

## Progression of Lung Inflammation and Damage in Rats After Cessation of Silica Inhalation

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Human epidemiologic studies have found that silicosis may develop or progress even after occupational exposure has ended, suggesting that there is a threshold lung burden above which silica-induced pulmonary disease progresses without further exposure. We previously described the time course of rat pulmonary responses to silica inhalation as biphasic, the initial phase characterized by increased but controlled pulmonary inflammation and damage. However, after a threshold lung burden was exceeded, rapid progression of silica-induced pulmonary disease occurred. To test the hypothesis that there is a threshold lung burden above which silica-induced pulmonary disease progresses without further exposure we initiated a study to investigate the relationship between silica exposure, the initiation and progression of silica-induced pulmonary disease, and recovery. Rats were exposed to silica (15 mg/m<sup>3</sup>, 6 h/day) for either 20, 40, or 60 days. A portion of the rats from each exposure were maintained without further exposure for 36 days to examine recovery. The major findings of this study are: (1) silica-exposed rats were not in pulmonary overload, and lung silica burden decreased with recovery; (2) pulmonary inflammation, damage and lipoidosis increased with recovery for rats exposed to silica for 40 and 60 days, but not 20 days; (3) histopathology revealed changes in silica-induced alveolitis, epithelial hypertrophy and hyperplasia, and alveolar lipoproteinosis consistent with bronchoalveolar lavage (BAL) endpoints; and (4) pulmonary fibrosis developed even when exposure was stopped prior to its initial development.

**Key Words:** silica inhalation; pulmonary inflammation; silica lung burden; recovery.

Aerosols of crystalline silica ( $\alpha$ -quartz) are produced in a number of industrial and agricultural processes, resulting in silica inhalation by exposed workers (IARC, 1997). Silica inhalation in humans has been directly linked to the disease

silicosis. The National Institute for Occupational Safety and Health (NIOSH) has estimated that at least 1.7 million workers in industrial occupations, and an unknown percentage of the 3.7 million workers employed in agricultural occupations, are exposed to silica (NIOSH, 2002).

Our laboratory has recently published a series of reports from a rat silica inhalation study designed to investigate the time course of rat pulmonary responses to silica inhalation (Porter *et al.*, 2001, 2002a,b). One of the major findings derived from this study was that the time course of rat pulmonary responses to inhaled silica was biphasic (Castranova *et al.*, 2002). During the first 41 days of silica exposure, we observed elevated but relatively constant levels of inflammation and damage, with no fibrosis. Subsequently, from 41 to 116 days of exposure, rapidly increasing pulmonary inflammation and damage with concomitant development of fibrosis occurred. This suggested that pulmonary defense mechanisms were initially able to compensate and control silica-induced pulmonary inflammation and damage, but after a certain threshold lung burden was exceeded, these control mechanisms no longer were adequate to prevent the progression of silica-induced pulmonary disease. Human epidemiologic studies have found that silicosis may develop or progress even after occupational exposure has ended (Hessel and Sluis-Cremer, 1987; Hnizdo and Murray, 1998; Hnizdo and Sluis-Cremer, 1993; Kreiss and Zhen, 1996; Miller *et al.*, 1998; Ng *et al.*, 1987), suggesting that there is a threshold lung burden above which silica-induced pulmonary disease progresses without further exposure in humans. Our rat silica inhalation model provided an opportunity to model this threshold phenomenon, which has been observed in humans, and to investigate the underlying mechanisms involved.

Therefore, we initiated a study to investigate the relationship between initial silica exposure, the initiation and progression of silica-induced pulmonary disease, and recovery after silica exposure. Three different exposure times were used in this study, namely 20, 40, and 60 days. These represent exposure times previously determined to result in elevated but controlled inflammation and damage without fibrosis (20 days), the tran-

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sition from controlled inflammation and damage to rapidly increasing inflammation and damage (40 days), and rapidly increasing inflammation and damage with fibrosis developing (60 days).

## MATERIALS AND METHODS

**Silica chemical analyses.** The silica used in this study was Min-U-Sil 5 (U.S. Silica, Berkeley Springs, WV). Bulk silica was examined by proton-induced x-ray emission (PIXE) spectrometry for inorganic contaminants and for desorbable organic carbon compounds by gas chromatography mass spectrometry. These analyses determined that the bulk silica was  $\geq 98.5\%$  pure quartz with low inorganic contamination ( $\leq 0.10\%$ ), and only trace amounts of desorbable organic carbon compounds were found. Aerosolized silica samples were examined for trace inorganic elements by PIXE spectrometry. Results indicate that the silica had only trace amounts of iron, calcium, titanium, and zinc (average total trace elements = 0.13%). Aerosolized silica samples were also analyzed for elemental and organic carbon using a thermal-optical analyzer. Total carbon averaged 0.21% (range 0.19–0.23%) of the silica aerosol.

**Rat silica aerosol exposure.** The exposure-recovery design of this study consisted of six groups: (1) 20 days exposure, 0 days recovery; (2) 20 days exposure, 36 days recovery; (3) 40 days exposure, 0 days recovery; (4) 40 days exposure, 36 days recovery; (5) 60 days exposure, 0 days recovery; and (6) 60 days exposure, 36 days recovery. For each of these groups, equal numbers of rats (198 rats/chamber) were exposed to filtered air (controls) or silica. The silica aerosol generation and exposure system used in this study has previously been described in detail (Porter *et al.*, 2001).

Pathogen-free male Fischer 344 rats (strain CDF, 75–100 g body weight) were purchased from Charles River (Raleigh, NC) and housed using individual cages in two 5 m<sup>3</sup> Hinners-type inhalation chambers during this study. One chamber was used for filtered-air exposures (control) and the other for exposure to 15 mg/m<sup>3</sup> silica. Exposures were conducted for 6 h per day, 5 days per week, for a total of 20, 40, or 60 exposure days. The rats were on a 12-h light-dark schedule and were exposed during the dark cycle to coincide with their most active period. After completion of the 20-, 40-, and 60-day exposures, half of the rats from each exposure group (air and silica) were used for sample collection; the remaining animals were maintained in normal housing with no further exposure for 36 days, after which they were also sampled. Water was available *ad libitum*, and food was available at all times except during exposures. The program of animal use was accredited by AAALAC International, and all procedures involving animals were performed under protocols approved by the NIOSH Institutional Animal Care and Use Committee (IACUC).

The silica aerosol concentration was monitored using two independent methods: a RAS-2 particle sensor allowed real-time monitoring, and gravimetric determinations were made at hourly intervals during each day of exposure. The gravimetric determinations indicated that the silica aerosol concentration ranged from 15.0 to 15.3 mg/m<sup>3</sup> silica during the study. Silica particle size averaged  $\leq 2 \mu\text{m}$  as determined with an Anderson 8-stage cascade impactor. The mass median aerodynamic diameter of the silica particles averaged 1.78  $\mu\text{m}$  (range 1.70–1.89  $\mu\text{m}$ ), and the geometric standard deviation averaged 1.93  $\mu\text{m}$  (range 1.87–2.00  $\mu\text{m}$ ).

