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LONG-TERM RESPONSE OF RATS TO SINGLE INTRATRACHEAL EXPOSURE OF LIBBY AMPHIBOLE OR AMOSITE

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In former mine workers and residents of Libby, Montana, exposure to amphibole-contaminated vermiculite has been associated with increased incidences of asbestosis and mesothelioma. In this study, long-term effects of Libby amphibole (LA) exposure were investigated relative to the well-characterized amosite asbestos in a rat model. Rat-respirable fractions of LA and amosite (aerodynamic diameter $\leq$2.5 μm) were prepared by water elutriation. Male F344 rats were exposed to a single dose of either saline, amosite (0.65 mg/rat), or LA (0.65 or 6.5 mg/rat) by intratracheal (IT) instillation. One year after exposure, asbestos-exposed rats displayed chronic pulmonary inflammation and fibrosis. Two years postexposure, lung inflammation and fibrosis progressed in a time- and dose-dependent manner in LA-exposed rats, although the severity of inflammation and fibrosis was smaller in magnitude than in animals exposed to amosite. In contrast, gene expression of the fibrosis markers Col 1A2 and Col 3A1 was significantly greater in LA-exposed compared to amosite-exposed rats. There was no apparent evidence of preneoplastic changes in any of the asbestos-exposed groups. However, all asbestos-exposed rats demonstrated a significant increase in the expression of epidermal growth factor receptor (EGFR) 2 yr after instillation. In addition, only LA-exposed rats showed significant elevation in mesothelin (Msln) and Wilms' tumor gene (WT1) expression, suggesting possible induction of tumor pathways. These results demonstrate that a single IT exposure to LA is sufficient to induce significant fibrogenic, but not carcinogenic, effects up to 2 yr after exposure that differ both in quality and magnitude from those elicited by amosite administration at the same mass dose in F344 rats. Data showed that LA was on a mass basis less potent than amosite.

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From 1920 to 1990, Zonolite Mountain in Libby, Montana, was the site of the largest active vermiculite ore mine in the United States. The vermiculite ore mined from this site contained a significant amount of amphibole asbestos, termed "Libby amphibole" (LA), which has been characterized as a complex mixture of both regulated and nonregulated amphibole types, primarily tremolite, winchite, and richterite and a small proportion of nonasbestiform accessory minerals (Meeker 2003). The mining, processing, use, and transport of this contaminated ore led to extensive contamination of the surrounding community. 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 (McDonald et al. 2004; Sullivan 2007; ATSDR 2002; Whitehouse et al. 2008; Weill et al. 2010). Studies by the Agency for Toxic Substances Disease Registry (ATSDR) of the United States indicated that the asbestosis mortality rate in Libby was 40- to 80-fold higher than the national average and lung cancer was 20–30% higher than expected (ATSDR 2002; 2003; Sullivan 2007).

In addition to epidemiological studies, several in vitro and in vivo investigations were conducted to assess the toxicity of LA and the development of asbestos-related disease (ARD). In vitro exposure to unprocessed samples of LA "6-mix" (containing significant quantities of nonrespirable material) induced both oxidative stress and apoptosis in murine alveolar macrophages (AM) (Blake et al. 2007; 2008) and oxidative DNA damage and chromosomal breaks in human lung epithelial cells (Pietruska et al. 2010). LA "6-mix" was also used for in vivo studies in mice and was shown to induce collagen synthesis and lung fibrosis, as well as to alter gene expression in pulmonary tissue, primarily of membrane-associated genes after a single intratracheal (IT) instillation (Putnam et al. 2008; Smartt et al. 2010). Webber et al. (2008) conducted studies using a size-fractionation water elutriation method developed to isolate rat-respirable fractions of LA (equivalent to $PM_{2.5}$) in which larger, nonrespirable particles and fibers are removed from the sample. Exposure of primary human airway epithelial cells to elutriated (i.e., respirable) LA resulted in a concentration-dependent increase in gene expression of many inflammatory mediators (Duncan et al. 2010). Our laboratory recently demonstrated similar gene expression changes up to 3 mo after a single IT instillation of elutriated LA in rats (Padilla-Carlin et al. 2011).

Although these studies have been critical in developing our understanding of the acute toxicity of LA, the long-term effects and contributions to ARD development in animal models are still poorly understood. To date, the only published in vivo study to assess the chronic results of exposure to LA demonstrated fibrosis and increased expression of membrane-associated genes 6 mo after IT instillation of unprocessed LA in mice (Putnam et al. 2008). Due to the long latent period of ARD development, examination of later time points would be beneficial in discerning the toxicity of LA. Furthermore, the use of elutriated rat-respirable samples may also provide a more realistic representation of the fibers that contribute to pulmonary disease produced by asbestos: those that are most capable of deposition in the airways and deep lung. The purpose of this current study was to evaluate the long-term inflammatory, fibrotic, and carcinogenic effects following a single IT exposure to respirable fractions of either LA or the positive control fiber amosite, which has been shown to produce significant adverse effects including asbestosis, cancer, and mesothelioma in rat models (Coffin et al 1982; Davis et al. 1978; Wagner et al. 1974). Markers of inflammation, lung injury, fibrosis, and mesothelioma, as well as pathologic changes in the lung were examined at both 1 and 2 yr following IT instillation of respirable fractions of LA or amosite in rats.

METHODS

Asbestos

Libby amphibole (LA) was collected from the Rainy Creek Complex located near Libby,

MT, by the U.S. Geological Survey (USGS, Denver, CO) in 2000 (LA2000). Physical and chemical characteristics of the LA2000 sample were reported previously (Meeker 2003). Standard amosite amphibole asbestos was used as a positive control and was obtained from the Research Triangle Institute (Research Triangle Park, NC).

Water Elutriation

LA and amosite samples were prepared as described previously (Duncan et al. 2010; Padilla-Carlin et al. 2011). Briefly, water elutriation was utilized to separate asbestos fibers with aerodynamic diameters $\leq$ 2.5 μm ($PM_{2.5}$) from larger fibers according to a settling velocity based upon the particle density and radius. These collected asbestos fibers are considered to be within the target respirable range for rodent species (Webber et al. 2008). All samples were determined to be negative for endotoxin (<0.06 EU/ml; Associates of Cape Cod, East Falmouth, MA).

Characterization of Elutriated Asbestos Preparations

Particle size distributions (length, width, and aspect ratio) were counted for approximately 1000 particles for the amosite and LA $PM_{2.5}$ samples using scanning electron microscopy (SEM) as described previously (Duncan et al. 2010; Padilla-Carlin et al. 2011). A complete report of particle dimensions in attached spreadsheets can be found online in the USGS report by Lowers and Bern (2009). These reports are summarized in supplemental tables in Duncan et al. (2010), and a condensed summary of the particle size distribution is presented in Table 1.

