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TIME COURSE OF PULMONARY RESPONSE OF RATS TO INHALATION OF CRYSTALLINE SILICA: NF-κB ACTIVATION, INFLAMMATION, CYTOKINE PRODUCTION, AND DAMAGE

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In vitro studies suggest that silica-induced lung disease may be linked to processes regulated by nuclear factor-κB (NF-κB) activation, but this has not been examined in vivo. Rats were exposed to a silica aerosol of 15 mg/m^3 (6 h/day, 5 days/wk) for 116 days, and bronchoalveolar lavage (BAL) was conducted at various times during the exposure. Silica-induced pulmonary inflammation and damage were determined by measuring BAL cell differentials and first BAL fluid lactate dehydrogenase (LDH) activity and serum albumin concentrations, respectively. NF-κB activation and production of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) by BAL cells were also measured. The results demonstrate that NF-κB activation occurred after 5 days exposure, and continued to increase thereafter. BAL cell production of IL-1 and TNF-α had increased incrementally by 10 and 30 days of exposure, respectively. This elevation continued through 79 days of exposure before further increasing at 116 days of exposure. Pulmonary inflammation and damage in

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silica-exposed rats were also significantly elevated at 5 days of exposure, further increased at a slow rate through 41 days of exposure, and dramatically increased thereafter. Taken together, the results indicate that the initial molecular response of NF-κB activation in BAL cells occurs in response to low levels of silica deposition in the lung and increases more rapidly versus exposure duration than silica-induced pulmonary inflammation, cellular damage, and cytokine production by BAL cells. This suggests that NF-κB activation in BAL cells may play an important role in the initiation and progression of silica-induced pulmonary inflammation, cellular damage, and fibrosis.

Several different lines of evidence suggest that nuclear factor-κB (NF-κB) may be an important regulator of pulmonary inflammation and fibrosis in silica-exposed animals. Silica is known to generate reactive oxygen species (ROS) (Vallyathan et al., 1995), and silicotic lungs are known to be in a state of oxidative stress (Vallyathan et al., 1997). Since NF-κB activation is known to occur in response to ROS (Sen & Packer, 1996), it was suggested that NF-κB activation may occur in vivo after silica exposure. Indeed, NF-κB activation was later demonstrated in silica-exposed rat primary alveolar macrophages (Rojanasakul et al., 1999) and bronchoalveolar lavage (BAL) cells isolated from rats exposed to silica by intratracheal (IT) instillation (Sacks et al., 1998).

Activated NF-κB functions in the regulation of cytokines implicated in the inflammatory and fibrogenic responses to silica. Using the mouse monocyte-macrophage cell line RAW 264.7, silica-induced NF-κB activation was linked with the expression of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) (Rojanasakul et al., 1999) and increased mRNA levels for interleukin-1 (IL-1) (Chen et al., 1995). In vivo studies in rats have established an association between NF-κB activation and cytokine expression in hypoxia (Leeper-Woodford & Detmer, 1999), in taurocholate pancreatitis (Satoh et al., 1999), and after exposure to a soluble fungal spore product (Nicholson et al., 1996), but to date not with silica.

A workshop on poorly soluble particles, such as silica, recently concluded that a significant gap in our knowledge exists concerning the temporal sequence of molecular, cellular, and histopathological changes that occur after exposure to poorly soluble particles (ILSI Risk Science Institute Workshop Participants, 2000). Therefore, we initiated a study whose comprehensive goal was to investigate, in detail, the temporal relationship between silica inhalation and the initiation and progression of adverse pulmonary responses in the rat model. In the first report from this investigation, we documented the relationship between the lung burden of silica and biochemical and histopathological evidence of pulmonary inflammation, damage, and fibrosis (Porter et al., 2001). We report here the temporal association between silica exposure and (1) pulmonary inflammation, (2) NF-κB activation and cytokine production from BAL cells, and (3) the development of pulmonary damage during silica inhalation.

METHOD

Silica Chemical Analyses

The crystalline silica used in this study was Min-U-Sil 5 (U.S. Silica, Berkeley Springs, WV) and was determined to be ≥98.5% quartz by PIXE spectrometry. Analyses of bulk silica for inorganic contaminants and desorbable organic carbon compounds indicated they were present at ≤0.10% and in trace amounts, respectively. Evaluation of aerosolized silica samples for trace inorganic elements by PIXE spectrometry indicated average total trace elements 0.13%, and elemental and organic carbon averaged 0.10%. Further details concerning the chemical analyses of the silica used in this study have been previously reported (Porter et al., 2001).