**Bronchoalveolar lavage.** Rats were euthanized with an ip injection of  $\geq 100$  mg sodium pentobarbital/kg body weight. Whole blood was collected from the renal vein using a Vacutainer blood collection tube with sodium ethylenediamine tetraacetate (Na<sub>2</sub>EDTA) as the anticoagulant. Blood cell differentials were determined using a Cell-Dyne 3500R hematology cell counter (Abbott Diagnostics, Abbott Park, IL). Bronchoalveolar lavage (BAL), with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free phosphate buffered saline (pH 7.4) plus 5.5 mM D-glucose (PBS), and the isolation of acellular first BAL fluid and BAL cells were conducted as previously described (Porter *et al.*, 2001). A tracheal cannula was inserted, and BAL was performed through the cannula using ice-cold PBS. The first lavage was 6 ml and was kept separate from the rest of

the lavage fluid; subsequent lavages used 8 ml of PBS until a total of 80 ml of lavage fluid was collected. The acellular supernatant from the first BAL and BAL cells were isolated by centrifugation (650  $\times$  g, 10 min, 4°C). The acellular supernatant from the first BAL (BAL fluid) was decanted for later analyses, while the acellular supernatants from the other lavage samples were decanted and discarded. BAL cells were resuspended in HEPES-buffered medium (10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], 145 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 5.5 mM D-glucose, pH 7.4), centrifuged a second time (650  $\times$  g, 10 min, 4°C), and after decanting the acellular supernatant, the final resuspension of the cells was made with HEPES-buffered medium. To assess pulmonary inflammation, cell counts of alveolar macrophages (AM) and polymorphonuclear leukocytes (PMN) were obtained using an electronic cell counter equipped with a cell sizer (Coulter Multisizer II, Coulter Electronics, Hialeah, FL), as previously described (Castanova *et al.*, 1990).

**BAL fluid phospholipid concentration.** BAL fluid phospholipid concentration was measured as the phosphorous present in lipid extracts of BAL fluid as previously described (Porter *et al.*, 2001).

**BAL fluid lactate dehydrogenase activity.** BAL fluid lactate dehydrogenase (LDH) activities were measured as a marker of cytotoxicity. LDH activities were determined by monitoring the LDH catalyzed oxidation of lactate to pyruvate coupled with the reduction of NAD<sup>+</sup> at 340 nm, using a commercial assay kit (Roche Diagnostic Systems, Somerville, NJ) and a Cobas Fara II Analyzer (Roche Diagnostic Systems, Somerville, NJ).

**BAL fluid serum albumin concentration.** BAL fluid albumin concentrations were determined as an indicator of the integrity of the blood-pulmonary epithelial cell barrier. BAL fluid albumin was determined colorimetrically at 628 nm based on albumin binding to bromocresol green, using a commercial assay kit (Sigma Chemical Company, St. Louis, Mo) and a Cobas Fara II Analyzer (Roche Diagnostic Systems, Somerville, NJ).

**Lung and lymph node silica burden.** Rats used for lung and lymph node silica burden determinations were not lavaged. Rats were euthanized with an ip injection ( $\geq 100$  mg/kg body weight) of sodium pentobarbital. The lungs and lymph nodes were removed, weighed, frozen at  $-80^\circ\text{C}$ , and then lyophilized. After lyophilization, the tissue samples were prepared for analysis by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) for silicon at 288.158, 251.432, and 252.411 nm, using matrix-matched standards for comparison, as previously described (Porter *et al.*, 2001). Replicate samples of Min-U-Sil 5 were carried through the entire procedure in conjunction with the tissue samples, and the average SiO<sub>2</sub> recovery of these samples was 99.5% (range 96.2–103.6%). The calculation of tissue SiO<sub>2</sub> from Si determinations made by ICP-AES analyses was done as follows. First, the mass of Si determined for a sample was divided by the atomic weight of Si to obtain the number of moles of Si in that sample. Next, to calculate the mass of SiO<sub>2</sub> in the sample, the number of moles of Si per sample was multiplied by the molecular weight of SiO<sub>2</sub>. The basis for the latter calculation is that the number of moles of Si equals the number of moles of SiO<sub>2</sub>.

**Lung and lymph node histopathology.** Lungs were preserved for histopathology by airway perfusion with 6 ml of 10% neutral buffered formalin (NBF). The tracheobronchial and parathymic lymph nodes, which are the lung-associated lymph nodes, were preserved by immersion in NBF. Lungs and lymph nodes were trimmed the same day and processed overnight in an automated tissue processor (Shandon Hypercenter XP, Shandon, Inc., Pittsburgh, PA). Tissues were embedded in paraffin using a commercial embedding system (Shandon Embedding Center, Shandon Inc., Pittsburgh, PA).

Paraffin blocks were sectioned at 5  $\mu\text{m}$  on a rotary microtome, placed on glass slides, and deparaffinized in an automated staining system (Shandon Linistain GLX, Shandon Inc., Pittsburgh, PA). Routine histopathologic assessments were made on slides stained with hematoxylin and eosin. Fibrosis was evaluated in sections stained with Masson's trichrome.

Semiquantitative histopathologic assessment of slides evaluated while blinded to exposure status, followed by nonparametric statistical analysis, is an established standard technique for evaluating morphologic changes in tissue

sections from toxicology studies (Haschek and Rousseux, 1998). Validation of the histopathological methods used in this study was established in a silica inhalation time-course previously conducted in this laboratory. Specifically, the determination of lung fibrosis by lung hydroxyproline assay and morphometric analysis of fibrotic nodules was consistent with semiquantitative histopathologic determination of fibrosis (Porter *et al.*, 2001; Scabilloni *et al.*, 2001).

In this study, slides were examined by a board-certified veterinary pathologist blinded to the exposure status of the individual rats. Tissue alterations were scored for severity (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe) as well as distribution (0 = none, 1 = focal, 2 = locally extensive, 3 = multifocal, 4 = multifocal and coalescent, and 5 = diffuse) as previously described (Hubbs *et al.*, 1997). To assure representative evaluation of lung pathology, multiple lung sections were taken from each rat, and the sum of the severity and distribution scores was determined for each section to give a pathology score for each section. The lung pathology score of that animal was the average of the pathology scores for the lung sections from that animal. For each exposure group, four sections were evaluated from half of the animals (from the left lung lobe, the right diaphragmatic lung lobe, the right cardiac lung lobe and the right apical lung lobe), to assure systematic variation did not occur among the lobes. When broad distribution of lesions was identified, two sections were evaluated (from the left lung lobe and the right diaphragmatic lung lobe) for the other half of the animals.

