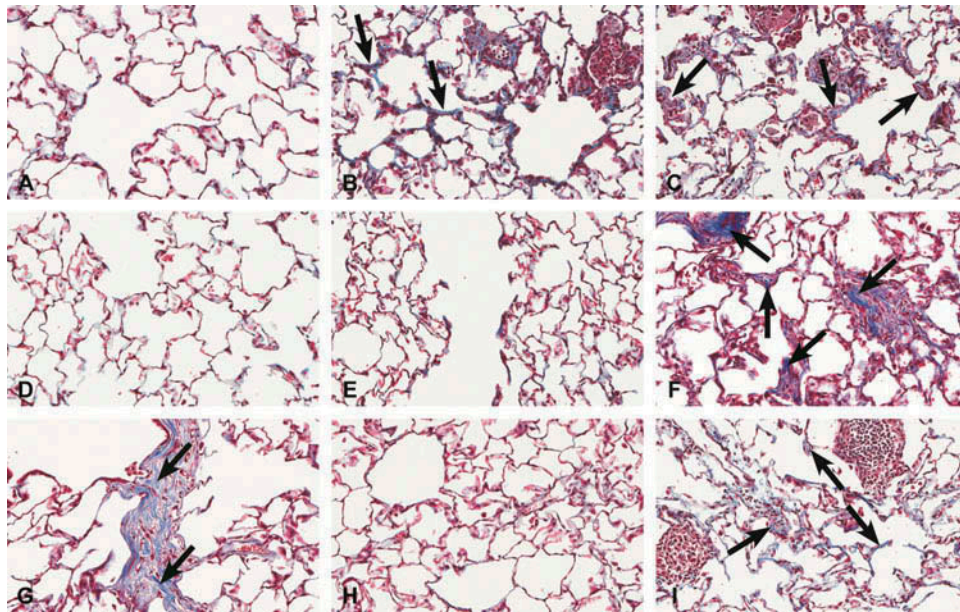


Figure 4. Masson's trichrome staining of the left lung 15 mo after a single IT exposure to DM (A), LA (0.5 and 1.5 mg/rat; B, C), ON cleavage fragments (0.5 and 1.5 mg/rat; D, E), SM (0.5 and 1.5 mg/rat; F, G), or ED (0.5 and 1.5 mg/rat; H, I). A dose-dependent increase in collagen accumulation (arrows) was seen in all groups with the exception of rats exposed to ON cleavage fragments, which did not develop fibrosis. Magnification 20 \times .

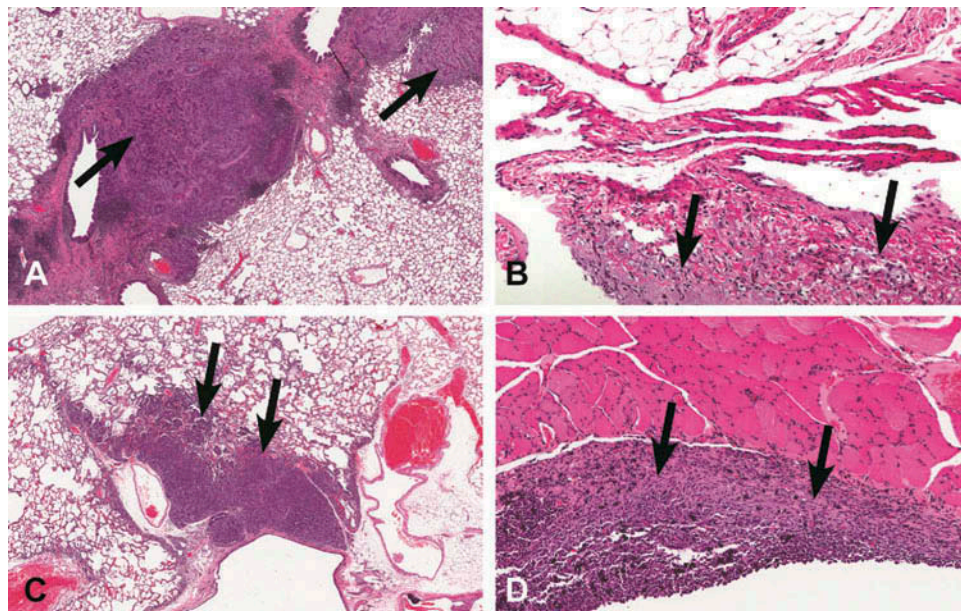


Figure 5. Hematoxylin and eosin staining of the left lung (A, C) and diaphragm (B, D) 15 mo following exposure to LA (A, B) or ED (C, D). (A) Metastatic bronchioloalveolar carcinoma (arrows) after exposure to 0.5 mg LA. (B) Fibrosis in the diaphragm (arrows) after exposure to 1.5 mg LA. (C) Bronchioloalveolar carcinoma (arrows) after exposure to 0.5 mg ED. (D) Fibrosis and subchronic inflammation in the diaphragm (arrows) after exposure to 1.5 mg ED. Magnification 2 \times (A, C) and 8 \times (B, D).