Animals and Experimental Design

All procedures were approved by the Institutional Animal Care and Use Committee (National Health and Environmental Effects Research Laboratory [NHEERL], U.S. Environmental Protection Agency [EPA]), and 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 Fischer 344 rats (Charles River Laboratories, Raleigh, NC), 6–8 wk of age, were double housed in polycarbonate cages with Beta Chip bedding, and acclimatized for at least 2 wk prior to use in this investigation. 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 221 ± 20 g (mean ± SD). The animals were placed into four groups: saline, amosite (0.65 mg/rat), low-dose LA (0.65 mg/rat), and high-dose LA (6.5 mg/rat). Eight animals from each group were necropsied 1 yr after instillation. Twenty-four animals were assigned to each group for the 2-yr necropsy. This group was designed to be larger to accommodate for the loss of animals due to strain- and/or age-related disease. Unscheduled deaths (primarily due to leukemia and various tumors, as typical for this strain) reduced this number to 11–15 rats per group; differing animal numbers for experiments are noted in the figure legends. Due to this increasing rate of mortality, the final necropsy was performed approximately 22 mo

TABLE 1. Particle Descriptive Statistics for Elutriated Libby Amphibole (LA) and RTI Amosite

Variable	Particles counted	Mean	Median	Minimum	Maximum	Std. dev.
LA length	1175	1.87	1.18	0.22	23.60	2.08
LA width	1175	0.29	0.27	0.02	3.56	0.23
Amosite length	967	2.73	2.60	0.21	103.58	11.22
Amosite width	967	0.28	0.28	0.02	2.95	0.21

Note. Data summarized from the USGS report by Lowers and Bern (2009).

after IT; however, results are still expressed as 2 yr post-IT.

Intratracheal 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. Asbestos suspensions in sterile saline were prepared and sonicated for 5 min, and then shaken by hand prior to each instillation. Animals received an IT instillation of either saline (negative control; 0.0 mg/rat), LA2000 (0.65 or 6.5 mg/rat), or amosite (positive control; 0.65 mg/rat) in a total volume of 250 μl.

Bronchoalveolar Lavage Collection

Rats were anesthetized with an overdose of Euthasol (Virbac Corp., Fort Worth, TX; pentobarbital sodium and phenytoin sodium; diluted 1:1 with 0.9% nonbacteriostatic saline) and euthanized by exsanguination of the abdominal aorta. For collection of bronchoalveolar lavage fluid (BALF), the trachea was cannulated, the left lung was ligated, and the right lung was lavaged with Ca^{2+}/Mg^{2+}-free PBS (pH 7.4; warmed to 37°C) 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 percentages 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*-acetylglucosaminidase (NAG) activity (Roche Diagnostics, Indianapolis, IN), and γ-glutamyl transferase (GGT) activity (Thermo Trace Ltd., Melbourne Australia). 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).

RNA Isolation and Real-Time PCR

The right caudal lung lobe was dissected, frozen immediately in liquid nitrogen, and stored at –80°C. Total RNA from the apical portion of the caudal lobe (approximately 30 mg of tissue) was then isolated using an RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions and subsequently treated with 1 μl RNAsin plus RNAse Inhibitor (ProMega, Madison, WI). RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Total RNA was then converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Real-time polymerase chain reaction (RT-PCR) was conducted using FastStart Universal Probe Master mix (Roche, Mannheim, Germany). Target cDNA was amplified using predesigned FAM dye-labeled TaqMan probes (ABI, Foster City, CA) for interleukin-1 beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), heme-oxygenase-1 (HO-1), macrophage inflammatory protein 2 (MIP2), collagen (Col1A1, Col1A2, and Col3A1), mesothelin (Msln), Wilms' tumor gene 1 (WT1), epidermal growth factor receptor (EGFR), and endogenous control 18S. Relative changes in mRNA expression were calculated following normalization to 18S.

Serum Mesothelin

Blood was collected through the abdominal aorta into a serum separator tube and centrifuged at 3500 rpm at 4°C for 10 min. Collected serum was stored at –80°C until time of analysis. Serum mesothelin (Msln) levels were determined using a rat N-ERC/Mesothelin Assay Kit enzyme-linked immunosorbent assay (ELISA; Immuno-Biological Laboratories) following provided manufacturer's instructions.

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 (H&E) or Masson's trichrome for collagen specific analysis. All lung sections were examined by a certified veterinary pathologist by light microscopy. The European Society of Toxicologic Pathology IN-HAND (International Harmonization of Nomenclature and Diagnostic criteria) classification method was used to evaluate the histopathological lesions (www.goreni.org). Semiquantitative scoring was conducted by evaluating various pathological indices using severity scores (0 = not present, 1 = minimal (<10% of examined area), 2 = mild (11–40%), 3 = moderate (41–80%); 4 = marked (81–100%)). Both the number of incidences and average scores for the incidences were evaluated for each pathological change, and an overall score was determined for each animal by adding the scores of all the animals within a group and then dividing the sum by the total number of animals.

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 post hoc test as necessary. Data are shown as mean ± SEM. Differences with $p < .05$ were considered statistically significant.

RESULTS

Inflammation and Lung Injury

To assess the possibility of continued inflammatory influx in asbestos-exposed rats, total cells were measured in BALF at 1 and 2 yr following a single IT exposure to saline, low LA (0.65 mg/rat), high LA (6.5 mg/rat), or amosite (0.65 mg/rat) (Figure 1, A–D). One year after exposure, total BALF cells (Figure 1A) remained similar to control levels in all asbestos-exposed groups, and consisted primarily of macrophages (approximately 75%) (Figure 1B). Two years after exposure, total cell levels had increased in all groups compared to cell numbers at 1 yr postexposure. This rise was due to increased numbers of neutrophils and lymphocytes (Figure 1, C and D, respectively), most likely due to age and not treatment, as the asbestos-exposed groups were not significantly different from the group exposed to saline. Consistent with this, expression levels of the inflammatory markers IL-6, MIP-2, TNF-α, and IL-1β were equal to those of controls at both 1and 2 yr after asbestos exposure (data not shown).

To assess chronic inflammation in the tissue, the left lung was fixed and stained for histopathological analysis. Histopathology of the left lung lobe (summarized in Table 2) indicates some intra-alveolar macrophage accumulation in the 2-yr saline-treated animals (Figure 2B), but not in the 1-yr saline-treated rats (Figure 2A). Although inflammatory influx was not augmented in BALF of animals 1 or 2 yrs post asbestos exposure, some level of granulomatous inflammation characterized by intra-alveolar or interstitial accumulation of macrophages associated with alveolar epithelial hyperplasia was observed in both 1- and 2-yr groups exposed to either LA or amosite (Figure 2, C–H). At 1 yr postexposure, most lung samples from low-LA (0.65 mg/rat) animals consisted of grade 1 (minimal) intra-alveolar accumulation of macrophages, associated with alveolar epithelial hyperplasia, which was located close to the terminal and respiratory bronchioles (Figure 2C). However, in some of the lungs, no pathological reaction was