**Statistical analyses.** For each exposure time, the difference between air- and silica-exposed rats at each recovery time point was tested, using appropriate contrasts in a two-way analysis of variance model with interactions (Figs. 1 and 4–8). For each exposure time, a one-way analysis of variance was conducted to determine if recovery after exposure to silica would result in significant differences in lung and lymph-node burdens (Figs. 2 and 3). Orthogonal polynomial contrasts were used to measure the linear and quadratic regression effects associated with exposure time to silica.

Due to the discrete nature of the histopathological scores, the nonparametric rank-sum test was used. Initially, pair-wise comparisons between air-exposed groups at each recovery time and between adjacent recovery time points were examined. No differences were determined for these comparisons, and thus the histopathological data from all the air-exposed rats were pooled. Subsequently, for each exposure time, the tests were limited to the pair-wise comparisons between the pooled air- and silica-exposed groups at each recovery time, and between adjacent recovery time points for silica-exposed rats (Tables 1 and 2).

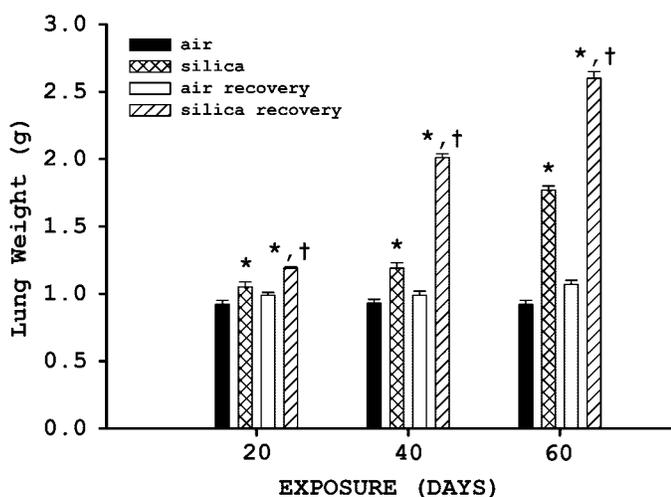


FIG. 1. Rat lung weights. Values represent mean  $\pm$  SE ( $n = 8$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

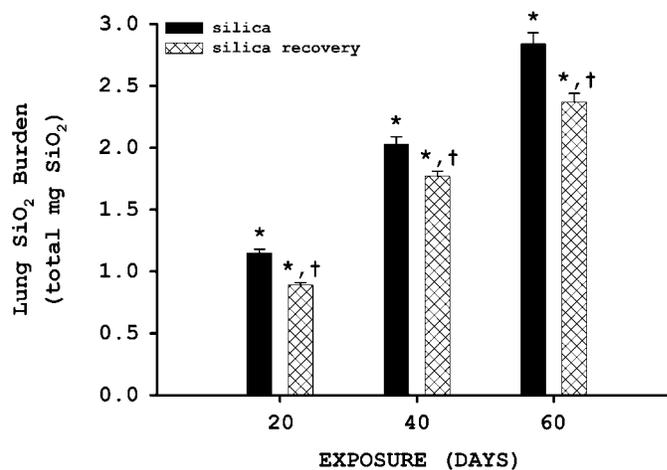


FIG. 2. Rat lung silica burden. Values represent mean  $\pm$  SE ( $n = 8$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. Silica was not detectable in any air-exposed rat lung, and thus no bar appears for air-exposed groups on the graph. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

## RESULTS

### Lung Weights

Silica-exposed rats had a significantly higher ( $p \leq 0.05$ ) lung weight in comparison to their corresponding air control groups for all exposure and recovery times (Fig. 1). For each exposure time, comparison of silica-exposed rats with 0 and 36 days recovery indicated that lung weight was significantly higher in the recovery groups, and the increase ranged from

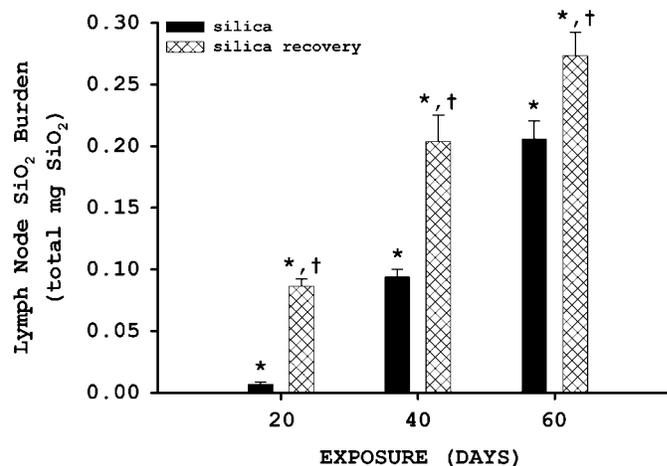


FIG. 3. Rat lymph node silica burden. Values represent mean  $\pm$  SE ( $n = 8$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. Silica was not detectable in any air-exposed rat lymph node, and thus no bar appears for air-exposed groups on the graph. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

TABLE 1  
Summary of Histopathological Alterations

Exposure (days)	Recovery (days)	Treatment	Alveolitis	Epithelial cell hypertrophy and hyperplasia	Lipoproteinosis
		air	0.00 ( <i>n</i> = 22) 0.50 ( <i>n</i> = 1) 1.00 ( <i>n</i> = 1)	0.00 ( <i>n</i> = 23) 0.50 ( <i>n</i> = 1)	0.00 ( <i>n</i> = 24)
20	0	silica	4.00 ( <i>n</i> = 2) <sup>a</sup> 4.25 ( <i>n</i> = 1) 4.50 ( <i>n</i> = 2) 4.75 ( <i>n</i> = 1)	4.00 ( <i>n</i> = 1) <sup>a</sup> 4.25 ( <i>n</i> = 1) 4.50 ( <i>n</i> = 3) 5.00 ( <i>n</i> = 1)	0.00 ( <i>n</i> = 3) 2.00 ( <i>n</i> = 2)
20	36	silica	4.50 ( <i>n</i> = 1) <sup>a,b</sup> 5.00 ( <i>n</i> = 4) 5.25 ( <i>n</i> = 1)	5.25 ( <i>n</i> = 2) <sup>a,b</sup> 5.50 ( <i>n</i> = 4)	2.50 ( <i>n</i> = 1) <sup>a,b</sup> 3.00 ( <i>n</i> = 1) 4.00 ( <i>n</i> = 3) 4.50 ( <i>n</i> = 1)
40	0	silica	5.00 ( <i>n</i> = 1) <sup>a</sup> 5.50 ( <i>n</i> = 4) 5.75 ( <i>n</i> = 1)	5.50 ( <i>n</i> = 3) <sup>a</sup> 5.75 ( <i>n</i> = 2) 6.00 ( <i>n</i> = 1)	4.00 ( <i>n</i> = 1) <sup>a</sup> 4.50 ( <i>n</i> = 2) 4.75 ( <i>n</i> = 2) 5.00 ( <i>n</i> = 1)
40	36	silica	5.75 ( <i>n</i> = 1) <sup>a,b</sup> 6.00 ( <i>n</i> = 1) 6.25 ( <i>n</i> = 2) 6.50 ( <i>n</i> = 1) 7.00 ( <i>n</i> = 1)	5.75 ( <i>n</i> = 1) <sup>a,b</sup> 6.00 ( <i>n</i> = 1) 6.25 ( <i>n</i> = 1) 6.50 ( <i>n</i> = 2) 7.00 ( <i>n</i> = 1)	6.50 ( <i>n</i> = 4) <sup>a,b</sup> 7.00 ( <i>n</i> = 2)
60	0	silica	6.00 ( <i>n</i> = 1) <sup>a</sup> 6.25 ( <i>n</i> = 1) 6.50 ( <i>n</i> = 1) 6.75 ( <i>n</i> = 1) 7.00 ( <i>n</i> = 2)	6.00 ( <i>n</i> = 1) <sup>a</sup> 6.25 ( <i>n</i> = 1) 6.50 ( <i>n</i> = 2) 6.75 ( <i>n</i> = 2)	5.50 ( <i>n</i> = 2) <sup>a</sup> 6.50 ( <i>n</i> = 1) 7.00 ( <i>n</i> = 3)
60	36	silica	6.50 ( <i>n</i> = 1) <sup>a,b</sup> 7.00 ( <i>n</i> = 4) 7.25 ( <i>n</i> = 1)	7.00 ( <i>n</i> = 4) <sup>a,b</sup> 7.50 ( <i>n</i> = 2)	6.50 ( <i>n</i> = 1) <sup>a,b</sup> 7.00 ( <i>n</i> = 2) 7.25 ( <i>n</i> = 3)

