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Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: http://www.tandfonline.com/loi/uteh20

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To cite this article: Jaime M. Cyphert, Danielle J. Carlin, Abraham Nyska, Mette C. Schladweiler, Allen D. Ledbetter, Jonathan H. Shannahan, Urmila P. Kodavanti & Stephen H. Gavett (2015) Comparative Long-Term Toxicity of Libby Amphibole and Amosite Asbestos in Rats After Single or Multiple Intratracheal Exposures, Journal of Toxicology and Environmental Health, Part A, 78:3, 151-165, DOI: 10.1080/15287394.2014.947455

To link to this article: http://dx.doi.org/10.1080/15287394.2014.947455

Published online: 15 Dec 2014.

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Journal of Toxicology and Environmental Health, Part A, 78:151–165, 2015
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ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287394.2014.947455

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COMPARATIVE LONG-TERM TOXICITY OF LIBBY AMPHIBOLE AND AMOSITE ASBESTOS IN RATS AFTER SINGLE OR MULTIPLE INTRATRACHEAL EXPOSURES

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In former mine workers of Libby, MT, exposure to amphibole-containing vermiculite was linked to increased rates of asbestosis, lung cancer, and mesothelioma. Although many studies showed adverse effects following exposure to Libby amphibole (LA; a mixture of winchite, richterite, and tremolite), little is known regarding the relative toxicity of LA compared to regulated asbestos, or regarding the risks associated with acute high-dose exposures relative to repeated low-dose exposures. In this study, pulmonary function, inflammation, and pathology were assessed after single or multiple intratracheal (IT) exposures of LA or a well-characterized amosite (AM) control fiber with equivalent fiber characteristics. Male F344 rats were exposed to an equivalent total mass dose (0.15, 0.5, 1.5, or 5 mg/rat) of LA or AM administered either as a single IT instillation, or as multiple IT instillations given every other week over a 13-wk period, and necropsied up to 20 mo after the initial IT. When comparing the two fiber types, in both studies LA resulted in greater acute neutrophilic inflammation and cellular toxicity than equal doses of AM, but long-term histopathological changes were approximately equivalent between fibers, suggesting that LA is at least as toxic as AM. In addition, although no dose-response relationship was discerned, mesothelioma or lung carcinomas were found after exposure to low and high dose levels of LA or AM in both studies. Conversely, when comparing studies, an equal mass dose given over multiple exposures instead of a single bolus resulted in greater chronic pathological changes in lung at lower doses, despite the initially weaker acute inflammatory response. Overall, these results suggest that there is a possibility of greater long-term pathological changes with repeated lower LA dose exposures, which more accurately simulates chronic environmental exposures.

Many adverse effects of asbestos exposure have been reported and extensively studied over the last century. Although much has been accomplished in the characterization of amphibole asbestos, mixtures of asbestiform (fibrous) minerals exist that are poorly characterized in terms of their relative toxicity compared to regulated forms of asbestos. The town of Libby, MT, was the setting of the first public health emergency finding by the U.S. Environmental Protection Agency (EPA) in June 2009 under the Comprehensive Environmental

Received 5 June 2014; accepted 18 July 2014.
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Response, Compensation, and Liability Act (or Superfund) (U.S. EPA, 2009). The findings recognized serious health impacts including excess cases of lung cancer and nonmalignant respiratory deaths reported in former vermiculite miners (McDonald et al., 2004; Sullivan, 2007; Whitehouse et al., 2008). The vermiculite mine in Libby contained a mixture of asbestiform amphiboles and other minerals, later coined "Libby amphibole" (LA), which contains winchite, richterite, and tremolite, as well as traces of other non-asbestiform minerals (Meeker et al., 2003). The complexity of LA may produce overall health effects that differ from the effects of its individual constituents, as well as other regulated amphiboles; therefore, it is critical to evaluate the comparative toxicity of LA to well-characterized asbestos.

While inhalation studies have been successful in elucidating the comparative toxicity of several types of amphibole and chrysotile asbestos (Davis et al., 1978; Wagner et al., 1974), the specialized equipment and large amounts of starting materials needed for each such study complicate this approach. Therefore, intratracheal (IT) instillations have become a standard alternative method. IT instillation introduces precise quantities of asbestos directly into the lungs using a route that is more controlled than inhalation and more physiologically relevant than intrapleural injection (Coffin et al., 1982; Driscoll et al., 2000). Although this method bypasses the nasal passages in an obligate nose-breathing animal, it permits delivery of a measured dose of fibrous particulates directly to the target site, which permits assessment of responses in a quantifiable manner at postexposure periods. Further, water elutriation was used to size-fractionate asbestos into a rat-respirable fraction ($PM_{2.5}$) to more accurately simulate an inhalation exposure (Duncan et al., 2010; Padilla-Carlin et al., 2011; Webber et al., 2008). A single IT instillation of LA-$PM_{2.5}$ was previously shown to be sufficient to induce significant acute (1 d and 3 mo) and chronic (1 and 2 yr) effects in vivo in the lung, including inflammation and fibrosis (Cyphert et al., 2012b; Padilla-Carlin et al., 2011); however, these studies assessed the toxicity of LA compared to a sample of amosite (AM) comprised of significantly longer fibers (i.e., RTI-AM) (1.9 $\pm$ 2.1 vs. 6.9 $\pm$ 12 μm, respectively). As such, no definitive conclusions could be reached as to the role of fiber type in relative LA toxicity compared to the reference sample. The purpose of this current study was twofold: to (1) assess the toxicity of LA compared to a well-characterized form of amosite asbestos, Union for International Cancer Control (UICC)-AM, with similar particle characteristics (i.e., length, width, aspect ratio, and particles or fibers/mg); and (2) determine whether toxicity differs if delivered in a single bolus dose versus multiple lower doses over time. Due to the similarities in fiber size distribution, it was hypothesized that exposure to respirable fractions of LA might induce an equal inflammatory response and long-term lung pathology as observed with UICC-AM. Further, the overall chronic effects would be dependent on the total dose of exposure and not length of time needed to accumulate the dose. To test these hypotheses, respiratory parameters, lung injury, inflammation, and pathology were assessed in Fischer 344 (F344) rats up to 20 mo following initiation of either single or multiple exposures to LA or UICC-AM. This is the first investigation to compare toxicological effects of a single high dose versus multiple lower dose LA exposures in comparison to a regulated asbestos sample of comparable size.

METHODS

Asbestos Samples

Libby amphibole (LA) was collected from the Rainy Creek Complex located near Libby, MT, in 2007 (LA2007) and was processed by the U.S. Geological Survey (USGS, Denver, CO) into finer materials by several methods, including crushing with a pneumatic press, horizontal grinder, ball mill grinder, and/or mortar and pestle. Union for International Cancer Control (UICC) amosite (AM) was used as a reference sample and was a generous gift from Dr. Philip Cook (U.S. EPA). The LA sample was size

fractionated by water elutriation as described previously (Cyphert et al., 2012b) to obtain a rat-respirable fraction using the method of Webber et al. (2008). The distribution of amphibole types in the 2007 LA collection is comparable to that in the 2000 LA collection (Lowers et al., 2012), which was used in earlier studies (Cyphert et al., 2012b; Duncan et al., 2010; Padilla-Carlin et al., 2011).