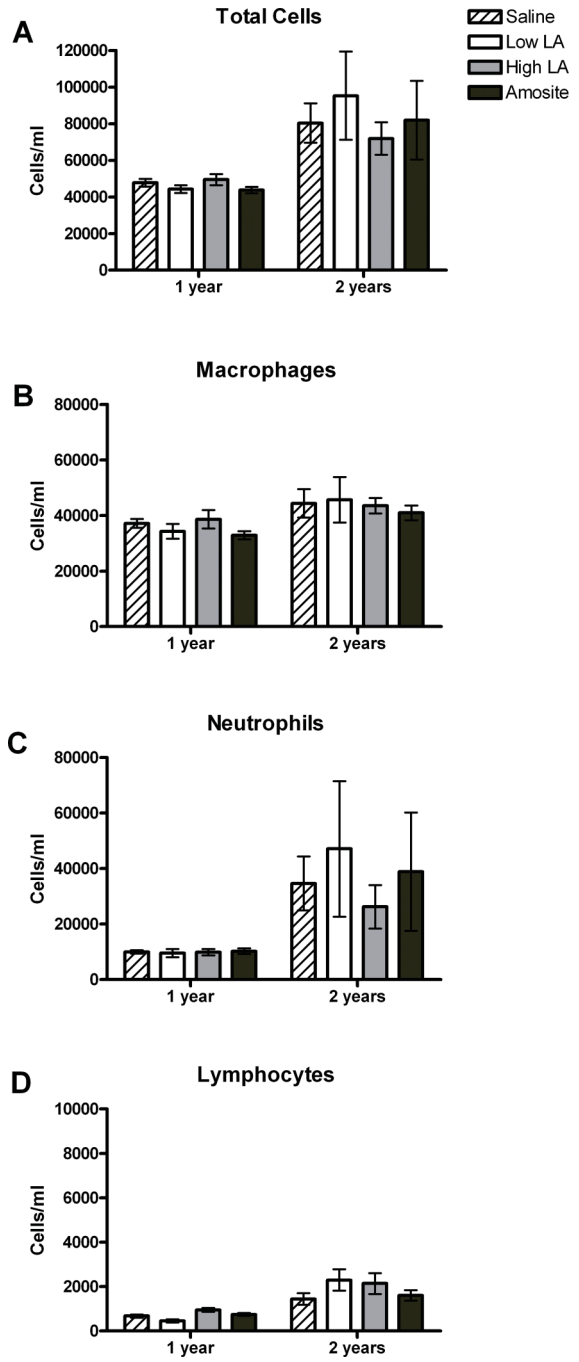

FIGURE 1. Total cells (A), macrophages (B), neutrophils (C), and lymphocytes (D) were counted in BALF both 1 and 2 yr following intratracheal instillation of saline, LA (0.65 or 6.5 mg/rat), or amosite (0.65 mg/rat). No significant differences among groups were found. For 1 yr: $n = 8$, all groups. For 2 yr: saline, $n = 11$; low LA, $n = 14$; high LA, $n = 14$; amosite, $n = 15$.

noted. High-LA (6.5 mg/rat) animals exhibited similar histopathological characteristics, albeit with a dose-dependent increase in severity up to grade 2 (mild, Figure 2E). Sporadically, the presence of macrophages in high-LA lungs was associated with grade 1 (minimal) presence of intra-alveolar neutrophils. In some cases, minimal interstitial mononuclear cell infiltration surrounded the intra-alveolar macrophages and in only one case a single multinucleated giant cell was noted, intermixed with the macrophages. Lungs of animals exposed to amosite (0.65 mg/rat) consisted of mostly grade 2 (mild) interstitial granulomatous reactions, consisting predominantly of fibroblasts, associated with various degrees of maturing collagen. In addition, within these granulomas, aggregates of macrophages were noted with the occasional presence of multinucleated giant cells. Characteristically, the interstitial granulomas were at the peribronchial location, or distributed at random in the lung parenchyma.

Two years post exposure, treatment-related changes in the lungs of the low- and high-LA-exposed rats (Figure 2, D and F, respectively) consisted of dose-related increased (minimal to mild) intra-alveolar and interstitial accumulation of macrophages, located in association with the terminal and respiratory bronchioles. Multinucleated giant cells were rarely seen, and were not considered a characteristic of this granulomatous reaction. The interstitial macrophage reaction was associated with the presence of spindle-shaped cells suggested to be fibroblasts, but no collagen deposition was noted by H&E stain. Alternatively, amosite-exposed lungs consisted of interstitial accumulation of fibroblasts, associated with the presence of only few macrophages, located close to the terminal and respiratory bronchioles (Figure 2H). The interstitial reaction in these animals was associated with relatively prominent collagen deposition, and multinucleated giant cells were frequently seen within these multifocal interstitial fibrotic nodules.

BALF was also analyzed for markers of lung injury, including lactate dehydrogenase (LDH) activity, *N*-acetylglucosaminidase (NAG) activity, γ-glutamyl transferase (GGT) activity, total protein content, and albumin. One year after asbestos exposure, markers of cellular toxicity (LDH), macrophage lysosomal activation (NAG), membrane damage (GGT), and epithelial permeability and vascular leakage (total

TABLE 2. Incidence Table of Pathologic Changes at 1 yr and 2 yr After a Single IT Exposure of Libby Amphibole (LA) or Amosite in F344 rats

Treatment and time point	Animals per group	Intra-alveolar AM aggregation	Intra-alveolar AM aggregation with interstitial fibroplasia	Alveolar epithelial hyperplasia	Interstitial granulomatous reaction[a]	Large giant cells
Saline						
1 yr	8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2 yr	11	0.5 (4)	0 (0)	0.5 (3)	0 (0)	0.2 (1)
LA (0.65 mg/rat)						
1 yr	8	0.5 (4)	0 (0)	0.4 (3)	0 (0)	0 (0)
2 yr	14	0.1 (2)	0.8 (7)	0.1 (1)	0.1 (2)	0.5 (5)
LA (6.5 mg/rat)						
1 yr	8	1.6 (7)	0 (0)	1.6 (7)	0 (0)	0.1 (1)
2 yr	14	0.1 (1)	1 (12)	0.2 (2)	1.4 (13)	0 (0)
Amosite (0.65 mg/rat)						
1 yr	8	0 (0)	0 (0)	0 (0)	1.3 (6)	0 (0)
2 yr	15	0 (0)	0.5 (7)	0.2 (2)	2.1 (14)	0.1 (1)

Note. Values represent the average histopathological score, with the number of observations in each group in parentheses.

[a]Characterized by interstitial AM aggregation, consisting primarily of fibroblasts and associated with varying degrees of maturing collagen and the occasional presence of multinucleated giant cells.

protein and albumin) were similar to those of saline-treated rats (Figure 3, A–D). The activity levels of these enzymes and total protein content were increased in the 2-yr animals; however, there was no significant difference between asbestos-exposed and control animals. In contrast, albumin was significantly elevated in both LA and amosite-treated animals 1 yr after exposure (Figure 3E). Although there is a trend of continued rise in albumin 2 yr after exposure to low and high LA, this did not reach statistical significance due to an age-associated increase of albumin in the saline-treated control animals.

Fibrosis

Collagen accumulation is a known marker of fibrosis; therefore, RT-PCR of a section of the right caudal lobe was performed to determine the relative gene expression changes of the collagen markers Col 1A1, Col 1A2, and Col 3A1 in response to asbestos exposure (Figure 4, A, B, and C, respectively). Compared to saline-exposed control rats, the expression of Col 1A1, Col 1A2, and Col 3A1 was unchanged in either LA or amosite-exposed rats 1 yr after a single IT-instillation. However, 2 yr postinstillation there was a trend of enhanced expression in all three collagen markers in all asbestos-exposed animals. Despite this relative increase in collagen expression across the board in both LA- and amosite-treated rats, only expression of Col 1A2 and Col 3A1 in the low-LA group reached statistical significance over saline-treated rats 2 yr postexposure.