Silica Aerosol Exposure of Rats

Specific-pathogen-free male Fischer 344 rats (strain CDF, 75–100 g) were purchased from Charles River (Raleigh, NC) and housed in an AAALAC-approved animal facility using individual cages in two 5-m^3 Hinners-type inhalation chambers. The rats were acclimated in the chambers for 1 wk prior to the start of the study. One chamber was used for filtered-air exposures (control) and the other for 15 mg/m^3 silica exposures. Exposures were conducted 6 h/day, 5 days/wk, for a total of 116 exposure days. Water was available ad libitum, and food was available at all times except during exposures. The rats were on a 12-h light–dark schedule and were exposed during the dark cycle to coincide with their most active period. As previously reported (Porter et al., 2001), the average weekly silica aerosol concentration ranged from 14.9 to 15.5 mg/m^3, and the mass median aerodynamic diameter of the silica aerosol was consistently ≤2 μm. Further information relating to the characterization of the silica aerosol and the silica aerosol generation and exposure system have been described previously (Porter et al., 2001).

Bronchoalveolar Lavage

Rats were euthanized with an ip injection of ≥100 mg sodium pentobarbital/kg body weight after 5, 10, 16, 20, 30, 41, 79, and 116 days of exposure. Whole blood was collected from the renal vein using a Vacutainer blood collection tube with sodium ethylenediamine tetraacetate (Na_2EDTA) as the anticoagulent. Blood cell differentials were determined using a Cell-Dyn 3500R hematology cell counter (Abbott Diagnostics, Abbott Park, IL). Bronchoalveolar lavage (BAL) and the isolation of the first acellular BAL fluid and BAL cells were conducted as described (Porter et al., 2001). BAL cells were resuspended in HEPES buffer [10 m*M* *N*-(2-hydroxyethyl)piperazine- *N′*-(2-ethanesulfonic acid), 145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 5.5 m*M* D-glucose, pH 7.4] and counted using an electronic

cell counter (Coulter MultiSizer II, Coulter Electronics, Hialeah, FL) with a cell-sizing attachment to distinguish between rat lavage cell types as previously described (Castranova et al., 1979). The polymorphonuclear leukocytes (PMN) designation excludes alveolar macrophages (AM), but may include some lymphocytes and AM-derived apoptotic bodies recently reported from this study (Millecchia et al., 2000; Mercer et al., 2001).

BAL Cell Nuclear Protein Extraction

Nuclear extracts of BAL cells were prepared by a three-step procedure. First, BAL cells were pelleted by centrifugation (650 × g, 10 min, 4°C) and resuspended in 500 µl lysis buffer [50 m*M* KCl, 0.5% Nonidet P-40 (NP-40), 25 m*M* HEPES (pH 7.8), 1 m*M* phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 100 µ*M* 1,4-dithiothreitol] on ice for 10 min. The cell lysate was centrifuged (12,000 × g, 1 min, 4°C) and the supernatant discarded. The nuclear pellet was next washed once with wash buffer [50 m*M* KCl, 25 m*M* HEPES (pH 7.8), 1 m*M* phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 100 µ*M* 1,4-dithiothreitol] and then resuspended in extraction buffer [500 m*M* KCl, 10% glycerol, 0.5% NP-40, 25 m*M* HEPES (pH 7.8), 1 m*M* phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 100 µ*M* 1,4-dithiothreitol]. After centrifugation (14,000 × g, 2 min, 4°C), the supernatant (nuclear protein extract) was removed and stored at –70°C. The protein concentration of the nuclear protein extract was determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL).

NF-κB Gel Shift Assay

An NF-κB binding sequence in the human interleukin-6 gene promoter (bases –74 to –54, TGGGATTTTCCCATGAGTCT) was used to synthesize an oligonucleotide for the NF-κB binding probe (Isshiki et al., 1990). Complementary single-stranded oligonucleotides were denatured at 80°C for 5 min and annealed at room temperature. The double-stranded probe was labeled with [^{32}P]ATP (Amersham, Arlington Heights, IL) using T4 kinase (Bethesda Research Laboratories, Gaithersburg, MD).

The DNA–protein binding reaction was conducted in a 24-µl reaction mixture including 1 µg poly(dI•dC), 3 µg nuclear protein extract, 3 µg bovine serum albumin, 4×10^4 cpm [^{32}P]-labeled oligonucleotide probe, and 12 µl reaction buffer [24% glycerol, 24 m*M* HEPES (pH 7.9), 8 m*M* Tris-HCl (pH 7.9), 2 m*M* EDTA, and 2 m*M* 1,4-dithiothreitol] (Ye et al., 1994). After the addition of the radiolabeled probe, the mixture was incubated for 20 min at room temperature, then resolved on a 5% acrylamide gel. The 5% acrylamide gel had been prerun at 170 V for 30 min, with 0.5 × Tris borate–EDTA at 200 V for 90 min. After completion of electrophoresis, the gel was dried and placed on Kodak X-OMAT film (Eastman Kodak, Rochester, NY), which was developed after an overnight exposure at –70°C.