Size distributions (length, width, and aspect ratio) were counted for at least 500 objects for each sample using transmission electron microscopy (TEM) at 10,000 to 20,000× as previously described (Cyphert et al., 2012a). Briefly, a minimum of four random grid openings on a minimum of two grids were analyzed. All objects with a minimum dimension of 0.2 μm were counted, measured for length and width, classified according to morphology, and analyzed by x-ray spectroscopy and x-ray diffraction to identify mineral type. Total surface area of all particles was measured in duplicate by krypton gas absorption using Brunauer–Emmett–Teller theory (Micromeritics Analytical Services, Norcross, GA). Table 1 summarizes the characteristics of total objects (particles and fibers) in the sample. Table 2 summarizes the characteristics of those objects that are defined as a fiber, that is, having an aspect ratio (AR) ≥ 5:1, without regard to object morphology.

TABLE 1. Physical Parameters of LA ($PM_{2.5}$) and UICC-AM Particles, Determined Using TEM Analysis (All Objects Counted)

Property	$LA_{2.5}$	Amosite
Total objects counted (*n*)	532	525
Total particles/mg (× 10^7)	86	71
Total surface area (m^2/g) ± SD	14.11 ± 1.0	4.83 ± 0.03
Length (μm)		
Mean ± SD	1.9 ± 3.0	2.1 ± 7.4
Median	0.8	0.8
Range	0.2–27.3	0.2–121.5
Width (μm)		
Mean ± SD	0.39 ± 0.29	0.43 ± 0 .34
Median	0.30	0.32
Range	0.07–3.0	0.05–3.05
Aspect ratio (μm)		
Mean ± SD	6.4 ± 11.7	5.6 ± 14.9
Median	2.0	2.2
Range	1.0–109.2	1.0–145.0

Note. Total surface area was measured by krypton gas adsorption using BET theory.

TABLE 2. Physical Parameters of a Subset of LA ($PM_{2.5}$) and UICC AM Objects Defined as Fibers (AR ≥ 5:1), Determined Using TEM Analysis

Property	$LA_{2.5}$	Amosite
Total objects counted (*n*)	138	90
Percent of sample defined as "fibers"	26	17
Length (μm)		
Mean ± SD	5.0 ± 4.5	7.5 ± 16.8
Median	3.6	1.7
Range	0.5–27.3	0.45–121.5
Width (μm)		
Mean ± SD	0.29 ± 0.19	0.26 ± 0.17
Median	0.23	0.22
Range	0.07–1.15	0.05–0.95
Aspect ratio (μm)		
Mean ± SD	19.5 ± 17.3	22.2 ± 31.0
Median	14.3	9.3
Range	5.0–109.2	5.0–145.0

Animals and Experimental Design

All procedures were approved by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory, 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, 12/12-h light–dark cycle). Healthy male F344 rats (Charles River Laboratories, Raleigh, NC, USA), 6–8 wk old (200 g average weight), were double housed in polycarbonate cages with beta-chip bedding and acclimatized for at least 2 wk prior to use in this investigation (total *n* = 504 rats). All animals received standard Purina rat chow (Brentwood, MO) and water ad libitum. Animal body weights were recorded every 2 wk and health status was monitored daily.

Animals were divided into two studies: multiple exposure (Study M) and single exposure (Study S) (Figure 1). Asbestos doses were prepared by weight and suspended in dispersion media (DM, containing glucose, rat serum albumin, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline [PBS]), as previously

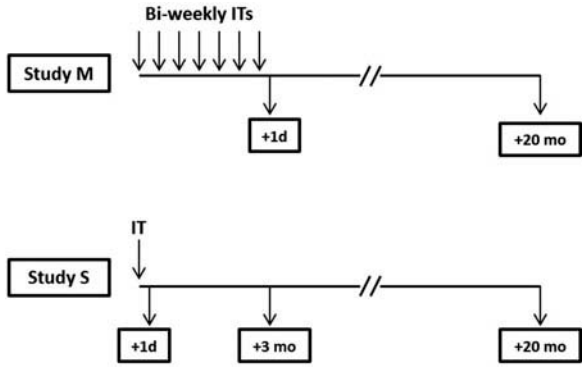

FIGURE 1. Schematic depicting the multiple (Study M) and single (Study S) exposure and necropsy schedules. In Study M, rats were exposed to 7 biweekly IT instillations of either LA or AM over the course of 13 wk and then necropsied either 1 d after the final exposure, or 20 mo after the initial exposure (17 mo after the final exposure). In Study S, rats were exposed to a single IT of either LA or AM and necropsied either 1 d, 3 mo, or 20 mo postexposure.

described (Cyphert et al., 2012a; Salazar et al., 2012). Animals in Study M (total $n = 224$ rats) were divided into 7 dose groups and were exposed via IT instillation every 2 wk for 13 wk for a total of 7 instillations, and necropsies were scheduled either 1 d ($n = 8$/group) from the final IT dose (3 mo from the first IT dose) or 20 mo (n = 24/group) from the first IT dose. Rats received a total dose of 0 (DM only), 0.15, 0.5, 1.5, or 5 mg LA, or 0.5 or 1.5 mg AM. Each instillation dose was one-seventh of the total dose and delivered in a 190-μl volume. For Study S (total $n = 280$ rats) animals were separated into 7 groups and received a total dose of 0 (DM only), 0.15, 0.5, 1.5, or 5 mg LA, or 0.15 or 0.5 mg AM in a single IT dose delivered in a 190-μl volume. Study S necropsies were scheduled either 1 d ($n = 8$/group), 3 mo ($n = 8$/group), or 20 mo ($n = 24$/group) following the single IT.

Pulmonary Function

Pulmonary function was monitored at the following times after exposure to asbestos samples: Study S: 5, 10, 16, and 20 mo post IT instillation; Study M: 1 wk and 6, 12, and 17 mo post final IT instillation (the latter 20 mo post initial IT instillation). Ventilatory parameters were measured using noninvasive whole-body plethysmography (Buxco Electronics, Wilmington, NC) in unrestrained rats monitored for 5 min in the plethysmograph chambers. Several ventilatory parameters were measured, including minute volume (product of breath frequency and tidal volume) and enhanced pause (P_{enh}, unitless), which often correlates with airflow obstruction (Hamelmann et al., 1997).