Note: Histopathology scores were determined as described in the Materials and Methods. Values represent histopathology score and number of animals with that score (*n*). Statistical analyses were done using nonparametric rank-sum test as described in the Materials and Methods.

<sup>a</sup>Significant difference ( $p \leq 0.05$ ) between silica-exposed group and air-exposed controls.

<sup>b</sup>Significant difference ( $p \leq 0.05$ ) between groups with 0 and 36 days recovery for a given silica-exposed group.

1.13-fold for 20-day exposure groups to 1.47-fold for the 60-day exposure groups (Fig. 1). The observed increases in lung weight may reflect the influx of inflammatory cells, edema, and increased collagen deposition, all of which were determined to have occurred in response to the silica inhalation.

#### Lung and Lymph Node Silica Burdens

Silica burden in the lungs increased with the duration of inhalation exposure (Fig. 2). At all three exposure times, silica lung burden of rats with 0 days recovery were significantly higher ( $p \leq 0.05$ ) than rats which were allowed to recover for 36 days. For rats exposed for 20 days, the recovery period resulted in a 22% decrease in silica lung burden, while rats allowed a recovery period after 40- and 60-day exposures had 13% and 16% reductions, respectively.

The lymph node silica burden determinations indicated that the relationship between burden and recovery was opposite of that observed for lung silica burden. Lymph node silica burden

of rats with 36 days recovery were significantly higher ( $p \leq 0.05$ ) than rats that had 0 days recovery for all three exposure times (Fig. 3). The largest increase in lymph node silica burden after recovery was the 12.7-fold increase for rats exposed for 20 days, followed by 2.2-fold and 1.3-fold increases determined for rats exposed for 40 and 60 days, respectively.

#### BAL Cell Differentials

Lavagable PMN yields from silica-exposed rats, regardless of whether the rats had 0 or 36 days recovery, were significantly higher ( $p \leq 0.05$ ) than the corresponding air control groups, with the response increasing with exposure duration (Fig. 4). For 20-day silica-exposed rats, no significant difference ( $p > 0.05$ ) was determined between PMN yields of rats with 0 and 36 days recovery. However, 36 days of recovery after 40 and 60 days of silica exposure resulted in significant ( $p \leq 0.05$ ) 1.7-fold and 1.4-fold increases in PMN yield, respectively.

TABLE 2  
Histopathological Fibrosis Summary

Exposure (days)	Recovery (days)	Treatment	Pulmonary	Tracheobronchial lymph node	Parathyroid lymph node
20	0	air	0.00 (24)	0.00 (n = 19)	0.00 (n = 23)
		silica	0.00 (4) 1.00 (2)	0.00 (n = 6)	0.00 (n = 6)
20	36	silica	3.50 (1) <sup>a,b</sup> 4.50 (4) 5.50 (1)	0.00 (n = 6) 2.00 (n = 1)	0.00 (n = 5)
		silica	2.00 (n = 1) <sup>a</sup> 2.50 (n = 1) 3.00 (n = 3) 4.00 (n = 1)	0.00 (n = 6)	0.00 (n = 6)
40	36	silica	3.25 (n = 1) <sup>a,b</sup> 4.50 (n = 2) 5.00 (n = 2) 6.00 (n = 1)	0.00 (n = 4) 4.00 (n = 1) 5.00 (n = 1)	0.00 (n = 2) <sup>a</sup> 4.00 (n = 1) 5.00 (n = 2)
		silica	3.50 (n = 1) <sup>a</sup> 4.25 (n = 2) 5.50 (n = 3)	0.00 (n = 4) 4.00 (n = 1)	0.00 (n = 3) 4.00 (n = 2)
60	36	silica	5.25 (n = 2) <sup>a</sup> 5.50 (n = 2) 6.50 (n = 1) 7.00 (n = 1)	6.00 (n = 2) <sup>a,b</sup> 7.00 (n = 4)	0.00 (n = 1) <sup>a</sup> 4.00 (n = 2) 6.00 (n = 1) 7.00 (n = 2)

Note: Histopathology scores were determined as described in the Materials and Methods. Values represent histopathology score and number of animals with that score (n). Variation in number of lymph nodes was due to the lymph nodes being too small to identify and remove at the time of necropsy from some animals. Statistical analyses were done using nonparametric rank-sum test as described in the Materials and Methods.

<sup>a</sup>Significant difference ( $p \leq 0.05$ ) between silica-exposed group versus air-exposed controls.

<sup>b</sup>Significant difference ( $p \leq 0.05$ ) between groups with 0 and 36 days recovery for a given silica-exposed group.