In addition to collagen mRNA expression, fibrosis was evaluated by staining the left lung lobe with Mason's trichrome collagen stain. At 1 yr postinstillation no staining was seen in saline treated animals (Figure 5A). Some significant staining was seen in some rats treated with low LA (Figure 5C), while more staining was apparent in animals treated with high LA (Figure 5E), consistent with minimal fibrosis. Amosite-treated rats showed enhanced collagen staining indicating mild fibrosis (Figure 5G). At 2 yr postinstillation, no collagen was noted in saline-exposed animals (Figure 5B), while staining was increased in all asbestos-treated groups, consistent with minimal, mild, and moderate fibrosis in low-LA-treated (Figure 5D), high-LA-treated (Figure 5F), and amosite-treated (Figure 5H) rats, respectively. Numerical results of collagen deposition are summarized in Table 3. Briefly, although fibrosis was both dose- and time-dependently increased in LA-exposed rats, the

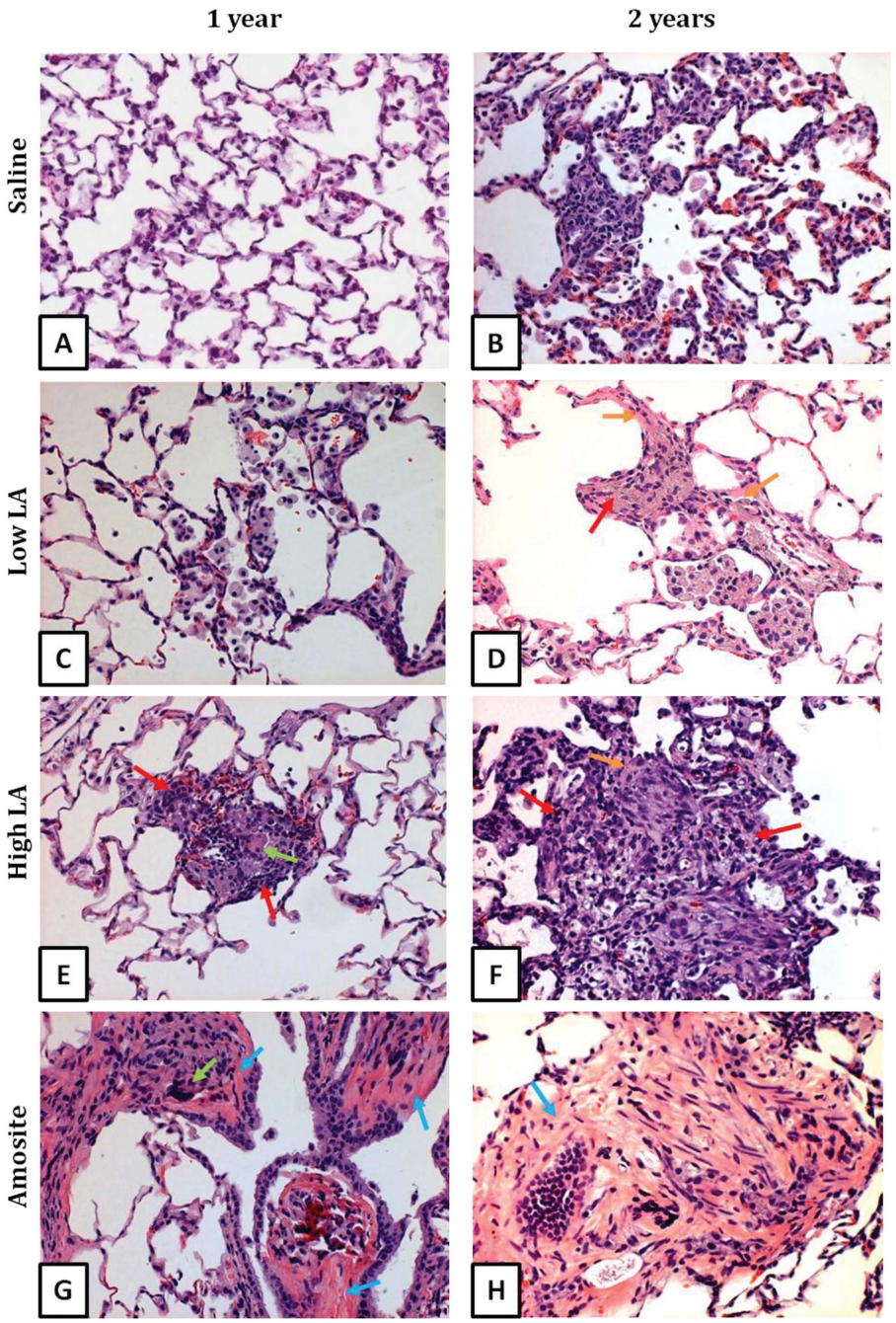

FIGURE 2. H&E staining of the left lung after a single IT exposure to saline (A, B), low-dose LA (0.65 mg/rat; C, D), high-dose LA (6.5 mg/rat; E, F), or amosite (0.65 mg/rat; G, H). Sections were taken 1 yr (left panel; A, C, E, G) and 2 yr postinstillation (right panel; B, D, F, H). At 1 yr (left panel), interstitial mononuclear cell infiltration (red arrows) and few multinucleated giant cells (green arrows) were noted intermixed with macrophages in both high-dose LA and amosite groups. Mature collagen (blue arrows) was present in amosite-treated animals. At 2 yr postinstillation (right panel) both low- and high-LA-dosed rats show interstitial accumulation of macrophages (red arrows) and fibrosis (orange arrows). Only amosite-dosed rats exhibited mature collagen (blue arrows). Magnification 20×.

degree of fibrosis was significantly greater in amosite-exposed animals at both 1 and 2 yr postinstillation.

Markers of Carcinogenicity

To assess the long-term effects of exposure to a single IT instillation of either LA