Bronchoalveolar Lavage Fluid (BALF)

Bronchoalveolar lavage fluid (BALF) was collected and analyzed as described previously (Cyphert et al., 2012b). Briefly, animals were deeply anesthetized with a mixture of sodium pentobarbital and sodium phenytoin (195 mg/kg and 25 mg/kg, respectively), the trachea was cannulated, left lung ligated, and right lung lavaged using a volume representing 60% of total lung capacity (35 ml/kg). Three washes were conducted using the same buffer aliquot. Total cell counts were determined using a Z1 Coulter Counter (Coulter, Inc., Miami, FL). Differential slides were stained with Leukostat (Thermo Fisher Scientific Co., Waltham, MA) and examined under light microscopy to determine total macrophages and neutrophils. The remaining BALF was analyzed for markers of lung injury, including protein content (Coomassie Plus Protein Assay Kit, Pierce, Rockford, IL), 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., Melbourne, Australia). All assays were conducted 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 after all necropsy time points.

In addition, in the 20-mo groups, the heart, pericardium, thymus, diaphragm, and rib cage (including parietal pleura) were removed, fixed in 10% formalin, and embedded in paraffin. For all tissues, longitudinal slices, 5 μm thick, were cut and stained with hematoxylin and eosin (H&E). Duplicate lung slices were also stained with Masson's trichrome for analysis of collagen accumulation. All tissue sections were examined by a certified veterinary pathologist by light microscopy as previously described (Cyphert et al., 2012a; Greaves et al., 2013), evaluating a variety of pathological indices using the following 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 analyses were 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 honestly significant difference or Dunnett's post hoc test as necessary. Data are shown as mean ± SEM. Differences with $p < .05$ were considered statistically significant.

RESULTS

Fiber Characteristics

It was previously reported that, at the same mass dose, exposure to LA resulted in less inflammation and fibrosis than exposure to RTI-AM in F344 rats (Cyphert et al., 2012b; Padilla-Carlin et al., 2011). However, this was possibly due to differences in fiber characteristics between the two samples, as the RTI-AM sample contains both longer fibers and greater aspect ratio than the LA sample (Duncan et al., 2010). To more accurately assess acute and long-term toxicity in the rat model, the effects of LA were compared to a standard, well-characterized reference sample with similar fiber characteristics. Transmission electron microscope (TEM) analysis revealed that UICC-AM particles and fibers were similar in length, width, and aspect ratio (AR) to LA that had been water-elutriated to derive a rat respirable ($PM_{2.5}$) sample, as shown in Tables 1 and 2, respectively. Further, each sample contained a comparable number of particles per milligram, as well as percent of particles defined as fibers (AR > 5:1). The only notable difference was the total surface area of LA particles, which was greater than that of AM sample (14.11 ± 1 compared to 4.83 ± 0.03 m^2/g).

Animal Weight and Pulmonary Function

Compared with DM control groups, there were no significant differences in group body weights following single (Study S) IT instillation of any dose of AM or LA (Table 3), including 1 wk after IT (data not shown). However, 1 wk after the 7 multiple (Study M) IT instillations, there was a significant decrease in body weight of rats exposed to the highest dose of LA (5 mg/rat), although there were no marked differences at later time points, nor were there differences in body weights between groups of rats instilled with the same mass dose of AM or LA. In Study S, minute volumes (MV) were greatest in rats exposed to the highest dose of LA (5 mg/ml) both at 5 and 20 mo post IT instillation (Table 3). Other groups also had significantly greater MV at 20 mo post IT instillation, although this was partly due to unusually low MV in the DM control group at this time. No marked changes in MV were found in Study M either 1 wk after final IT instillation or 20 mo after initial IT instillation. In contrast, a dose-dependent increase in enhanced pause (P_{enh}) was found 1 wk after multiple IT exposures to LA or AM. The AM P_{enh} response was equivalent to LA P_{enh} response at the same mass dose of LA. No exposure-related changes in P_{enh} were observed 20 mo after the initial IT in Study M, nor at 5 or 20 mo after IT in Study S.

Acute Inflammation and Lung Injury

Both acute and long-term inflammation were assessed in BALF after either multiple or

TABLE 3. Animal Body Weights and Respiratory Parameters After Either Multiple (Study M; Cumulative Dose Shown) IT or Single (Study S) IT Instillation Exposure of F344 rats to Libby Amphibole (LA), Amosite (AM), or Control Dispersion Media (DM)

Dose and time point (*n*)	Body weight (g)	Minute volume (ml/min)	Penh (unitless)
Study M			
DM			
1 wk (24)	350 ± 3	375 ± 6	0.45 ± 0.01
20 mo (16)	441 ± 16	396 ± 12	0.54 ± 0.04
AM (0.5 mg/rat)			
1 wk (24)	349 ± 4	385 ± 7	0.48 ± 0.01
20 mo (15)	457 ± 8	381 ± 14	0.47 ± 0.03
AM (1.5 mg/rat)			
1 wk (24)	343 ± 5	400 ± 11	0.55 ± 0.02*
20 mo (15)	450 ± 14	397 ± 12	0.71 ± 0.26
LA (0.15 mg/rat)			
1 wk (24)	352 ± 3	371 ± 11	0.43 ± 0.02
20 mo (16)	447 ± 9	359 ± 13	0.43 ± 0.03
LA (0.5 mg/rat)			
1 wk (24)	349 ± 4	365 ± 17	0.47 ± 0.03
20 mo (20)	452 ± 9	354 ± 14	0.51 ± 0.09
LA (1.5 mg/rat)			
1 wk (24)	348 ± 4	388 ± 10	0.55 ± 0.01*
20 mo (14)	438 ± 14	343 ± 15	0.55 ± 0.05
LA (5.0 mg/rat)			
1 wk (24)	331 ± 4*	388 ± 10	0.70 ± 0.02*
20 mo (15)	439 ± 10	399 ± 12	0.43 ± 0.01
Study S			
DM			
5 mo (24)	381 ± 4	400 ± 26	0.45 ± 0.02
20 mo (18)	447 ± 10	313 ± 24	0.48 ± 0.07
AM (0.15 mg/rat)			
5 mo (24)	378 ± 4	413 ± 14	0.44 ± 0.02
20 mo (20)	458 ± 9	383 ± 13	0.37 ± 0.03
AM (0.5 mg/rat)			
5 mo (24)	372 ± 3	404 ± 15	0.48 ± 0.03
20 mo (18)	448 ± 9	425 ± 7*	0.44 ± 0.02
LA (0.15 mg/rat)			
5 mo (24)	382 ± 4	390 ± 13	0.45 ± 0.01
20 mo (18)	452 ± 9	418 ± 13*	0.47 ± 0.02
LA (0.5 mg/rat)			
5 mo (24)	379 ± 5	418 ± 12	0.47 ± 0.02
20 mo (17)	464 ± 9	414 ± 18*	0.45 ± 0.02
LA (1.5 mg/rat)			
5 mo (24)	378 ± 4	436 ± 7	0.48 ± 0.01
20 mo (12)	455 ± 10	362 ± 30	0.44 ± 0.03
LA (5.0 mg/rat)			
5 mo (24)	372 ± 4	491 ± 20*	0.52 ± 0.01
20 mo (14)	462 ± 7	468 ± 14*	0.45 ± 0.02