In some cases, the DNA–protein binding assay was modified to examine the specificity of NF-κB binding. The DNA–protein assay was conducted as described except the [^{32}P]-labeled oligonucleotide probe was initially omitted from the assay and one of the following was added: (1) unlabeled double-stranded oligomer of the human interleukin-6 gene promoter (bases –74 to –54, TGGGATTTTCCCATGAGTCT), (2) double-stranded oligomers containing an activator protein-1 (AP-1) binding sequence (Ye et al., 1996a, 1996b), (3) antibody to p50, (4) antibody to p65, or (5) antibody to SP-1. After 15 min incubation on ice, the [^{32}P]-labeled oligonucleotide probe was added to the DNA–protein binding assay and the assay was completed as described earlier.

BAL Fluid Lactate Dehydrogenase Activity

BAL fluid lactate dehydrogenase (LDH) activities were determined by monitoring the LDH catalyzed oxidation of pyruvate coupled with the reduction of NAD at 340 nm using a commercial assay kit (Roche Diagnostics Systems, Somerville, NJ).

BAL Fluid Serum Albumin Concentration

BAL fluid serum albumin concentrations were determined colorimetrically at 628 nm based on serum albumin binding to bromcresol green using a commercial assay kit (Sigma Chemical Company, St. Louis, MO).

BAL Cell Culture

BAL cells were cultured at a density of 1×10^6 total phagocytes/ml in 12-well plates using Eagle's minimum essential medium (EMEM) media supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml penicillin and streptomycin at 37°C in 5% CO_2. Total phagocytes was defined as the sum of AMs and PMNs in a BAL cell sample. After 18 h in culture, the samples were transferred to centrifuge tubes, centrifuged (650 × g, 10 min, 4°C), and the cell-free supernatants (BAL cell-conditioned media) were removed. The BAL cell-conditioned media were aliquoted into multiple samples and frozen at –80°C for later analyses.

TNF-α Determination

Tumor necrosis factor-α (TNF-α) was determined in BAL cell-conditioned media using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BioSource, Camarillo, CA). Total TNF-α production was calculated by multiplying TNF-α (pg/10^6 phagocytes) by the total number of lavaged phagocytes.

IL-1 Determination

The interleukin-1 (IL-1) activity of BAL cell-conditioned media was determined according to a previously described method (Kang et al., 1992;

Ma et al., 1999). Thymocytes were obtained from male CD-1 mice (6–10 wk of age) and suspended in RPMI-1640 medium supplemented with 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum, and 2×10^{-5} *M* mercaptoethanol. Thymocytes (1×10^6 cells/0.1 ml per well) and 0.1-ml aliquots of the BAL cell-conditioned media were added in quadruplicate to 96-well microculture plates. Cell cultures were incubated at 37°C in 5% CO_2 for 30 h. The cultures were pulsed for 22 h with 1.0 μCi [^{3}H]thymidine (11.3 Ci/mmol thymidine), and the thymocytes were harvested using a 96-well cell harvester apparatus. The radioactivity in the collecting glass fiber filters was determined, and the amount of IL-1 activity in the BAL cell-conditioned media was expressed as disintegrations per minute (dpm). Total IL-1 production was calculated by multiplying IL-1 (dpm/10^6 phagocytes) by the total number of lavaged phagocytes.

Statistical Analyses

The difference between air-exposed and silica-exposed rats at each time point was tested using appropriate contrasts in the two-way analysis of variance model with interaction (Searle, 1971). A log transformation of the response variable was used to satisfy assumptions of normality and constant variance. To characterize toxicity changes over time, data were fitted to a linear regression model that included any statistically significant polynomial terms up to a cubic. Predicted values and associated confidence intervals from the regression model (Myers, 1990) were used to identify significant differences between exposure times (i.e., time points with nonoverlapping confidence intervals) within the silica-exposed animals. This approach was preferable to doing pairwise tests of mean values, as the regression model fits a single curve to the dose-response relationship over the entire range of data.