### Blood Neutrophil Counts

Increases in blood neutrophils paralleled increases in lavageable PMNs. All the silica-exposed rats had significantly

higher blood neutrophils than the corresponding air control groups, except for the 20-day silica-exposed rats with 0 days recovery (Fig. 5). Furthermore, for 20-day silica-exposed rats, no significant difference ( $p > 0.05$ ) was determined between

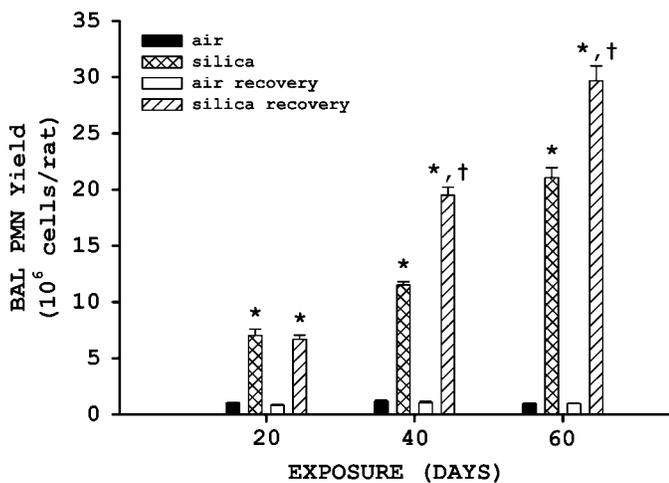


FIG. 4. BAL PMN yield. Values represent mean  $\pm$  SE ( $n = 11$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

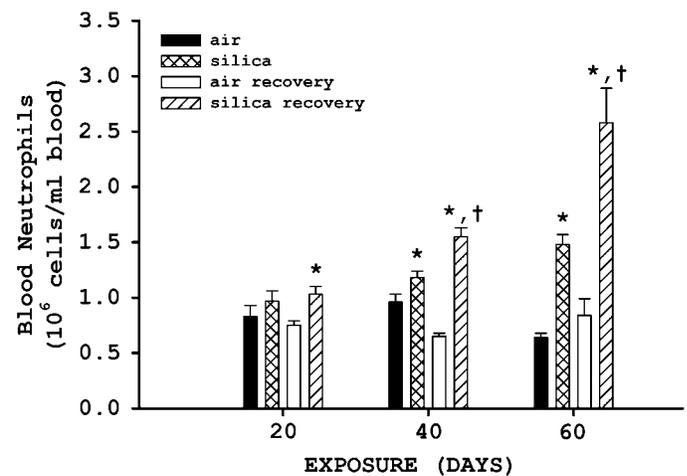


FIG. 5. Rat blood neutrophils. Values represent mean  $\pm$  SE ( $n = 8-11$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

blood neutrophils of rats with 0 and 36 days recovery. However, 36 days of recovery after 40 and 60 days of silica exposure resulted in significant increases ( $p \leq 0.05$ ) of 31.7% and 57.4%, respectively, above the 0 day recovery level.

#### BAL Fluid Lactate Dehydrogenase

Silica-exposed rats had significantly higher ( $p \leq 0.05$ ) BAL fluid LDH activities than the corresponding air control groups at every exposure-recovery time examined, with response increasing with exposure duration (Fig. 6). For each exposure time, comparison of silica-exposed rats with 0 and 36 days recovery indicated that BAL fluid LDH activity was significantly higher ( $p \leq 0.05$ ) in the recovery groups, with the increases ranging from 1.4-fold to 1.7-fold.

#### BAL Fluid Albumin

Silica-exposed rats had significantly higher ( $p \leq 0.05$ ) BAL fluid albumin concentrations than the corresponding air control group at every exposure-recovery time, with response increasing with exposure duration (Fig. 7). Comparison of silica-exposed rats with 0 and 36 days recovery determined that the increase in BAL fluid albumin for the recovery group after a 20-day exposure was not significant ( $p > 0.05$ ), but the increase was significant ( $p \leq 0.05$ ) for the 40- and 60-day silica-exposed rats.

#### BAL Fluid Phospholipids

Except for rats exposed for 20 days with 0 days recovery, all of the silica-exposed rats had significantly higher BAL fluid phospholipid concentrations than corresponding air controls, with the response increasing with exposure duration (Fig. 8). For 20- and 40-day silica-exposed rats, BAL fluid phospholipid

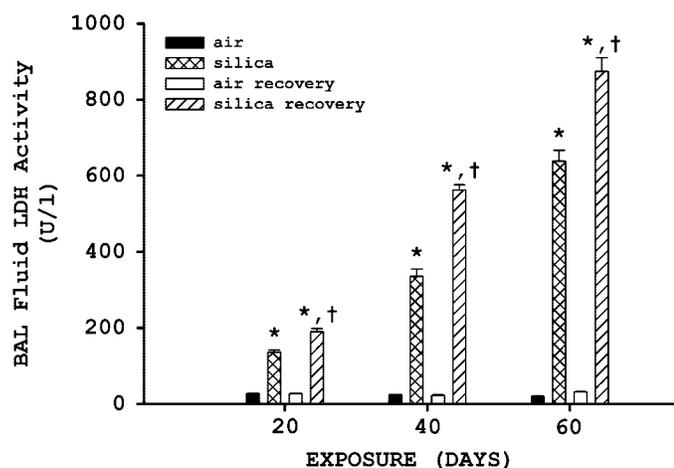


FIG. 6. BAL fluid LDH activities. Values represent mean  $\pm$  SE ( $n = 11$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

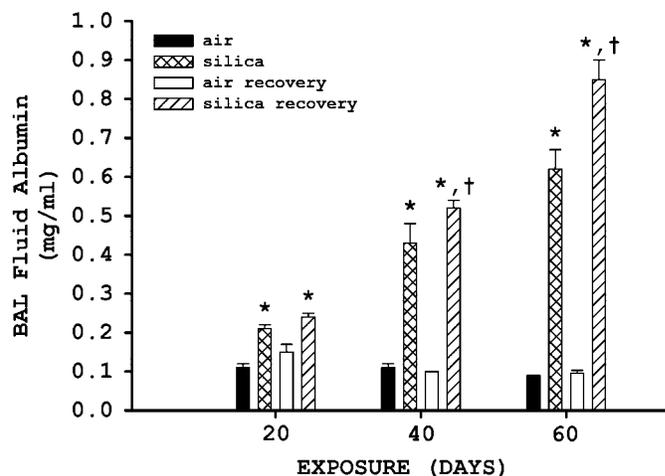


FIG. 7. BAL fluid albumin concentrations. Values represent mean  $\pm$  SE ( $n = 11$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

concentrations were significantly higher in rats allowed 36 days recovery in comparison to those with 0 days recovery. For 60-day silica-exposed rats, BAL fluid phospholipid concentrations measured in rats with 0 and 36 days recovery were not significantly different ( $p > 0.05$ ).