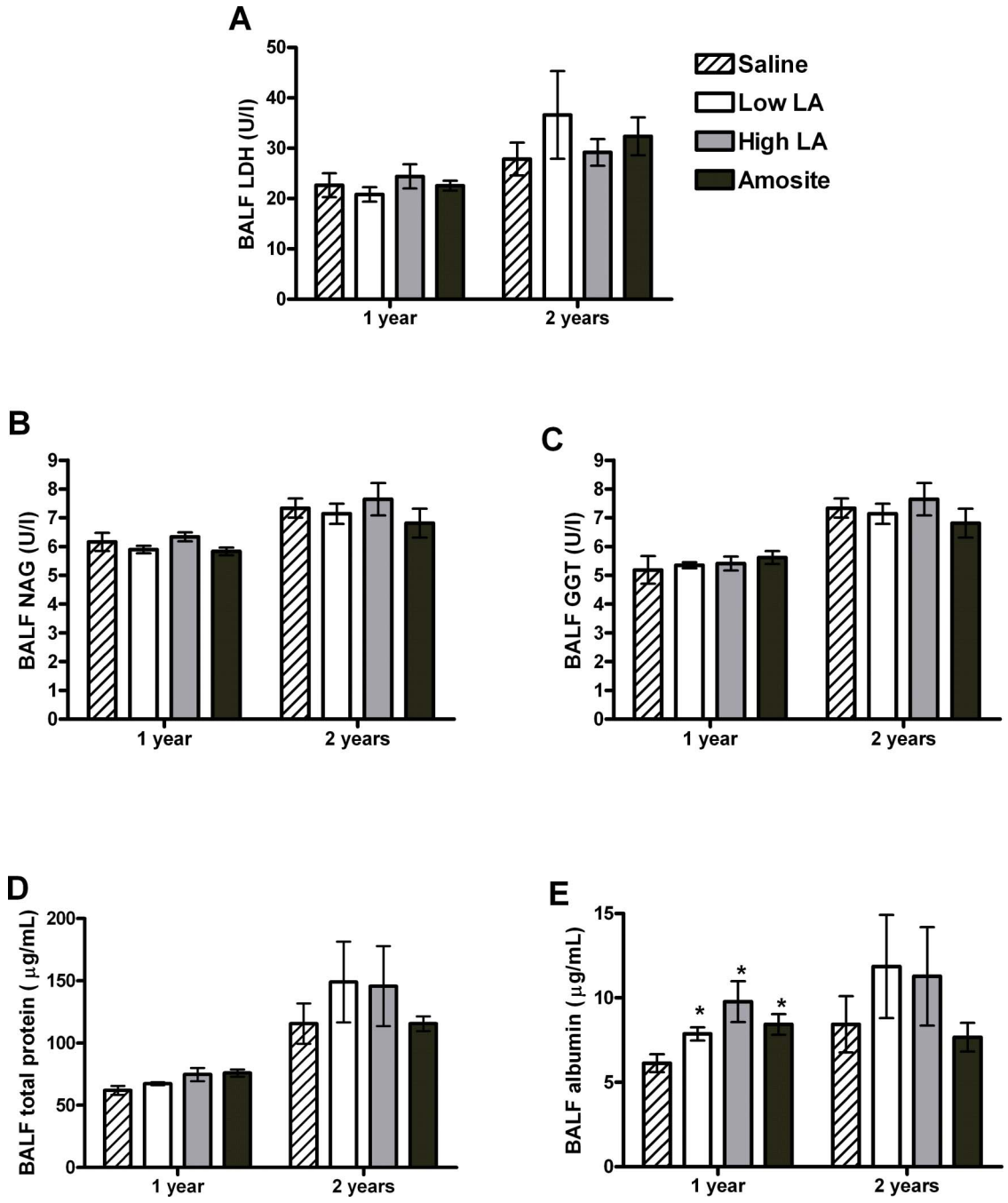

FIGURE 3. BALF was analyzed for markers of cellular toxicity (LDH, A), macrophage lysosomal activation (NAG, B), membrane damage (GGT, C), and epithelial permeability/lung damage (total protein and albumin, D and E, respectively) 1 yr and 2 yr following intratracheal instillation of saline, LA (0.65 or 6.5 mg/rat), or amosite (0.65 mg/rat). At 1 yr after exposure, only BALF albumin was significantly increased in all asbestos-treated animals; all other parameters had returned to control levels. By 2 yr postinstillation all BALF parameters were at control levels. Asterisk indicates significant at $p < .05$ compared to saline controls of the same age. For 1 yr: $n = 8$, all groups. For 2 yr: saline, $n = 11$; low LA, $n = 14$; high LA, $n = 14$; amosite, $n = 15$.

or amosite asbestos, the development of lung cancer and/or mesothelioma was determined 1 and 2 yr after exposure. No potential preneoplastic changes were seen pathologically in H&E-stained sections of the left lung lobe from any of the saline or asbestos-exposed groups at either 1 or 2 yr postexposure. However, mRNA expression of several mesothelioma markers

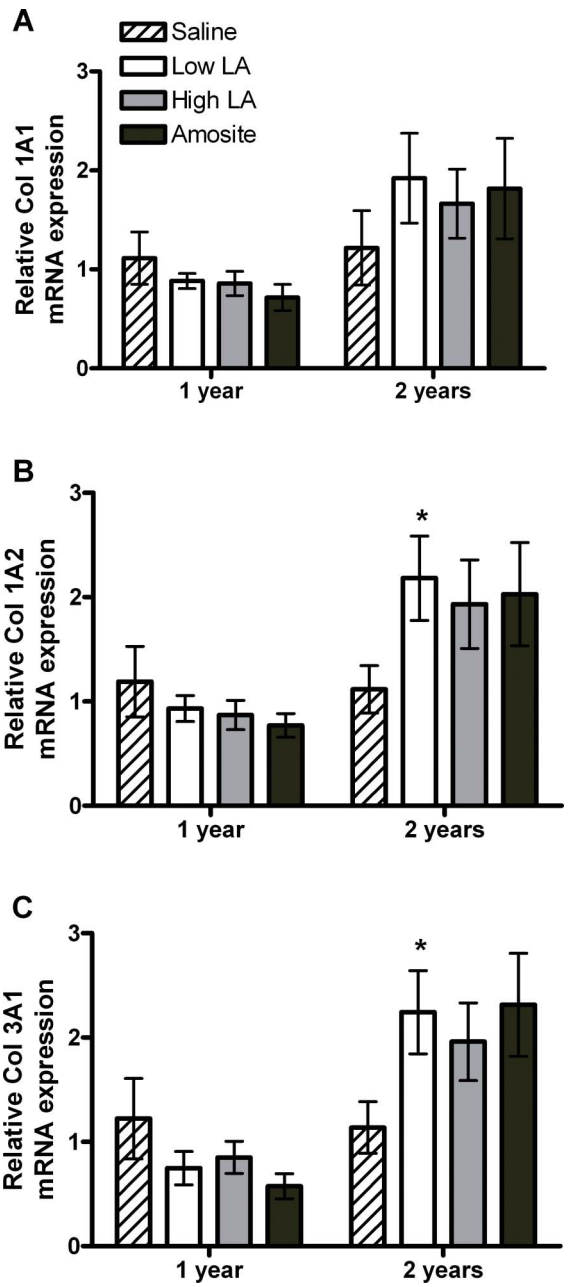

FIGURE 4. mRNA expression of markers for fibrosis 1 yr (left panel) and 2 yr (right panel) following intratracheal instillation of saline, LA (0.65 or 6.5 mg/rat), or amosite (0.65 mg/rat). Total RNA was collected from the apical portion of the caudal lobe, and expression of Col 1A1 (A), Col 1A2 (B), and Col 3A1 (C) was determined by RT-PCR. Two years after instillation, there was a trending increase of all three collagen markers in all asbestos-exposed rats; however, only the low-dose group reached statistical significance. For all groups $n = 6$; asterisk indicates significant at $^*p < .05$ compared to saline.

and serum Msln was also analyzed. RT-PCR of a section of the right caudal lobe was performed to determine the relative gene expression changes of the mesothelioma-associated genes Msln, Wilms' tumor gene 1 (WT1), and EGFR in response to asbestos exposure (Figure 6, A, B, and C respectively). In addition, serum Msln levels were determined by ELISA (Figure 7). At 1 yr after exposure, the expression of WT1 mRNA in animals exposed to low LA was significantly increased by threefold relative to saline-treated controls (Figure 6B). This rise in gene expression was significantly greater than the expression in both high-LA and amosite groups, which was not different than in the saline-challenged rats. No marked changes were seen in Msln or EGFR expression at 1 yr postexposure. Consistent with the expression of Msln mRNA, serum Msln was also unchanged 1 yr after exposure in asbestos-treated rats compared to controls (Figure 7).

Relative to saline controls, gene expression of all three mesothelioma markers were increased in lung tissue 2 yr after a single exposure to LA. Although there was some elevation in Msln expression in animals exposed to amosite, only rats that received a low-dose treatment of LA showed significantly increased Msln mRNA expression at this time (Figure 6A). Conversely, in the serum, Msln was significantly elevated in 43% of animals from all groups, including the saline controls, compared to levels at 1 yr postexposure (Figure 7). Similar to the 1-yr time point, WT1 expression was increased threefold in rats that had been exposed to low LA, while expression in high-LA-treated or amosite-treated rats was no different than in saline controls (Figure 6B). EGFR expression was enhanced 2.5-fold in all asbestos-treated rats compared to saline controls 2 yr after instillation (Figure 6C).