Note. Groups were assessed 1 week (Study M) or 5 months (Study S) after final IT instillation, and 20 months after first IT instillation (both studies). Values shown are means and standard errors. Number in parentheses represents number of rats assessed for body weight. For assessment of all respiratory parameters, $n = 6$ rats/group. Asterisk indicates significant at $p < .05$ compared to dispersion media (DM) controls at the same time point within the same study.

single IT instillation of AM or LA (Figure 2). In both studies, at 1 d post instillation, both AM and LA induced dose-dependent inflammation characterized by increased neutrophils that was significantly greater in LA-exposed rats compared to rats exposed to an equal dose of AM (Figure 2B). Macrophage numbers were either equivalent or reduced compared to DM controls at all time points after asbestos exposure in both studies, with one exception: A single high dose of LA (5 mg/ml) also resulted in a twofold increase in macrophages in the BALF 1 d after instillation (Figure 2A). In Study S, neutrophilic inflammation was still elevated compared to DM controls in all groups at 3 mo post instillation, and the response to 0.5 mg/ml LA was still greater than for AM at the same dose; however, the magnitude of the response was markedly reduced. Chronic inflammation was not evident in either study, as both macrophage and neutrophil levels in all asbestos-exposed groups matched those in the control groups 20 mo after initial exposure.

Similar patterns of AM and LA responses were seen in other markers of acute inflammation and lung injury (Figure 3). Multiple or single exposures to AM or LA resulted in similar trends of dose-dependent rise in membrane damage (GGT), cellular toxicity (LDH), and epithelial permeability (total protein) markers 1 day after instillation (Figure 3A, 3C, and 3D, respectively). Significant increases of these markers persisted out to 3 mo post instillation in Study S, but returned to baseline levels by 20 mo in both studies. However, only the macrophage lysosomal marker, NAG, was elevated significantly following exposure to high doses of LA in both studies (Figure 3B), and this effect persisted up to 20 mo post-instillation. Although exposure to LA induced significantly more neutrophilic inflammation than the same dose of AM in both studies, only LDH levels followed the same trend; all other markers of lung injury were either equivalent between LA and AM at the same dose, or lower in the LA group 1 d after dosing (GGT at 1.5 mg/rat in Study M and total protein at 0.5 mg/rat in Study S).

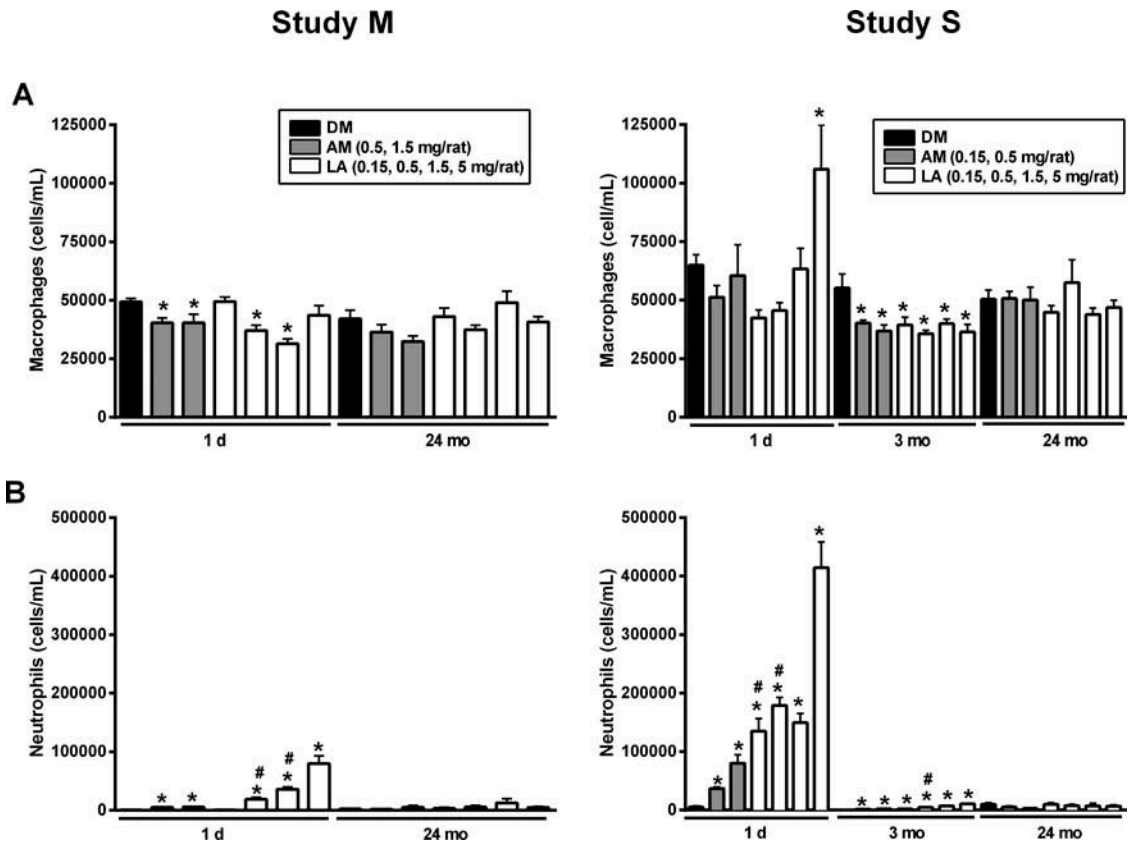

FIGURE 2. Total macrophages (A) and neutrophils (B) were measured in the BALF following either multiple (left panel) or single (right panel) IT instillation of LA (0.15, 0.5, 1.5, or 5 mg/rat) or AM (0.15, 0.5, or 1.5 mg/rat). Values shown are means and standard errors. A dose dependent increase in both macrophages and neutrophils was seen 1 d after exposure in Study S, while only neutrophils increased dose-dependently 1 d after final exposure in Study M. Asterisk indicates significant at $p < .05$ compared to dispersion media (DM) controls at the same time point; $^{\#}p < .05$ compared to an equivalent dose of AM at the same time point.

Survival and Histopathology

Following exposure to LA, AM, or DM, groups of rats were held up to 20 mo after initial instillation. All treatment groups from this time point in both studies had some early deaths (most were euthanized moribund while some were found dead in cages), ranging from 4 to 12 of 24 rats per group (83–50% survival), but survival was not related to dose or asbestos type (Table 4, first column). The most common cause of early death was mononuclear cell leukemia (MCL), a common cancer in F344 rats (Haseman et al., 1998). There was no relationship of MCL to exposure group.