RESULTS

BAL and Blood Cell Differentials

The number of PMNs isolated from silica-exposed rats by BAL was significantly higher ($p \leq .05$) than controls at every exposure time examined (Table 1). The degree of pulmonary inflammation was relatively stable from 5 to 30 days of exposure. However, the number of PMNs harvested by BAL progressively increased at 41 days of exposure and beyond. For the silica-exposed rats, blood neutrophils were significantly higher ($p \leq .05$) than control at every exposure time of 10 days or longer. The degree of systemic inflammation remained relatively stable from 10 to 41 days of exposure. However, at 79 and 116 days of exposure, blood neutrophils were significantly higher in comparison to shorter exposure times (Table 1).

The number of AMs isolated from silica-exposed rats was significantly higher ($p \leq .05$) than controls at exposure days 41 and 79, and increased

TABLE 1. Lavageable polymorphonuclear leukocytes and blood neutrophils

Exposure day	PMN (10^6 cells/rat)[a]		Neutrophils (10^6 cells/ml blood)[d]	
	Air	Silica	Air	Silica
5	1.66 ± 0.21	5.83 ± 0.74[b]	0.91 ± 0.08	1.08 ± 0.07
10	0.89 ± 0.09	6.01 ± 0.42[b]	1.00 ± 0.05	1.30 ± 0.06[b]
16	0.70 ± 0.07	8.63 ± 0.62[b]	0.99 ± 0.06	1.30 ± 0.08[b]
20	1.49 ± 0.46	7.30 ± 0.31[b]	0.90 ± 0.05	1.27 ± 0.09[b]
30	0.92 ± 0.10	7.23 ± 0.46[b]	0.92 ± 0.05	1.28 ± 0.07[b]
41	0.97 ± 0.07	11.08 ± 0.73[b,c]	0.99 ± 0.07	1.39 ± 0.07[b]
79	1.37 ± 0.11	45.22 ± 4.17[b,c]	1.12 ± 0.15	2.83 ± 0.15[b,e]
116	2.34 ± 0.26	143.16 ± 14.93[b,c]	0.82 ± 0.05	2.12 ± 0.19[b,e]

[a]The polymorphonuclear leukocytes (PMN) designation excludes alveolar macrophages (AM) but may include some lymphocytes (Castranova et al., 1979) and AM-derived apoptotic bodies recently reported from this study (Millecchia et al., 2000; Mercer et al., 2001). Values are means ± SE (n = 9–10 rats for each air-exposed and silica-exposed group at each exposure time).
[b]Significantly different from control ($p \le .05$).
[c]Significantly higher ($p \le .05$) than the preceding exposure time.
[d]Values are means ± SE (n = 11–15 rats for each air-exposed and silica-exposed group at each exposure time).
[e]Significantly higher ($p \le .05$) than rats exposed to silica for ≤41 days.

further at day 116 (Table 2). A significantly higher ($p \le .05$) number of blood monocytes was determined in silica-exposed rats in comparison to controls at 79 and 116 days of exposure (Table 2).

BAL Fluid LDH Activity

LDH activity in BAL fluid of silica-exposed rats was significantly higher ($p \le .05$) in comparison to air-exposed control rats at every exposure time

TABLE 2. Lavageable alveolar macrophages and blood monocytes

Exposure day	AM (10^6 cells/rat)		Monocytes (10^6 cells/ml blood)	
	Air	Silica	Air	Silica
5	6.86 ± 0.63	6.05 ± 0.65	0.33 ± 0.05	0.23 ± 0.05
10	5.24 ± 0.49	6.08 ± 1.00	0.15 ± 0.01	0.15 ± 0.03
16	4.80 ± 0.46	6.41 ± 0.70	0.17 ± 0.04	0.15 ± 0.03
20	6.84 ± 0.78	6.17 ± 0.33	0.24 ± 0.05	0.36 ± 0.06
30	5.41 ± 0.41	6.79 ± 0.85	0.13 ± 0.03	0.12 ± 0.02
41	4.75 ± 0.28	8.46 ± 0.98[a]	0.19 ± 0.05	0.18 ± 0.05
79	4.45 ± 0.31	8.45 ± 1.31[a]	0.13 ± 0.03	0.24 ± 0.02[a]
116	5.63 ± 0.52	27.17 ± 3.23[a,b]	0.16 ± 0.03	0.42 ± 0.06[a]

Note. Values are means ± SE (n = 9–10 rats for each air-exposed and silica-exposed group at each exposure time).
[a]Significantly different from control ($p \le .05$).
[b]Significantly higher ($p \le .05$) than rats exposed to silica for ≤41 days.

examined (Figure 1). For silica-exposed rats, the elevation of LDH activity was relatively constant through the first 30 days of exposure. Thereafter, LDH activity steadily increased with significant differences ($p \leq .05$) detected in LDH activity progressively from 41 to 116 exposure days (Figure 1).