indicator of asbestos exposure in humans, and is found after exposures to low levels of LA (Christensen et al., 2013). Lung function in Libby workers with localized pleural thickening was decreased in comparison to those without

localized pleural thickening (Clark et al., 2014; Lockey et al., 2015). Pleural thickening and lung fibrosis are clearly associated with significant decrements in lung function in asbestos-exposed workers (Wilken et al., 2011).

In our study, rats did not display a significant reduction in minute volume or airway hyperresponsiveness (AHR) to MCh aerosol challenge after exposure to LA, although a plethysmography index of ventilatory changes that often correlates with airway resistance (Penh) was significantly increased 1 wk after exposure. In contrast, high-dose SM chrysotile exposure significantly elevated minute volume and MCh AHR within 1 wk of treatment, which continued for at least 15 mo. The observed ventilatory responses with SM exposure correlated with the greatest degree of lung interstitial fibrosis among tested samples, indicating an increased lung physiological dead space not participating in gas exchange. Despite the observation that localized pleural thickening is the most sensitive indicator of asbestos exposure in humans, no changes in parietal pleura of the ribs were found after exposure to any sample, and only a few cases of minimal fibrosis of the diaphragm were observed 15 mo after IT exposure to LA or ED. Such changes may require longer times after exposure to develop and potentially contribute to decrements in lung function. SM and LA fibers had comparable lengths, aspect ratios, and fiber/mass, although the surface area of SM was fourfold greater than that of LA or other NOA samples (Table 1; Cyphert et al., 2012a). Fiber surface area may be a key physical determinant of lung cellular toxicity (Duncan et al., 2014). Despite the overall similarity in physical dimensions, SM exposure produced greater changes than LA in pulmonary function (minute volume, Penh, and lung resistance) on an equivalent mass basis over the duration of this study.

Bronchioloalveolar carcinomas are lesions with a noninvasive growth pattern in which there is no evidence of stroma, vascular, or lymphatic invasion (Ebbert et al. 2010; Brambilla et al., 2001), and represent about 4% of all non-small-cell lung carcinomas (Raz et al., 2006). Previously, Churg and Wiggs (1984) indicated that amphibole fibers may be more carcinogenic than chrysotile fibers. In this study, low-dose LA (0.5 mg) induced a single bronchioloalveolar carcinoma 15 mo after exposure. Cyphert et al. (2015) showed that LA also produces mesotheliomas 20 mo after IT exposure, at doses of 0.15 or 5 mg given once or in multiple doses over a 13-wk period. The fact that carcinoma

is observed 15 mo after exposure to the lower dose (0.5 mg) of LA further informs the adverse health risks of this amphibole, despite the lack of a clear dose-response relationship for LA-induced carcinoma.

Sumas Mountain chrysotile induced significant granulomatous inflammation as well as interstitial fibrosis and markers of membrane damage and cytotoxicity (GGT and LDH activity levels) 15 mo after exposure, and these lesions were unchanged from those found 3 mo after dosing (Cyphert et al., 2012a). Prior reports support a greater fibrogenic potential of chrysotile compared with amphibole fibers (Davis et al., 1978; Hirano et al., 1988; Wagner et al., 1974), even when controlled for fiber number. Although no apparent carcinomas were detected in SM-exposed rats, other investigators noted increased incidence of carcinomas after chronic inhalation of chrysotile (Wagner et al., 2008; Davis et al., 1978). Our data showing that SM chrysotile produces persistent lung inflammation, restrictive lung function changes, and more severe long-term lung fibrosis compared with LA, ED, and cleavage fragment ON samples suggest that continued exposure and health monitoring in areas of SM deposits are strongly advised.