DISCUSSION

It is well known that individuals exposed to asbestos often develop lung disease later in life

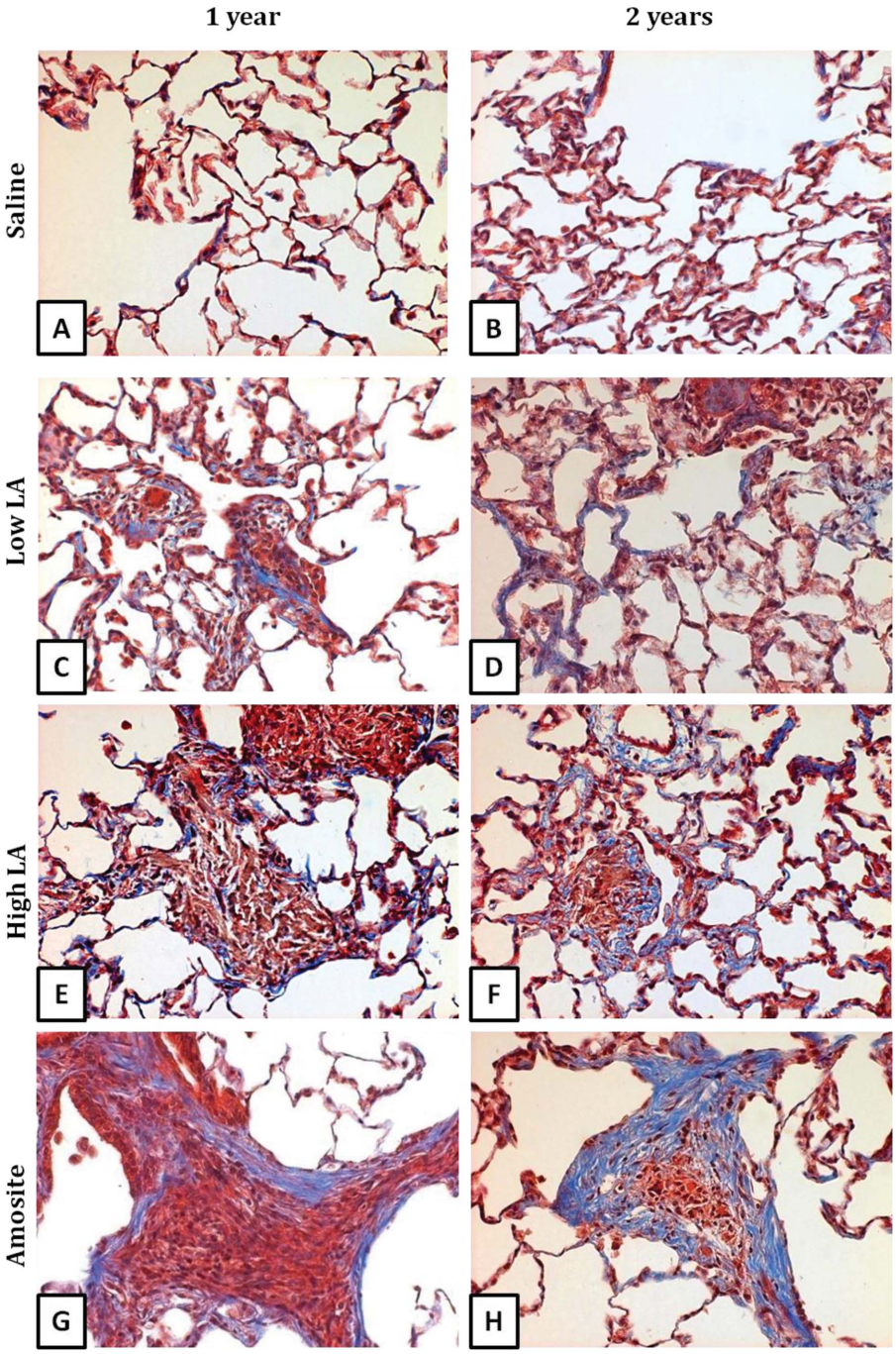

FIGURE 5. Masson's trichrome staining for collagen of the left lung lobe after a single IT exposure to saline (A, B), low-dose LA (0.65 mg/rat; C, D), high-dose LA (6.5 mg/rat; E, F), or amosite (0.65 mg/rat; G, H). Sections were taken 1 yr (left panel; A, C, E, G) and 2 yr postinstillation (right panel; B, D, F, H). Collagen deposition is noted as blue staining. Magnification 20×.

(Broaddus et al. 2011; Mossman et al. 2011). Development processes of pleural plaques, diffuse pleural thickening, asbestosis, lung cancer, and mesothelioma often have a long latency period, developing anywhere from 10 to 40 yr after exposure (Currie et al. 2009). To examine the development of ARD, experimental studies have analyzed the long-term (>6 mo) effects of different types of amphibole (actinolite, amosite, anthophyllite, crocidolite, tremolite)

TABLE 3. Average Fibrosis Score Following a Single IT Exposure of Saline, Low Libby Amphibole (LA), High LA (0.65 and 6.5 mg/rat, Respectively), or Amosite (0.65 mg/rat)

	Time after instillation	
Group	1 yr	2 yr
Saline	0 ± 0 (8)	0 ± 0 (11)
Low LA	0.25 ± 0.16 (8)	0.67 ± 0.11 (18)*
High LA	0.88 ± 0.13 (8)#	1.88 ± 0.13 (16)*Ψ
Amosite	1.50 ± 0.33 (8)#	3.0 ± 0 (17)*Ψ

Note. Significant differences indicated by: #, $p < .05$ compared to 1 yr saline; asterisk, $p < .05$ compared to 2 yr saline; Ψ, $p < .05$ compared to 1 yr same group.

Note. A score of 1, 2, or 3 was assigned to each animal to indicate minimal, mild, and moderate fibrosis respectively. Values represent the average fibrosis score ± SEM with the number of animals per group in parentheses.

and/or serpentine asbestos (chrysotile) in animal models via different exposure routes (Wagner et al. 1974; 1982; 1984; Davis et al. 1978; Coffin et al. 1982; Hirano et al. 1988), although to date there have been no published long-term studies of LA.

While inhalation studies have been successful in inducing the development of fibrosis and various forms of cancer, they were complicated by the fact that large amounts of starting material were needed. Moreover, intrapleural studies utilized a nonphysiological route of exposure, as well as high doses of asbestos. Alternatively, IT instillations have been used to introduce precise quantities of asbestos directly to the lungs using a route that is more controlled than inhalation and more physiologically relevant to occupational and/or environmental exposures than intrapleural injection. However, a caveat to IT studies is the possibility of fiber agglomeration, which is not seen after inhalation exposure (Driscoll et al. 2000; Bernstein et al. 1997). IT instillation of LA in both mice and rats results in an immediate acute inflammatory response that progressively develops into fibrosis (Smartt et al. 2010; Padilla-Carlin et al. 2011). Previous work from our laboratory showed that rats exposed to LA develop immediate, dose-dependent granulomatous inflammation, denoted by intra-alveolar or interstitial accumulation of macrophages, accompanied by