BAL Fluid Serum Albumin Concentration

Silica-exposed rats had significantly higher ($p \leq .05$) levels of serum albumin in BAL fluid at all exposure times in comparison to air-exposed controls (Figure 2). BAL fluid serum albumin levels in silica-exposed rats at 5 and 10 days of exposure were significantly lower ($p \leq .05$) than those determined between 16 and 41 days of exposure, followed by significant progressive increases ($p \leq .05$) at 79 and 116 days of exposure (Figure 2).

NF-κB Activation in BAL Cells

The DNA binding activity of NF-κB was investigated using nuclear extracts of air-exposed and silica-exposed BAL cells with a gel shift assay (Figure 3, A and B). Analyses of the gel shift assays indicated that (1) NF-κB activation in BAL cells isolated from air-exposed controls remained low and relatively constant throughout the exposure, (2) NF-κB activation in BAL

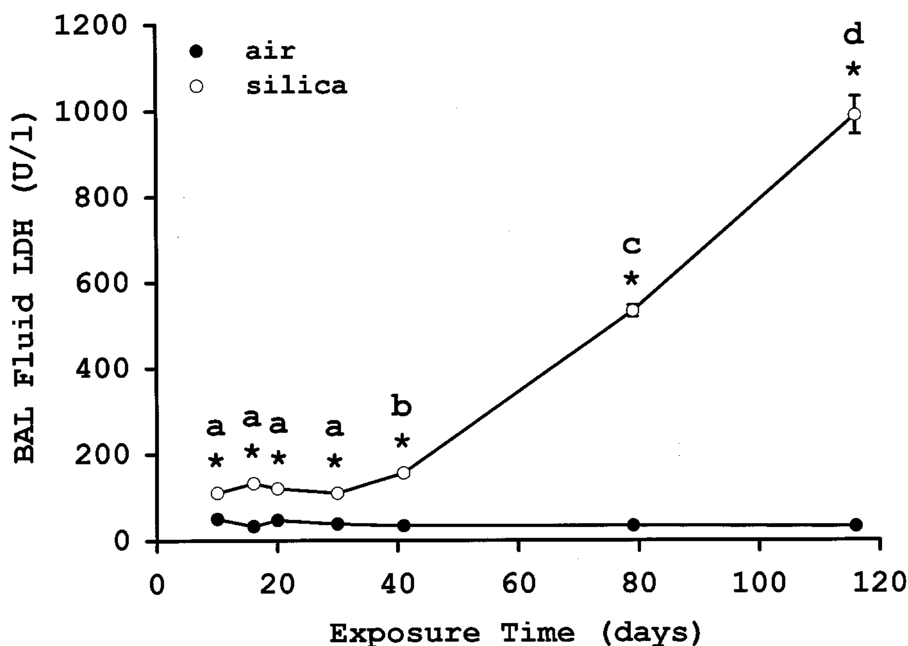


FIGURE 1. BAL fluid lactate dehydrogenase activities. Values represent means ± SE (n = 15 rats for each air-exposed and silica-exposed group at each exposure time). An asterisk indicates significant difference ($p \leq .05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq .05$).

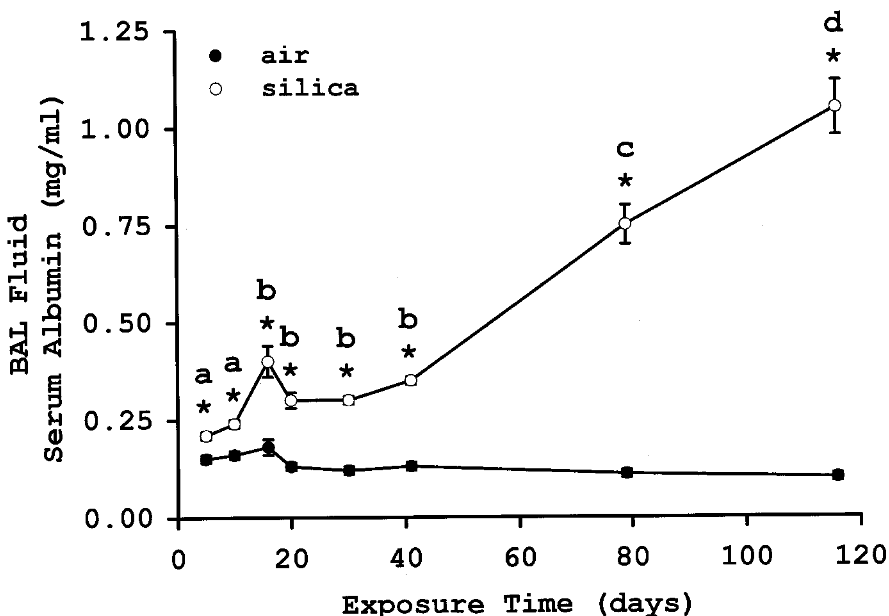


FIGURE 2. BAL fluid serum albumin concentrations. Values represent means ± SE (n = 15 rats for each air-exposed and silica-exposed group at each exposure time). An asterisk indicates significant difference ($p \leq .05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq .05$).