Histopathological changes in the lung were evaluated throughout the time course of both studies (Table 4). Other tissues including diaphragm, pleura, thymus, heart, and pericardium were evaluated 20 mo after exposure. In both studies, the primary histopathological response 1 d after instillation of amphibole was a dose-dependent increase in intra-alveolar macrophages and neutrophils localized to the centriacinar regions (with LA) or to the peribronchial regions (with AM). Although the neutrophilic inflammatory response was greater following a single instillation of amphibole (Figure 2), in Study M inflammation was also associated with other pathological changes including alveolar multinucleated giant cells (especially after exposure to AM), alveolar epithelial hyperplasia and minimal interstitial fibrosis. In addition, 1 day after multiple instillations of LA, the presence of

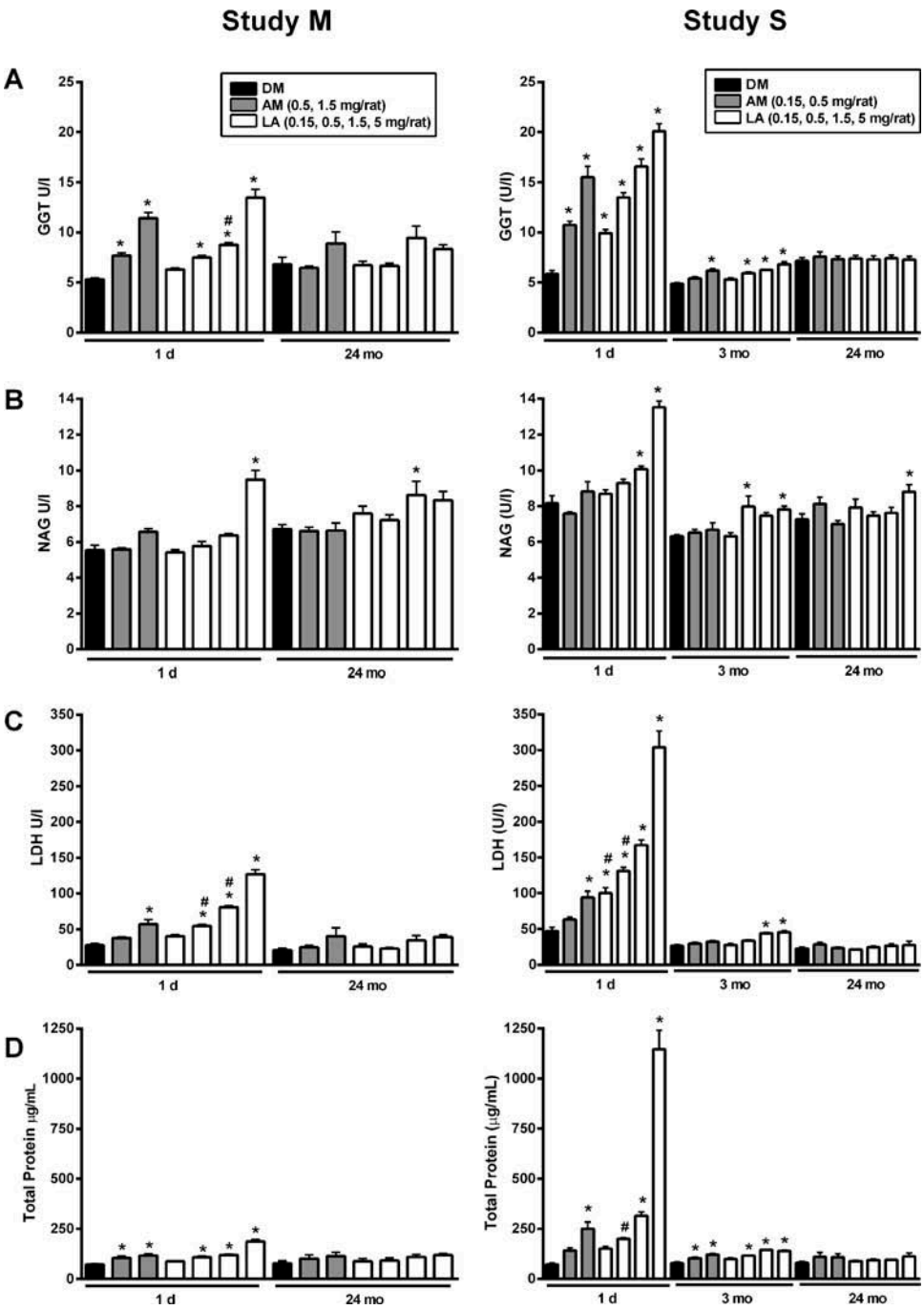

FIGURE 3. Markers of membrane damage (GGT, A), macrophage lysosomal secretion (NAG; B), cellular toxicity (LDH, C), and epithelial permeability/lung damage (total protein; D) were measured in the BALF following either multiple (left panel) or single (right panel) IT instillation of LA (0.15, 0.5, 1.5, or 5 mg/rat) or AM (0.15, 0.5, or 1.5 mg/rat). Values shown are means and standard errors. A dose-dependent increase of biomarkers was seen in both studies at either 1 d or 3 mo post-exposure. Asterisk indicates significant at $p < .05$ compared to DM controls at the same time point; $^{\#}p < .05$ compared to equivalent dose of AM at the same time point.

TABLE 4. Incidence Table of Pathological Changes in the Lung at Various Time Points After Either Multiple (Study M) or Single (Study S) IT Instillation Exposure of F344 rats to Libby Amphibole (LA), Amosite (AM), or Control Dispersion Media (DM)