A comprehensive analysis of amphibole material from a number of locations in El Dorado Hills, CA, by the USGS showed that it consisted of tremolite, actinolite, and magnesiohornblende, with mainly fibrous or prismatic morphologies, and cleavage fragments were not considered to be a significant component (Meeker et al., 2006). Our ED sample was collected about 3–5 miles from the center of sampling by USGS in El Dorado Hills, and about 58% of all ED particles analyzed were tremolite, although 88% of fibers (aspect ratio ≥ 5) were tremolite, and no actinolite or magnesiohornblende was detected (Cyphert et al., 2012a). The ED sample (Table 1; Cyphert et al., 2012a) had shorter fibers with lower aspect ratios than the USGS samples (Meeker et al., 2006). Water elutriation used to obtain the rat-respirable ED sample is likely to remove large or long fibers, possibly contributing to the differing characteristics found with the USGS sample analysis. Despite the low prevalence of longer fibers, ED induced neutrophil recruitment to the lung 1 d and 3 mo post-

exposure (Cyphert et al., 2012a), and neutrophils were higher (though not significantly) 15 mo post-exposure, suggesting persistent particulate-induced inflammation. Further, markers of cytotoxicity and lung injury (LDH, albumin and protein) were significantly elevated at 15 mo, and a case of bronchioloalveolar carcinoma was found after low-dose (0.5 mg) ED exposure. These results indicate the potential of significant chronic toxicity of ED material. Further studies of amphiboles from El Dorado County need to be conducted in order to gain a more accurate understanding of potential health impacts of this NOA.

The toxicity of cleavage fragments having dimensions equivalent to asbestiform fibers is uncertain (Harper, 2008; U.S. EPA, 2003). The lung cancer risk in workers exposed to cleavage fragments was equivocal, leading to a determination that the occupational recommended exposure limit of 0.1 f/cm^3 for asbestiform fibers also would apply to elongated mineral particles (including cleavage fragments), as long as they are derived from certain amphibole minerals such as tremolite and ferroactinolite, and meet the definition of a fiber with an aspect ratio of 3:1 or greater and length greater than $5 \mu\text{m}$ (National Institute for Occupational Safety and Health [NIOSH], 2011). However, few lab studies support significant biological effects of cleavage fragments. Unlike asbestiform crocidolite and chrysotile fibers, riebeckite and antigorite cleavage fragments do not activate signaling pathways or genes involved in carcinogenesis (Mossman, 2008). The ON sample used in this study was a nearly pure sample of ferroactinolite cleavage fragments, which was previously demonstrated to induce acute inflammation 1 d after exposure (Cyphert et al., 2012a), a prototypical response to a predominantly particulate sample (with fewer longer fibers) that may be cleared relatively rapidly from the lung. The lack of any degree of fibrosis or tumors in any ON-exposed rats 15 mo after exposure in the present study indicates a reduced long-term toxicity of this class of asbestos-derived mineral.

IT administration of NOA provides accurate delivery of precise doses directly to the conducting airway and respiratory regions of the lung, facilitates comparisons of dose-related and temporal responses to multiple samples, and is useful for

ranking sample toxicity, especially when quantities are limited as in this study. However, this technique also bypasses nasal passages and delivers material at a high dose rate to the lung, potentially producing inhomogeneous distribution and altering normal clearance pathways, inflammation, and other pathological responses (Driscoll et al., 2000). Although concentrated deposits of fibers or particles were not readily observed in lung sections after exposure to any of the samples, the possibility that aggregated fibers induced focal inflammation and granulomas after SM, ED, or LA exposure cannot be excluded. However, ON at the same IT doses induced no interstitial granulomatous inflammation or fibrosis, and minimal macrophage accumulation and alveolar epithelial hyperplasia, suggesting that the particular physical and chemical characteristics of the fiber samples, and not the IT doses employed, were critical in granuloma formation and the fibrotic response.

This study was designed to evaluate the long-term comparative toxicological effects of site-specific NOA. Our results showed that (i) ED produced persistently increased lung injury parameters; (ii) SM produced persistent AHR and deficits in pulmonary function; (iii) a dose-dependent rise in lung fibrosis was observed with all NOA samples except for ON, with SM producing the greatest degree of fibrosis, and (iv) both LA and ED produced bronchioloalveolar carcinoma at the low exposure dose. Collectively, these findings demonstrate that all NOA samples except cleavage fragment ON possessed some degree of chronic toxicity. Further studies are needed to systematically evaluate the potential acute and chronic adverse health effects of site-specific NOA.

Disclaimer

The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does the mention of trade names of commercial products constitute endorsement or recommendation for use.

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