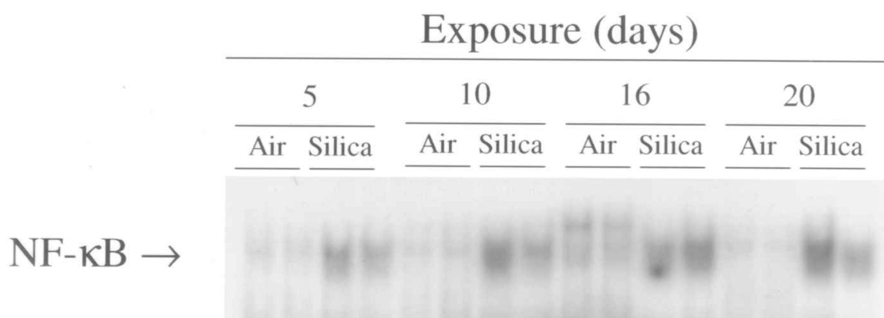
cells of silica-exposed rats was greater than the basal level observed in air-exposed rats at every exposure time, and (3) NF-κB activation in silica-exposed rats increased progressively throughout the silica exposure.

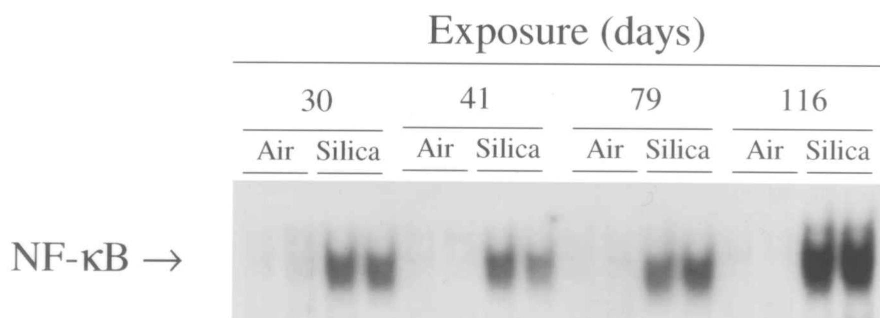
A supershift assay was conducted on the nuclear extracts from BAL cells isolated from a rat exposed to silica for 116 days (Figure 4). In comparison to the reference NF-κB band (Figure 4, lane 1), unlabeled oligonucleotide containing the NF-κB binding site reduced NF-κB binding (Figure 4, lane 2), whereas an oligonucleotide containing an AP-1 binding site (Figure 4, lane 3) did not effect NF-κB binding. Antibodies to p50 (Figure 4, lane 4) and p65 (Figure 4, lane 5) protein subunits of NF-κB caused the NF-κB band to shift, while an antibody to SP-1 had no effect (Figure 4, lane 6). Taken together, these results support the finding that NF-κB is activated, and its binding is specific in BAL cells isolated from silica-exposed rats.

TNF-α Production by BAL Cells

TNF-α was determined in BAL cell-conditioned media and was used to calculate total TNF-α production from lavagable BAL cells. In comparison to air-exposed controls, TNF-α production from BAL cells isolated from rats exposed to silica for 30, 79, and 116 days was significantly higher ($p \leq .05$) (Figure 5). In addition, BAL cell TNF-α production from 10 to 79 days of sil-