Dose and time point (*n*)	A-PMNL	A-M	A-G	A-H	PI	PLG	IF	Tumors[a]
Study M								
DM								
1d (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
20 mo (16)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
AM (0.5 mg/rat)								
1 d (8)	2 (0.3)	8 (1.0)	5 (0.6)	8 (1.0)	0 (0)	0 (0)	0 (0)	0 (0)
20 mo (15)	0 (0)	14 (1.1)	15 (1.0)	15 (1.1)	1 (0.1)	2 (0.1)	15 (1.0)	0 (0)
AM (1.5 mg/rat)								
1 d (8)	2 (0.3)	8 (2.0)	8 (1.0)	8 (1.0)	0 (0)	0 (0)	5 (0.6)	0 (0)
20 mo (15)	0 (0)	15 (1.0)	15 (1.7)	15 (1.1)	0 (0)	3 (0.2)	15 (1.7)	0 (0)
LA (0.15 mg/rat)								
1 d (8)	0 (0)	7 (0.9)	3 (0.4)	7 (0.9)	0 (0)	0 (0)	0 (0)	0 (0)
20 mo (16)	1 (0.1)	11 (0.8)	3 (0.3)	8 (0.6)	1 (0.1)	0 (0)	8 (0.5)	1 (M)[b]
LA (0.5 mg/rat)								
1 d (8)	7 (0.9)	8 (1.0)	4 (0.5)	8 (1.0)	0 (0)	1 (0.1)	0 (0)	0 (0)
20 mo (20)	0 (0)	20 (1.1)	1 (0.1)	20 (1.1)	3 (0.2)	1 (0.1)	15 (0.8)	0 (0)
LA (1.5 mg/rat)								
1 d (8)	5 (0.9)	6 (1.5)	4 (0.5)	6 (1.5)	0 (0)	5 (0.6)	0 (0)	0 (0)
20 mo (15)	2 (0.3)	15 (1.7)	2 (0.2)	15 (1.2)	1 (0.1)	2 (0.1)	14 (1.4)	0 (0)
LA (5.0 mg/rat)								
1 d (8)	7 (1.1)	7 (2.6)	7 (0.9)	7 (2.6)	0 (0)	5 (0.6)	6 (0.8)	0 (0)
20 mo (16)	2 (0.4)	16 (2.1)	1 (0.1)	16 (2.1)	3 (0.2)	11 (0.8)	16 (2.0)	1 (M)[c]
Study S								
DM								
1d (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3 mo (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
20 mo (18)	1 (0.1)	4 (0.2)	2 (0.1)	1 (0.1)	0 (0)	0 (0)	0 (0)	0 (0)
AM (0.15 mg/rat)								
1 d (8)	7 (1.0)	8 (1.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3 mo (8)	0 (0)	7 (0.9)	8 (1.0)	3 (0.4)	0 (0)	0 (0)	5 (0.6)	0 (0)
20 mo (20)	0 (0)	11 (0.8)	5 (0.3)	9 (0.6)	0 (0)	0 (0)	11 (0.6)	1 (C)[d]
AM (0.5 mg/rat)								
1 d (8)	8 (2.0)	8 (2.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3 mo (8)	0 (0)	8 (1.0)	8 (1.9)	8 (1.0)	0 (0)	0 (0)	8 (1.5)	0 (0)
20 mo (18)	1 (0.2)	3 (0.2)	3 (0.2)	3 (0.3)	0 (0)	3 (0.2)	18 (1.1)	1 (A)[e]
LA (0.15 mg/rat)								
1 d (8)	8 (1.0)	8 (1.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3 mo (8)	0 (0)	6 (0.8)	4 (0.5)	1 (0.1)	0 (0)	0 (0)	0 (0)	0 (0)
20 mo (18)	0 (0)	5 (0.5)	0 (0)	3 (0.3)	1 (0.1)	1 (0.1)	1 (0.2)	0 (0)
LA (0.5 mg/rat)								
1 d (8)	8 (2.0)	8 (2.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3 mo (8)	0 (0)	8 (1.0)	1 (0.1)	8 (2.0)	0 (0)	1 (0.1)	0 (0)	0 (0)
20 mo (17)	1 (0.1)	15 (0.9)	0 (0)	10 (0.6)	0 (0)	1 (0.1)	0 (0)	0 (0)
LA (1.5 mg/rat)								
1 d (8)	8 (3.0)	8 (3.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3 mo(8)	0 (0)	8 (2.0)	7 (1.1)	8 (2.0)	0 (0)	5 (0.6)	7 (0.9)	0 (0)
20 mo (12)	0 (0)	12 (1.7)	0 (0)	12 (1.8)	3 (0.3)	1 (0.1)	12 (1.4)	0 (0)
LA (5.0 mg/rat)								
1 d (8)	8 (3.0)	8 (3.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
3 mo (8)	0 (0)	8 (2.0)	8 (2.0)	8 (2.0)	0 (0)	7 (1.0)	8 (2.0)	0 (0)
20 mo (14)	0 (0)	14 (2.0)	8 (0.6)	7 (1.0)	0 (0)	14 (1.3)	14 (2.3)	1 (M)[f]

Note. Number in parentheses represents animals that survived to designated time point (out of 8 per group at early time points and out of 24 per group at 20 mo. In all other columns, values represent number of animals with observed lesions with the average histopathological score in parentheses for all animals. A-PMNL = intra-alveolar polymorphonuclear leukocyte infiltration; A-M = intra-alveolar accumulation of macrophages; A-G = presence of alveolar multinucleated giant cells; A-H = alveolar epithelial hyperplasia; PI = pleural inflammation; PLG = presence of multinucleated giant cells in peribronchial lymphoid tissues; IF = interstitial fibrosis.

[a]Several tissue types in addition to the left lung lobe were evaluated for incidence of mesothelioma (M), carcinoma (C), and adenoma (A) including diaphragm, pleura, thymus, heart, and pericardium.

[b]Located in the diaphragm.

[c]Located in the pleura.

[d]Located in the left lung lobe and classified as malignant.

[e]Located in the left lung lobe.

[f]Located in the pericardium and classified as malignant.

multinucleated giant cells within bronchial-associated lymphoid tissue (BALT) also was noted, similar in degree to Study S 3 mo after exposure.

In Study S, the intra-alveolar macrophage accumulation decreased in severity by 3 mo, but was associated with alveolar epithelial hyperplasia and minimal interstitial fibrosis similar to that seen in Study M at 1 d; however, there were no longer any intra-alveolar neutrophils present in the lungs of rats exposed to either amphibole. Alveolar multinucleated giant cells were present in all exposed rats but more abundant after instillation of AM. The presence of multinucleated giant cells within BALF was only seen 3 mo after a single exposure to LA and was similar in severity to that seen 1 d after multiple exposures.

Most histopathological changes related to inflammation declined or were stable up to 20 mo after exposures to LA or AM. However, interstitial fibrosis was markedly elevated at 20 mo after exposure and greater in rats from Study M compared to Study S at lower doses (0.15 and 0.5 mg/rat). Interstitial fibrosis was comparable between groups of rats exposed to the same mass dose of LA or AM in Study M. In addition to the progressive lung pathology described here, after multiple exposures to LA, two cases of benign mesothelioma were noted on the diaphragm (0.15 mg LA) and from the parietal pleura (5 mg LA) (Figures 4A and 4B). These tumors were characterized by ramified finger-like projections, and in both cases cauliflower-like nodules were noted. In Study S, one case of malignant mesothelioma was seen after exposure to LA (5 mg). The tumor was located on the pericardium over the base of the heart and had invaded to the underlying myocardium (Figures 4C and 4D). Criteria for the diagnosis of mesothelioma were as described previously (Greaves et al., 2013). Unlike the benign mesotheliomas seen in Study M, this tumor showed anaplasia and invasiveness and therefore was diagnosed as malignant. No cases of mesothelioma were seen after exposure to AM in either study; however, two