#### Pulmonary Histopathology

Pulmonary inflammatory changes observed in histopathologic sections were consistent with the BAL fluid findings. Alveolitis was observed in all exposed rats, appeared at the

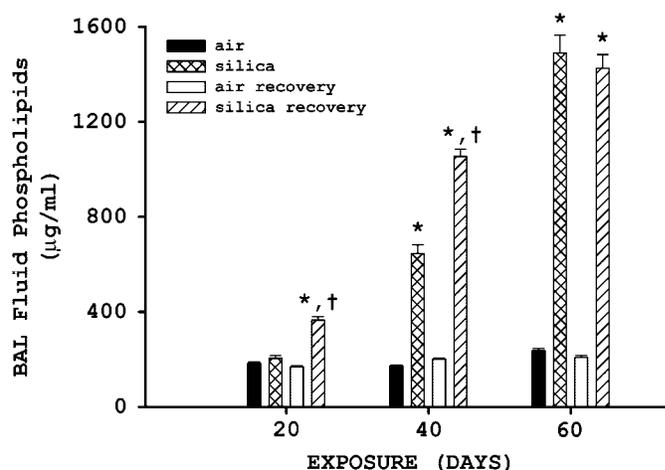
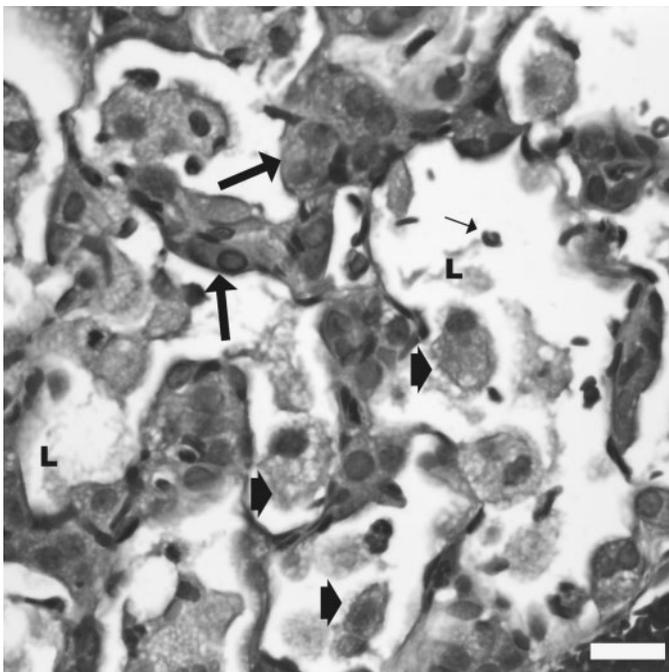


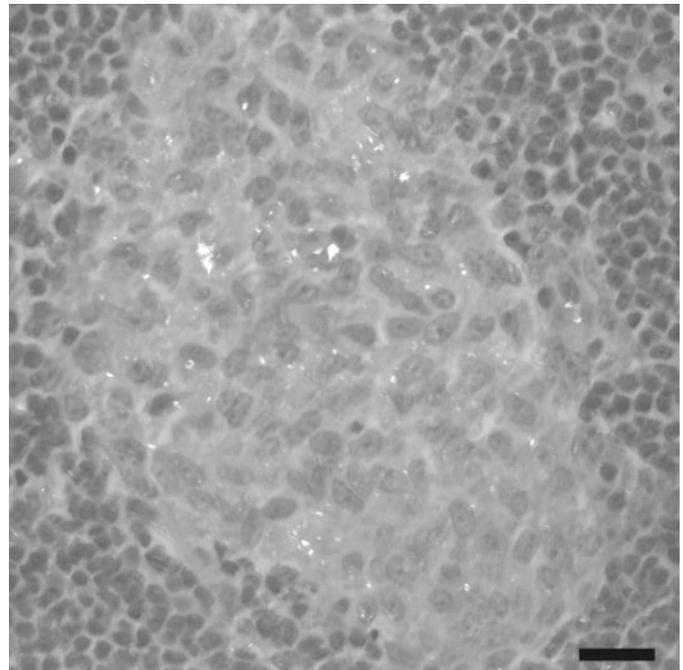
FIG. 8. BAL fluid phospholipid concentrations. Values represent mean  $\pm$  SE ( $n = 10-11$ ). An asterisk (\*) indicates a significant difference ( $p \leq 0.05$ ) versus corresponding air-exposed controls. A dagger (†) indicates a significant difference ( $p \leq 0.05$ ) between rats with 0 and 36 days recovery within a silica exposure group.

earliest time point (20 days), and was consistently associated with hypertrophy and hyperplasia of the alveolar epithelium (Fig. 9). Fibrosis and lipoproteinosis were not consistently present in rats exposed to silica for 20 days without recovery, but were significantly increased in the 20-day exposure group that was allowed to recover for 36 days. Rats exposed to silica for 40 or more days, regardless of recovery status, had significantly increased fibrosis and lipoproteinosis. The pathology scores for alveolitis, alveolar epithelial cell hypertrophy and hyperplasia, lipoproteinosis, and pulmonary fibrosis all increased with time, even when exposure was discontinued (Tables 1 and 2, Figs. 9 and 10).

Morphologic alterations were also observed in the lymphatic system and involved both the lymph nodes and the irregularly distributed patches of mucosal lymphoid tissue distributed beneath the bronchial epithelium known as bronchus-associated lymphoid tissue (BALT). Although the irregular distribution of the BALT resulted in it being present in some but not all sections of lung, and thus prevented accurate quantitative scores from being obtained, silica-exposed rats developed changes in BALT that were not seen in any control rats. Specifically, granulomatous inflammation of BALT was first observed as a minimal focal change in a single lung lobe of one rat exposed to silica for 20 days and was present in at least one



**FIG. 9.** Photomicrograph from the alveolar region of the lung of a rat exposed to silica for 60 days and then allowed to recover for 36 days. Characteristic changes include proliferation and hypertrophy of alveolar epithelial cells (large arrows) and alveolar lipoproteinosis (L). The presence of neutrophils (small arrow) and increased numbers of alveolar macrophages (arrowheads) are characteristic of histiocytic and suppurative alveolitis (H&E stained section; magnification bar is 20  $\mu$ m).



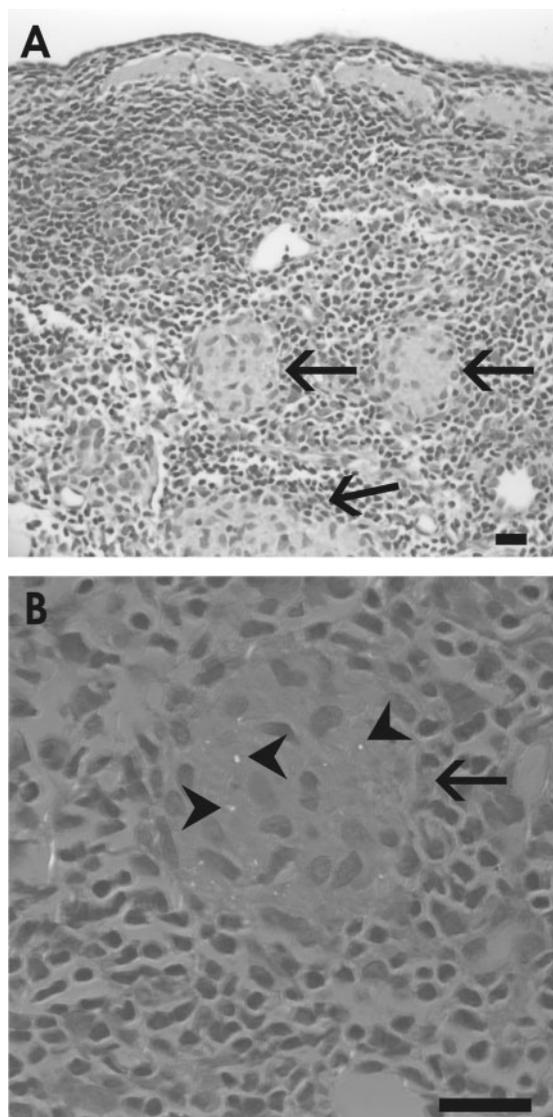
**FIG. 10.** Photomicrograph of the tracheobronchial lymph node of a rat as visualized under polarized light. This rat was exposed to silica for 60 days and then allowed to recover for 36 days. The cells occupying the center of the microscopic field are epithelioid macrophages. The white crystalline material within these cells is the crystalline silica, which is visualized in the polarized light (H&E stained section; magnification bar is 20  $\mu$ m).