A





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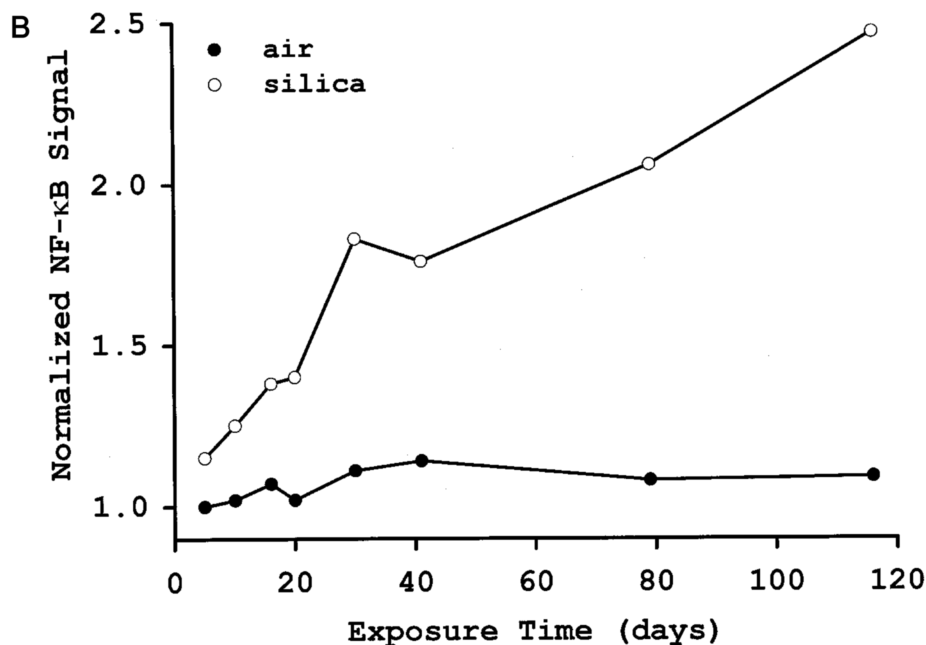

FIGURE 3. NF-κB gel shift assay. (A) Autoradiograph of the NF-κB gel shift assay conducted on nuclear extracts of BAL cells obtained from air-exposed and silica-exposed rats. (B) Intensity of NF-κB bands were quantitated with a phosphoimager. NF-κB band intensity was divided by a nonspecific band intensity to control for protein loading. Data are from duplicate samples (A) and are representative of several determinations.

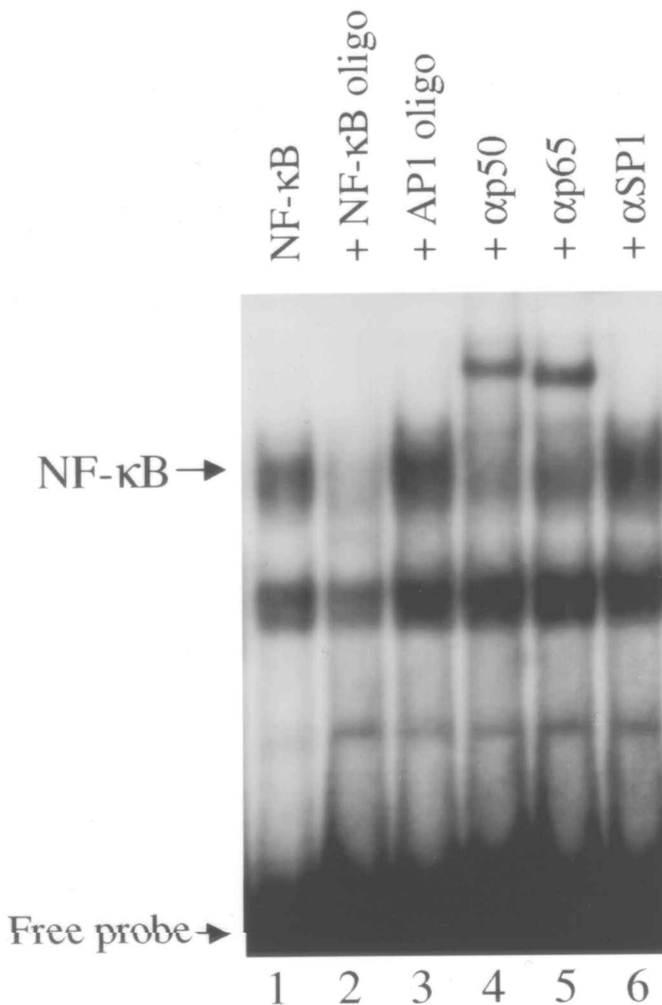

FIGURE 4. NF-κB gel supershift assay. Autoradiograph of an NF-κB gel super shift assay conducted on nuclear extracts of BAL cells obtained from a silica-exposed rat at 116 days exposure. Data are representative of several determinations. Lane 1, labeled NF-κB probe alone; lane 2, unlabeled NF-κB probe was included in the DNA–protein binding assay; lane 3, unlabeled AP-1 oligonucleotide probe was included in the DNA–protein binding assay; lane 4, antibody to the p50 subunit of NF-κB was included in the DNA–protein binding assay; lane 5, antibody to the p65 subunit of NF-κB was included in the DNA–protein binding assay; lane 6, SP-1 was included in the DNA–protein binding assay. Data are representative of several determinations.

ica exposure was significantly lower ($p \leq .05$) than that determined at 116 days of silica exposure (Figure 5).