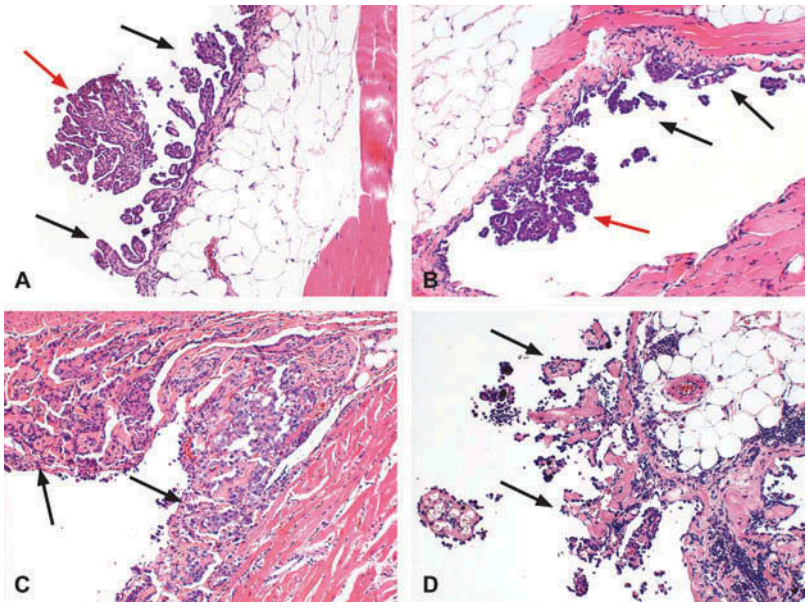

FIGURE 4. Exposure to LA induces development of mesothelioma. Multiple IT instillations (Study M) of 5.0 and 0.15 mg/rat LA resulted in the development of benign mesothelioma in the pleura (A) and diaphragm (B), respectively, 20 mo after exposure. In these two cases the mesothelial proliferation was localized to one site, composed of a main complex ramified nodule. No local invasion was noted. Additionally, after 1 instillation of 5 mg/rat LA (Study S), a single rat developed malignant pericardial mesothelioma (C) that exhibited local invasion into the myocardium and spread to the mediastinal fat surrounding the heart (D). Black arrows denote the presence of mesothelial proliferations. Red arrows indicate cauliflower-like ramified nodules. Magnification 10×.

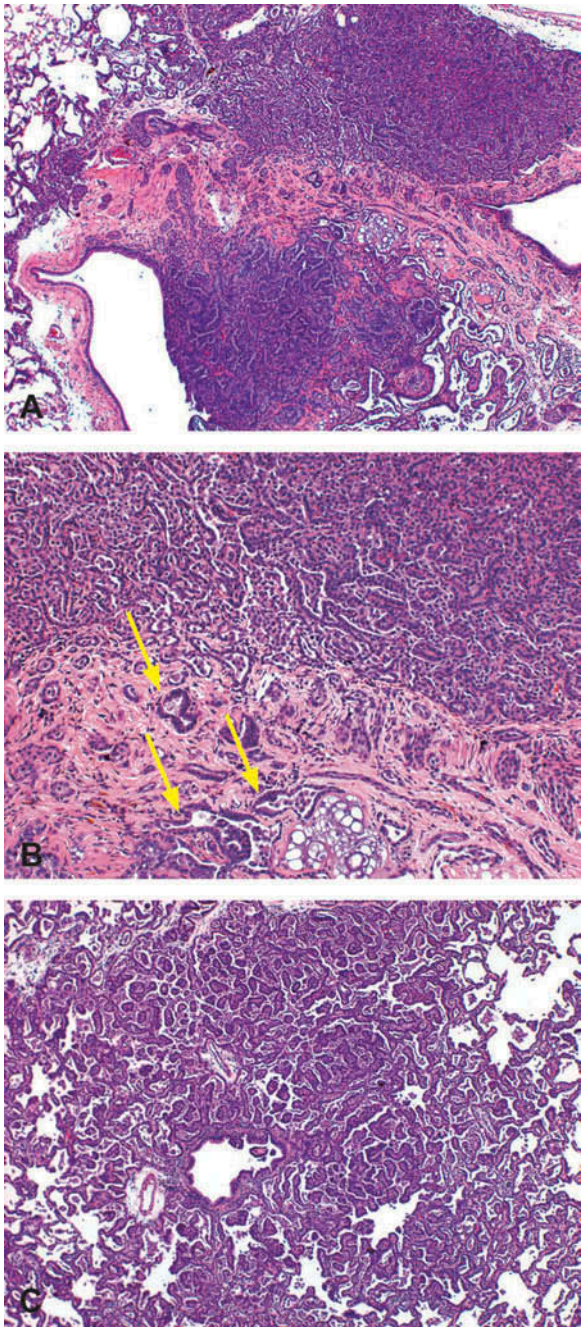

FIGURE 5. Exposure to amosite induces development of lung cancer. A single instillation (Study S) of 0.15 or 0.5 mg/rat UICC amosite induced development of bronchiolo-alveolar carcinoma (A,B) or adenoma (C), respectively. The carcinoma was classified as malignant due to its local invasiveness (B, yellow arrows). Magnification 4× (A, C) and 10× (B).

cases of bronchiolo-alveolar tumors were noted in Study S: a malignant carcinoma (0.15 mg AM; Figures 5A and 5B), and a benign adenoma (0.5 mg AM; Figure 5C).

DISCUSSION

The toxicity of asbestos or asbestiform minerals has often been attributed to their fiber characteristics. It was shown that fiber dimensions may play a role in the level of inflammatory, fibrotic, or carcinogenic responses to fibers (Cyphert et al., 2012a; O'Neill, 2008; Padilla-Carlin et al., 2011; Stanton et al., 1981). For this reason, UICC-AM was chosen as the control fiber, as it demonstrated particle size distributions similar to those of elutriated LA used in this study (Duncan et al., 2014). TEM analysis showed LA and UICC-AM had equivalent fiber lengths, widths, and aspect ratios. The only difference noted was a greater surface area for LA compared with AM, as determined by krypton gas absorption. Previous studies showed that fiber surface area generally correlated with both fiber length and overall potency (Aust et al., 2011; Duncan et al., 2014). Further, a greater surface area allows for a high capacity to absorb and accommodate biomolecules, which has been linked to cellular and genetic toxicity (Nagai et al., 2011). Differences in surface chemistry and fiber chemical composition were not assessed in either study, and although the average length and aspect ratio of the two samples were similar, the greater surface area of the LA sample may be a confounding factor and may contribute to any increased potency reported.

Consistent with similar physical characteristics, LA and UICC-AM produced essentially equivalent inflammatory and fibrotic changes in the lung, although LA resulted in greater acute PMN accumulation and LDH activity levels in both studies. While macrophage accumulation and interstitial fibrosis were roughly equal following exposure to each fiber type in both studies, greater fibrosis was seen after AM exposure in Study S at 3 mo compared to equivalent doses of LA. However, similar doses of LA (0.25, 0.5, or 1 mg) previously were found to produce interstitial fibrosis at 3 mo equivalent to that noted after AM exposure in this study (Cyphert et al., 2012a; Shannahan et al., 2012).