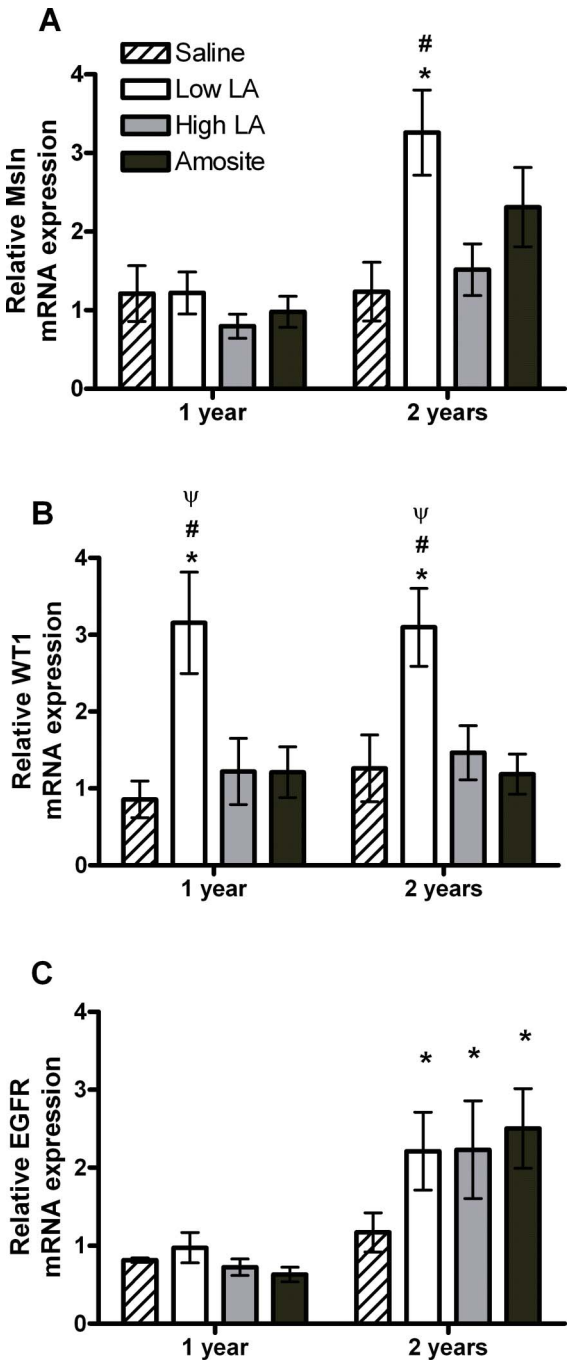

FIGURE 6. mRNA expression of mesothelioma markers 1 yr and 2 yr following IT of saline, LA (0.65 or 6.5 mg/rat), or amosite (0.65 mg/rat). Total RNA was collected from the apical portion of the caudal lobe, and expression of Msln (A), WT1 (B), and EGFR (C) was determined by RT-PCR. One year postinstillation, rats exposed to low-dose LA had a threefold increase in expression of WT1. Two years after exposure, EGFR was significantly increased in all asbestos-exposed groups, but only the low-dose LA group showed a significant increase in Msln and WT1. For all groups, $n = 6$; asterisk indicates significant at $p < .05$ compared to saline; #, significant at $p < .05$ compared to high LA; and Ψ, significant at $p < .05$ compared to amosite.

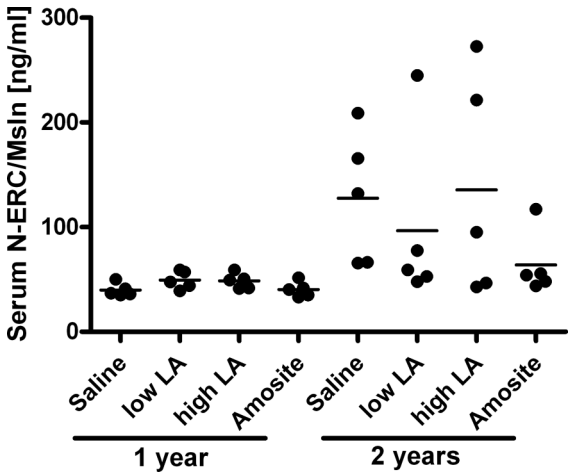

FIGURE 7. Serum N-ERC/Msln protein levels 1 and 2 yr following a single IT of saline, LA (0.65 or 6.5 mg/rat), or amosite (0.65 mg/rat). Blood was collected from the abdominal aorta, and serum N-ERC/Msln levels were determined by ELISA. One year post IT, N-ERC levels were consistent with saline-exposed controls and clustered closely together. Two years post IT, serum N-ERC/Msln levels were elevated in some rats of all exposure groups, including controls, and the variability between rats was increased compared to the 1-yr animals. Each point represents an individual rat. For all groups, $n = 5$.

an influx of neutrophils that gradually waned over the 3-mo period. While rats exposed to amosite also demonstrated immediate granulomatous inflammation, this inflammation rapidly reverted to interstitial AM aggregation that was associated with proliferating fibroblasts ultimately resulting in collagen deposition (granulomatous reaction) by 3 mo postinstillation (Padilla-Carlin et al. 2011). These patterns of acute inflammation were reflected in the chronic inflammation noted 1 and 2 yr post instillation of LA or amosite. At 1 yr post-IT, rats exposed to LA demonstrated dose-dependent intra-alveolar and interstitial AM aggregation that developed into graulomatous reactions, characterized by interstitial AM aggregation, fibroblast proliferation, and collagen deposition by 2 yr. Again, a more severe chronic inflammation was seen in rats exposed to amosite at the same mass dose. Most amosite-exposed rats demonstrated at 1 yr post-IT granulomatous reaction that increased in severity over time, resulting in significant collagen deposition and fibrosis over the entire time course of this study. Visible fiber agglomeration was not noted histologically either at early time points, or 1–2 yr after IT instillation.

The development of asbestos-induced lung fibrosis depends on the complex actions and interactions of several cell types, the cytokines released by these cells, and the extracellular matrix (Bienkowski and Gotkin 1995). Many studies showed that asbestos exposure increased tissue collagen content as well as expression of collagen genes (Hirano et al. 1988; Morimoto et al. 1997; Putnam et al. 2008; Smartt et al. 2010). In the current study, both amphiboles produced dose- and time-dependent elevation in lung collagen deposition up to 2 yr after exposure, although the response was greater in animals exposed to amosite. These results are consistent with greater fiber lengths and aspect ratios observed in elutriated amosite fibers compared with those of elutriated LA fibers (Duncan et al. 2010; Lowers and Bern 2009); specifically, fibrosis correlated best with the number of fibers $\geq$10 μm in length (Table 4). Although significant collagen deposition was seen in all groups 1 yr after instillation, the expression of type I and type III mRNA was not elevated compared to controls. Interestingly, at 2 yr postexposure, although both amphiboles appeared to induce comparable expression of type I and III mRNA, only exposure to low LA induced a statistically significant rise in the expression of Col1A2 and Col3A1. Although this could simply reflect the variability of the aged animals, it is also possible that the expression of genes in this group was delayed compared to high LA and amosite due to the lower dose and lesser fibrogenicity, respectively. Alternatively, the lower dose of LA may be less potent in inducing the release of cytokines that are responsible for negative feedback inhibition of gene expression in response to collagen deposition.

Other studies noted similar discrepancies between collagen deposition and gene expression at more acute time points. Smartt and colleagues (2010) found that a single IT exposure to LA or crocidolite in mice produced both accumulation of collagen in lung tissue, as well as an initial increase in the expression

TABLE 4. Correlation (R^2) of Fibrosis Scores at 1 and 2 yr Post IT Instillation of Amosite (0.65 mg/rat) or Libby Amphibole (LA; 0.65 or 6.5 mg/rat) or Saline Vehicle Only With the Number of Particles (≥ 0.2 μm) and Fibers (≥ 5, 10, 20 μm) Calculated in These Sample Doses

	Time after instillation	
Length (μm)	1 yr	2 yr
≥0.2	0.0747	0.0925
≥5.0	0.3367	0.3645
≥10	0.9677	0.9632
≥20	0.7999	0.7617

Note. Number and length of fibers in each size category were calculated from the data provided in Lowers and Bern (2009).

of collagen genes. Although both exposures resulted in similar expression of Col1A1 and Col3A2 mRNA at each time point, crocidolite exposure resulted in greater collagen deposition that persisted through 3 mo despite gene expression returning to control levels at this point. Putnam et al. (2008) demonstrated that collagen deposition was still elevated 6 mo after crocidolite exposure. In addition, Padilla-Carlin et al. (2011) showed previously that during early time points (up to 3 mo) after a single IT exposure of LA or amosite in rats, expression of type I mRNA in the lung was either unchanged or even downregulated compared to controls, although mild fibrosis (grade 2) was evident only in amosite-treated animals by 3 mo postinstillation. Consistent with this, expression of type 1 collagen was found to be unchanged 1 mo after a single IT of chrysotile in rats (Morimoto et al. 1997); however, lung collagen content is significantly increased 1 mo after chrysotile exposure and continues to rise over time (Hirano et al. 1988). These studies indicate that persistence and accumulation of collagen may or may not correlate with the expression of collagen genes at later time points, but rather may reflect changes in protein synthesis and/or collagen degradation.