section of lung from every rat exposed to silica for 40 or 60 days and allowed to recover for 36 days (Fig. 11); no granulomatous inflammation of BALT was observed in the lung of any air-exposed rat. Lymphoid hyperplasia of BALT was also observed in some sections of lung from rats exposed to silica for 40 or 60 days and allowed to recover for 36 days, but was not present in the lungs of air-exposed rats.

Morphologic alterations within the lymph nodes lagged behind the changes seen in the lungs. These alterations consisted of granulomatous inflammation, lymphoid hyperplasia, and fibrosis. Fibrosis appeared after granulomatous inflammation, and the fibrosis pathology score continued to increase during recovery. Thus, after 60 days of silica exposure, a 36 day recovery period increased the fibrosis scores in the tracheobronchial lymph node four-fold from 0-day recovery and increased the fibrosis score in the parathyroid lymph nodes more than three-fold (Table 2).

## DISCUSSION

In any pulmonary toxicology study involving toxic particles, the issue of pulmonary overload needs to be considered. Pulmonary overload is defined as a condition when the pulmonary dust burden is sufficiently high that AM particle clearance is impaired, resulting in nonspecific pulmonary inflammation,



**FIG. 11.** Histopathologic changes in silica-exposed BAL. (A) Photomicrograph of the hypertrophied BAL in a silica-exposed rat. This rat was exposed to silica for 60 days and then allowed to recover for 36 days. Foci of granulomatous inflammation (arrows) are scattered amidst the lymphocytes (reference bar is 20  $\mu\text{m}$ ). (B) A higher magnification photomicrograph of the foci of granulomatous inflammation (arrow) within BAL under polarized light showing the birefringent crystalline silica particles (arrowheads), which are localized within epithelioid macrophages in these foci (reference bar is 20  $\mu\text{m}$ ).

interstitial and alveolar accumulation of particles, and epithelial cell proliferation (Donaldson, 2000; Miller, 2000). In this study, pulmonary silica burden increased in a linear manner with increasing exposure time and decreased with 36 days recovery (Fig. 2). The reduction in lung burden after 36 days recovery correlated with concomitant increases in lymph node silica burden in these rats (Fig. 3). These observations indicate that silica particle clearance from the lung had not been impaired, and thus the rats were not in pulmonary overload. This

determination is consistent with our previous rat silica inhalation study, in which we demonstrated that rats similarly exposed to a respirable silica aerosol (15  $\text{mg}/\text{m}^3$  silica, 6 h/day, 5 days/week) were not in pulmonary overload after 116 days exposure; i.e., lung burden had reached an equilibrium state, and volumetric burden of silica in the lung did not exceed 6% of the pulmonary macrophage volume, which is proposed to be required in the initial phase of overload (Oberdorster *et al.*, 1992; Porter *et al.*, 2001).

Lavagable PMNs were determined as an indicator of silica-induced pulmonary inflammation. In comparison to air-exposed controls, PMNs were significantly higher in silica-exposed rats for all exposure and recovery times (Fig. 4). For rats exposed for 20 days, there was no difference in the number of PMNs isolated between the 0- and 36-day recovery groups. However, after inhalation exposure to silica for 40 and 60 days, rats with 36 days recovery had higher PMN counts than those rats which had 0 days recovery, indicating that recruitment of PMNs into the lung continued without further silica exposure. The finding that PMN recruitment into the lung continued after silica exposure was stopped is consistent with previous reports. One study reported continued PMN recruitment into pulmonary spaces 24 weeks after exposure to 10  $\text{mg}/\text{m}^3$  silica (6 h/day, 5 days/week) for 4 weeks (Henderson *et al.*, 1995). In another study, rats exposed to 10 or 50  $\text{mg}/\text{m}^3$  silica (7 h/day, 5 days/week) for 32 or 75 days had increased BAL PMNs after a 64-day recovery period in comparison to BAL PMNs measured in rats with 0 days recovery (Donaldson *et al.*, 1990). In the present study, the recruitment of PMNs into the lung was sufficiently high that blood neutrophils, the primary source for BAL PMNs, exhibited parallel increases (Fig. 5). Also, lavagable PMNs and blood neutrophils for silica-exposed rats at 20 and 40 days exposure are similar in magnitude to those reported from our earlier silica inhalation study at 20 and 41 days exposure, indicating consistency of the dose-response relationship in this animal model (Porter *et al.*, 2002b).

It was expected that silica deposition in the lung would result in some cytotoxic damage. This damage may be via direct radical production from silica particles (Vallyathan *et al.*, 1995) and/or silica-induced cellular-mediated radical production (Blackford *et al.*, 1994). To gauge the extent of silica-induced damage, BAL fluid albumin was measured as a marker of damage to the pulmonary blood-gas barrier, and LDH as an indicator of cytotoxicity. BAL fluid LDH activities (Fig. 6) and albumin concentrations (Fig. 7) from silica-exposed rats were significantly higher than the corresponding air-exposed controls at every exposure-recovery time. The general pattern of increases in BAL fluid LDH and albumin were similar; BAL LDH activities were significantly higher in rats with 36 days recovery than in rats with 0 days at all three exposure times, whereas increases for BAL albumin only occurred after 40 and 60 days of silica exposure. Thus, after the cessation of silica exposure, silica-induced damage to the lung progressed. LDH is an intracellular enzyme, and thus its presence in BAL fluid

is presumed to be from cells that have undergone cytotoxic damage. However, rat plasma also has measurable levels of LDH (Franken *et al.*, 2000), and thus leakage of LDH from the blood into the lung, evidenced by increased albumin in the lung, may also contribute to BAL fluid LDH levels. However, this contribution is probably small in relation to the contribution from cytotoxic damage to the lung phagocytic and epithelial cells.