Different study dosing regimens produced seemingly distinct differences in animal growth

rates and kinetics of inflammatory and respiratory responses. Despite a sevenfold higher dose of amphibole in Study S with a single IT instillation compared to the initial dose in the multiple IT instillation Study M, significant changes in body weight were only observed in Study M 1 wk after final dosing. At this same time, increases in P_{enh}, which often correlates with airflow obstruction (Hamelmann et al., 1997), were observed only in Study M, which may be due partly to repeated exposure promoting chronic irritation and inflammation in the airways. In contrast, changes in minute volume were only observed in rats exposed to AM or LA in Study S, which may indicate physiological compensation for the greater acute inflammatory response initiated by the larger single doses. In Study S, while BALF total cells and inflammatory markers 1 d after instillation were severalfold higher than 1 d after the last of multiple instillations in Study M, both regimens produced the same trend in dose response and effects observed with each fiber types. Similarly, 1-d lung tissue intra-alveolar neutrophils and macrophages were higher in Study S, but again Study M showed the same trend in response. On the other hand, peribronchial lymphoid and intra-alveolar giant cells, alveolar epithelial hyperplasia, and interstitial fibrosis were greater in Study M 1 d after final dosing, but were not seen 1 d after a single instillation. Controlling for time after first instillation with Study M 1-d and Study S 3-mo time points, intra-alveolar macrophages, peribronchial lymphoid giant cells, and alveolar hyperplasia were equivalent between studies. Conversely, there was a greater degree of interstitial fibrosis and accumulation of intra-alveolar giant cells 3 mo after a single IT of AM (0.5 mg) or high doses of LA (1.5 and 5 mg). This degree of fibrosis was also comparable with what was reported 3 mo after a short-term inhalation exposure to AM (Bernstein et al., 2010).

In both studies, BALF markers returned to baseline levels by 20 mo. In lung tissue 20 mo from first IT instillation, multiple exposures produced greater increases in interstitial fibrosis, accumulation of intra-alveolar giant cells, and alveolar epithelial hyperplasia at lower doses of LA than a single IT dose, but the response was equal at higher doses of LA in both studies. High lung burdens of poorly soluble particles or durable fibers may induce inflammation, fibrosis, or tumor development through lung particle overload or overproduction of reactive oxidant species (Oberdorster, 2002). These observations suggest that repeated fiber exposure, especially at low concentration, might not be effective in inducing antioxidant defenses, thus leading to exacerbated lung pathobiological effects relative to a single IT dose. It is also possible that clearance mechanisms might be impaired at first exposure, producing greater fiber retention after subsequent exposures and thus greater lung injury. However, since greater effects of repeated exposures were primarily seen at lower doses of LA, the former hypothesis is more likely to explain these differences. Similar to LA, multiple exposures to the one dose of AM common to both studies (0.5 mg) produced more intra-alveolar giant cells and alveolar epithelial hyperplasia than a single IT dose, but the degree of fibrosis was equal between studies. The degree of fibrosis induced by 1.5 mg AM in Study M was similar to that reported 18 mo following 6 mo of inhalation exposure to 11 mg/m^3 AM (Wagner et al., 1974). However, an amosite sample with significantly longer fiber lengths and greater aspect ratios (RTI-AM) produced a greater degree of interstitial fibrosis than a sample of LA with fiber size characteristics similar to that reported in this study (Cyphert et al., 2012b).

In addition to inflammation and interstitial fibrosis, both fiber types produced some tumor development, although of distinctly different types. UICC-AM produced both benign bronchioloalveolar adenoma and malignant bronchioloalveolar carcinoma, while LA produced benign and malignant mesotheliomas. Bronchioloalveolar adenomas are benign tumors originating in the epithelium; they are the most common form of lung cancers in nonsmokers and are thought to be precursors to malignant bronchial adenocarcinomas, of which bronchioloalveolar carcinomas (BAC) are considered a subset (Arenberg, 2007). On the other hand, mesothelioma is an extremely

rare neoplasm arising from mesothelial lining of the pleura, peritoneum, or pericardium that is associated with exposure to industrial pollutants, of which asbestos is the principle carcinogen. Natural incidence of malignant mesothelioma in humans is 7–40 per 1 million in Western nations (0.0007–0.004%) (Robinson and Lake, 2005), but rates after exposure to LA are significantly higher (Antao et al., 2012; Dunning et al., 2012; Horton et al., 2006; Sullivan, 2007). Although malignant mesothelioma usually remains latent in the human body for 20–50 yr before it appears, benign mesothelioma can manifest sooner and is often an indication of more serious asbestos-related diseases in the future (Ahmed et al., 2013; Myers, 2012). In addition to being rare in the human population, mesothelioma also has extremely low rates of natural occurrences in F344 rats. The National Toxicology Program (NTP) reported that intrathoracic mesothelioma occurred in only 0.037% of 66,941 control or treated F344 rats (Willson et al., 2003). In the present study, although only 3 rats were found with mesothelioma (2 benign), this is a higher rate than the NTP finding, whether counting all rats in the study at 20 mo (0.9% total, 0.3% malignant) or only LA-exposed rats (1.6% total, 0.5% malignant). Tumors were produced in response to the lowest (0.15 mg) as well as the highest doses (5 mg) of asbestos, indicating potential for tumorigenesis at low dose levels. The total number of tumors counted was relatively low in all groups and no significant inferences can be made with regard to fiber type or dose.

In conclusion, these studies were designed to evaluate and compare the effects of LA and AM, an amphibole known for its toxicity, as well as to compare an acute versus a chronic exposure to the same mass dose of amphibole asbestos. Although sample mass was the dose metric chosen here for evaluation of noncancer and cancer effects, evaluation of other dose metrics such as fiber length or surface area may be more appropriate and may provide greater insight into fiber toxicity. In both studies, although LA resulted in greater acute neutrophilic inflammation and cellular toxicity (LDH) than equal doses of AM, the resulting long-term histopathological changes were approximately equivalent between fibers. Both fibers induced similar levels of pulmonary interstitial fibrosis and resulted in tumor development, albeit different types, suggesting that LA is at least as toxic as AM. Interestingly, the same total mass dose given over multiple exposures instead of a single bolus resulted in greater chronic pathological changes in lung despite the weaker acute inflammatory response. Overall, the results of the two protocols suggest that there is a possibility of greater long-term pathological changes with repeated lower dose exposures, as is typical of chronic environmental exposures to inhaled fibers.

FUNDING

This work was supported by a U.S. EPA/University of North Carolina Toxicology Research Program Training Agreement (CR 933237 and 83515201-0). The authors thank Judy Richards for assistance with biochemical analysis, and Drs. David Berry, Ian Gilmour, Maureen Gwinn, Gary Hatch, and Ronald Hines for manuscript review.

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, not does the mention of trade names of commercial products constitute endorsement or recommendation for use.

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