Asbestos exposure is a well-known risk factor in the development of lung cancer and mesothelioma. Although multiple IT instillations and intrapleural injections of some types of asbestos were found to induce development of mesothelioma in rats (Coffin et al. 1982; Wagner et al. 1982; 1984), this response has not been reported after a single IT instillation of LA. This is the first published study to evaluate the in vivo carcinogenicity of LA exposure in laboratory animals. Due to the overwhelming epidemiological data demonstrating the carcinogenic effects of LA in individuals residing in Libby, MT (ATSDR 2002; McDonald et al. 2004; Sullivan 2007; Weill et al. 2010; Whitehouse et al. 2008), it was postulated that there would be similar carcinogenic effects in rats exposed to LA. However, no preneoplastic changes (such as mesothelial-cell hyperplasia), lung tumors, or mesotheliomas were noted histologically either 1 or 2 yr following IT instillation of LA in this study. Similarly, no carcinogenic changes were associated with the positive control amosite, despite the documented ability of this fiber to induce lung cancer and/or mesothelioma in rats (Coffin et al. 1982; Davis et al. 1978; Wagner et al. 1974). Coffin et al. (1982) showed that IT instillation of amosite resulted in tumor and mesothelioma development. However, their experiments employed a dose of asbestos that was mid-range (3 mg/rat) to the doses of asbestos used in the current study, approximately 5 times more animals were used per group. Even with large number of animals to screen, lung tumors and mesotheliomas were detected in only 0.7 and 1.4%, respectively, of rats exposed to amosite by IT instillation. Based on these numbers, less than 1 out of 24 rats exposed to amosite in the 2-yr cohort would be predicted to develop a lung tumor and/or mesothelioma; however, our high dose of LA was roughly twofold higher than the IT dose given in that study, so there was a possibility we could detect carcinogenic effects using fewer animals. Another caveat to our study design is the length of time after exposure. Historical studies highlight the need to extend past the 2-yr time point as much as the lifetime of the animal (Coffin et al. 1982; Wagner et al. 1982; 1984), which in some cases was approximately 2.5 yr after initial exposure. However, termination of the experiment at 22 mo was chosen, when mortality in the control saline-exposed

group reached >50%. This truncated protocol might explain the lack of sensitivity for detecting carcinogenic effects. It is also possible that more detailed examination of pleural tissues might reveal the presence of mesotheliomas.

Despite the absence of histological carcinogenic effects, LA or amosite produced some changes in the mesothelioma-specific biomarkers Msln, WT1, and EGFR by 2 yr postinstillation, suggesting the possible induction of pathways leading to tumor development. Mesothelin (Msln) is normally only expressed in the mesothelial cells lining the pleura, pericardium, and peritoneum, but may be highly expressed in several types of cancer and mesothelioma (Hassan et al. 2004)). Similarly, WT1 is restricted mostly to mesenchymally derived tissues (Park et al. 1993), although transcripts have been detected by Northern analysis in mesothelioma cell lines derived from rats exposed to asbestos, as well as cell lines derived from human patients with mesothelioma (Amin et al. 1995; Walker et al. 1994). Finally, EGFR protein expression is increased in vitro in rat pleural mesothelial cells after exposure to known carcinogenic asbestos fibers (Faux et al. 2001). In the present study, rats exposed to LA fibers underwent upregulation of all three genes in the lungs at 2 yr post IT, although surprisingly Msln and WT1 were only elevated in those rats exposed to low LA. WT1 was the only biomarker upregulated at the earlier 1-yr time-point, again only in low-LA animals. This pattern of expression was similar to that of fibrosis markers discussed earlier. Although the fibrosis and cancer marker gene expression data shown here may support the so-called "low-dose hypothesis" linked to other types of exposures (vom Saal and Hughes 2005), these results were not consistent with the dose-dependent enhancement of chronic inflammation and fibrosis in LA-exposed rats. More likely, the expression of Msln and WT1 may not reflect protein levels in the tissues, but simply delayed responses due to less potent effects of the lower dose of LA or variations in animals due to age. Unlike expression of Msln and WT1, EGFR expression was enhanced by 2 yr in all groups exposed to asbestos. Significant correlations between EGFR immunohistochemical expression and corresponding mRNA levels (Destro et al. 2006) and elevated serum levels (Gaafar et al. 2010) have been previously documented.

In addition to gene expression, the levels of Msln protein in serum, which was shown to be a reliable biomarker for mesothelioma, were determined (Onda et al. 2006; Robinson et al. 2003; Scherpereel et al. 2006). Similar to Msln mRNA expression, serum N-ERC/Msln levels remained at control levels in all asbestos-exposed rats 1 yr after exposure, consistent with previous studies in our lab (personal communication). However, by 2 yr postexposure, levels of serum N-ERC/Msln were elevated in all groups, including saline-exposed controls, despite the absence of any evidence of mesothelioma in lung. This might be explained by the spontaneous testicular cancers, characterized as adenoma/mesothelioma, common in aged Fischer 344 rats (Haseman et al. 1998). Of the 20 animals analyzed for serum Msln levels, 19 were reported to have "enlarged testes" and/or visual testicular tumors at the time of necropsy. Nine of these 19 animals, or approximately 47%, had elevated serum N-ERC/Msln levels compared to levels at 1 yr postexposure. As the specific pathology of the testicular tumors was not evaluated, it was not possible to determine whether the rise in serum N-ERC/Msln was specific to testicular mesothelioma or other malignancies. Finally, the elevation of this serum marker in all groups due to strain-related, not treatment-related, spontaneous tumors may mask any increase of the marker due to the development of lung or pleural mesothelioma.

In conclusion, a single IT exposure to LA results in progressive chronic inflammation in the lung and significant dose-dependent fibrosis, but no histological evidence of carcinogenic effects in the lungs. Similarly, no marked carcinogenic effects in the lungs were noted after exposure to amosite. On an equal mass basis, the degree of chronic inflammation and fibrosis was greater following amosite exposure, suggesting that this fiber exerts a greater toxicity compared to LA. However, these changes may

be attributed to other dose metrics including fiber number, surface area, fiber length, or biopersistance (Cook et al. 1982; Davis et al. 1978; Stanton et al. 1981; Wagner et al. 1984). In this study, fiber number cannot explain the differential toxicity of the two types of amphiboles as both $PM_{2.5}$ samples used had similar number of particles per unit weight (LA: 8.55×10^8/mg; amosite: 7.87×10^8/mg) (Duncan et al. 2010; Lowers and Bern 2009). The greater effects observed after exposure to amosite might, however, be explained by the greater length of this fiber compared to LA (Duncan et al. 2010; Lowers and Bern 2009; Padilla-Carlin et al. 2011). Fibrosis scores at both 1 and 2 yr correlated well with fibers lengths >10 μm (R^2 = .9677 and .9632, respectively). However, fiber length did not correlate well with gene expression of any biomarkers analyzed in this study. In addition, at earlier time points, different parameters (BALF protein and lung tissue fibrosis) correlate best with different fiber lengths (Padilla-Carlin et al. 2011), suggesting alternate roles for fibers of different lengths in the overall toxicity of an asbestos sample. Collectively, these studies highlight the complexity of the assessment of the toxicity of asbestos fibers and suggest the use of additional dose metrics to better elucidate the comparative fibrogenicity and carcinogenicity of amphiboles.

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