Alveolar lipoproteinosis is a well-documented response to inhaled silica and is believed, at least in part, to result from enhanced production and secretion of phospholipids and surfactant proteins by alveolar Type II cells (Crouch, 1991; Hook, 1991). BAL phospholipid levels were determined in this study as an index of alveolar lipoproteinosis. Silica-exposed rats had significantly higher BAL fluid phospholipid concentrations than corresponding air controls, except for the 20-day exposure group with 0-day recovery (Fig. 8). BAL fluid phospholipid concentrations increased with recovery when rats were exposed for 20 and 40 days, but no progression was observed for rats exposed for 60 days. In comparison to air-exposed control rats, statistically significant alveolar epithelial cell hypertrophy and hyperplasia was observed in silica-exposed rats at the earliest time point, after 20 days of exposure, and progressed significantly with exposure duration and recovery time (Table 1). Since alveolar lipoproteinosis is characterized by an overproduction of surfactant phospholipids and surfactant proteins, both of which are products of alveolar type II cells, it is not surprising that statistically significant alveolar lipoproteinosis correlated with alveolar epithelial cell hypertrophy and hyperplasia.

Pulmonary fibrosis is a major component of accelerated and chronic silicosis in humans (Green and Vallyathan, 1996). In this rat study, pulmonary fibrosis increased with the number of days of exposure. Interestingly, for rats exposed for 20 days, fibrosis developed during recovery even though exposure was discontinued prior to its initial development. Specifically, the pathology score for pulmonary fibrosis was low and statistically indistinguishable from control values after 20 days exposure. However, despite continued lung clearance of silica, pulmonary fibrosis increased more than 10-fold when the rats exposed for 20 days were allowed 36 days of recovery (Table 2). Furthermore, the fact that the PMN influx did not progress in the rats exposed to silica for 20 days, whereas fibrosis did, suggests that lavagable PMNs do not predict fibrogenicity. This is consistent with the lack of PMN influx seen in human chronic silicotic lavage despite the progressive nature of the fibrosis (IARC, 1997).

Unresolved pulmonary inflammation has been implicated as an important factor in the development of pulmonary fibrosis (Cotran *et al.*, 1999). Unresolved inflammation may have played a role in the development of the pulmonary fibrosis in the 20-day exposure group, because the degree of inflammation, as indicated by BAL PMNs, did not lessen during a 36-day recovery period (Fig. 4) despite a decrease in the lung

silica burden (Fig. 2). This mechanism appears to be even more significant for the rats exposed to silica for 40 and 60 days, as recovery resulted in significantly greater pulmonary inflammation (i.e., BAL PMNs) in comparison to corresponding exposure groups with no recovery.

Clearance of silica from the lung to the lung-associated lymph nodes not only resulted in a significant silica burden in these lymph nodes (Fig. 3); it also resulted in the appearance of granulomatous inflammation in the bronchus-associated lymphoid tissue and the lung-associated lymph nodes and the subsequent development of lymph node fibrosis after a time delay (Table 2). Granulomatous inflammation has been previously described in the lung-associated lymph nodes of rats exposed to both respirable crystalline and amorphous silica, where these foci have been termed either quartz-typical areas, granulomas, or granulomatous lymphadenitis (Bruch *et al.*, 1975; Friedetzky *et al.*, 1998; Huang *et al.*, 2001; Rosenbruch, 1992). Previous studies indicate that the inflammatory cells within these foci express the macrophage marker ED-1 (Friedetzky *et al.*, 1998); hence these foci were designated as granulomatous inflammation in this study. As with previous studies (Rosenbruch, 1992), fibrosis of lung-associated lymph nodes developed in foci of granulomatous inflammation in our study. Though the lymph node fibrosis was delayed relative to pulmonary fibrosis, the pathology scores for fibrosis in the lymph nodes were comparable to fibrosis scores in the lungs of rats exposed to silica for 60 days with 36 days recovery. Lymph node fibrosis is also important because it may impair subsequent clearance of inhaled silica via the lymphatics and is believed to play a role in increasing the susceptibility to silicosis progression (Murray *et al.*, 1991; Seaton and Cherie, 1998).

Several major observations were made in this study. First, parameters of silica-induced pulmonary inflammation and damage increase with duration of silica inhalation (i.e., with increasing silica lung burden). Secondly, the parameters of pulmonary inflammation and damage continued to progress during the recovery period with no further silica exposure. This progression was most obvious after 40 and 60 days exposure and occurred in spite of the fact that silica lung burden decreased with recovery, in part due to the transport of silica from the lung to lung-associated lymph nodes. Finally, histopathological assessment of pulmonary fibrosis indicated that fibrosis developed even when exposure was discontinued prior to its initial development.

Based on results of our previous silica inhalation studies, we proposed the hypothesis that there is a critical lung burden and, when it is exceeded, lung inflammation and damage progress without further exposure. The observations in this study support this hypothesis. However, the mechanism(s) responsible for the progression of silica-induced pulmonary disease, despite decreasing silica lung burden, remains to be established. One possible mechanism is that the silica lung burden had not decreased sufficiently to reduce the expression of biochemical

mediators, such as nitric oxide and/or cytokines such as interleukin-1 and tumor necrosis factor- $\alpha$ , which have been implicated in silica-induced lung disease (Porter *et al.*, 2002a,b).

Lastly, one should consider the human relevance of this rat silica inhalation model. Human epidemiologic studies have found that, even after occupational exposure has ended, silicosis may develop or progress (Hessel and Sluis-Cremer, 1987; Hnizdo and Murray, 1998; Hnizdo and Sluis-Cremer, 1993; Kreiss and Zhen, 1996; Miller *et al.*, 1998; Ng *et al.*, 1987), suggesting that in humans there maybe a threshold silica burden above which silica-induced pulmonary disease would progress without further exposure. A recent study of coal miners who had relatively low working lifetime dust exposures, and whose exposures were almost entirely under the current U.S. exposure limits for coal and silica, determined that relatively low occupational exposures to silica in mixed dust are associated with pulmonary responses, including inflammation and fibrosis (Kuempel *et al.*, 2003). When considered together, these human studies suggest that relatively low silica exposures may pose a serious health risk because silica-induced disease, once initiated even at low threshold lung burdens, can progress even in the absence of further exposure. The findings of our rat inhalation model are consistent with the pattern of pulmonary responses reported in humans and, thus, add support to this proposal. The similarity in the rat silica inhalation model presented here and previously reported human pulmonary responses suggests that this rat model can be used in future investigations of the mechanisms which may be responsible for these phenomena (Kuempel *et al.*, 2002).

The observations reported in this study clearly raise some questions, which can be further investigated in subsequent studies. One such hypothesis would be to investigate whether the same degree of fibrosis would result if the total number of exposure days plus recovery days were equivalent. For example, would a rat exposed to silica for 20 days with 40 days recovery develop a similar degree of fibrosis in comparison to a 40-day silica-exposed rat with 20 days recovery? Another line of possible investigation would be to determine if the levels of biochemical mediators implicated in silica-induced lung disease (e.g., nitric oxide, interleukin-1 and/or tumor necrosis factor- $\alpha$ ) also increase after cessation of silica exposure and thus parallel the changes in pulmonary inflammation and the development of fibrosis determined in this study.

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