IL-1 Production by BAL Cells

IL-1 was determined in BAL cell-conditioned media and was used to calculate total IL-1 production from lavagable BAL cells. BAL cells isolated from silica-exposed rats had significantly higher ($p \leq .05$) levels of IL-1 production

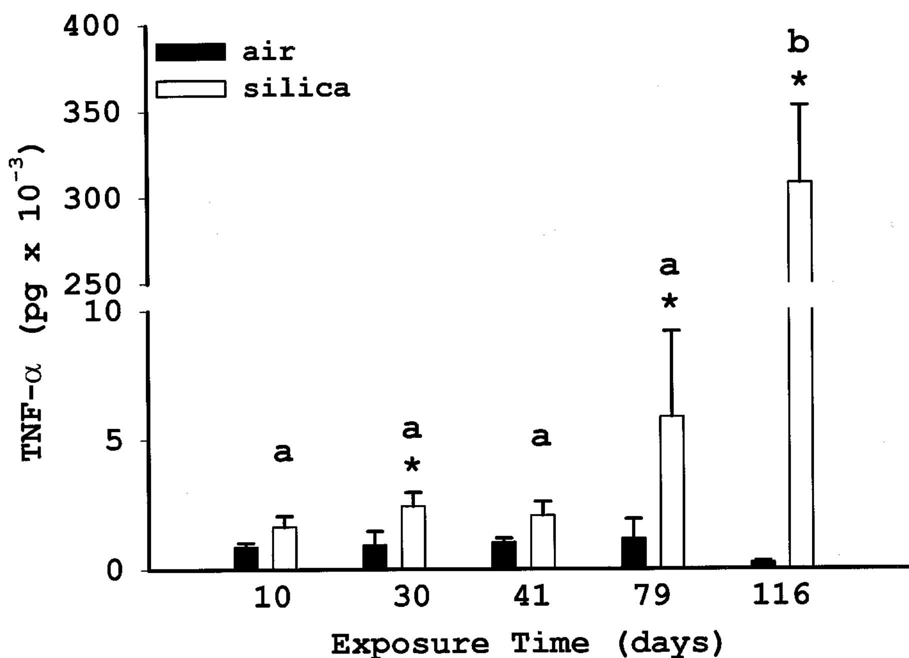

FIGURE 5. TNF-α production by BAL cells. Values represent means ± SE (n = 5 rats for each air-exposed and silica-exposed group at each exposure time). An asterisk indicates significant difference ($p \leq .05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq .05$).

at all exposure times in comparison to air-exposed controls (Figure 6). BAL cell IL-1 production from 10 to 79 days of silica exposure was significantly lower ($p \leq .05$) than that determined at 116 days of silica exposure (Figure 6).

DISCUSSION AND CONCLUSIONS

In a previous report from this study, we described the temporal relationships between silica exposure, lung silica burden, and the development of pulmonary fibrosis (Porter et al., 2001). In the present report, we examined the mechanisms underlying the development of pulmonary fibrosis, primarily focusing on silica-induced NF-κB activation, BAL markers of inflammation and damage, and the expression of inflammatory and fibrogenic cytokines by BAL cells.

Previous silica inhalation studies have been conducted using acute (Driscoll et al., 1991; Mohr et al., 1992; Friedetzky et al., 1998), subchronic (Henderson et al., 1995; Dey et al., 1996; Johnston et al., 2000) and chronic (Muhle et al., 1991) exposures to silica. However, none of these studies established the temporal sequence of molecular, cellular, and histopathological changes that occur after exposure to silica. The contribution of our studies is the description of a detailed time course of changes in a full array

of pulmonary parameters from transcriptional signaling to fibrosis during inhalation of silica.

Silica-induced inflammation, as indicated by elevated levels of PMNs in BAL cells, was noted at the earliest time point evaluated, that is, after 5 days of exposure (Table 1). This increased level of PMNs remained relatively constant through 30 days of exposure, after which PMNs progressively increased at a dramatic rate throughout the remainder of the silica exposure. Similar rapid increases in PMNs after intratracheal (IT) instillation of silica (Yuen et al., 1996) and progressive increases in PMNs in response to silica inhalation (Henderson et al., 1995) have been reported, although in the latter case the progressive increases occurred after cessation of the silica exposure. In the present study, the recruitment of PMNs into the lung was so extensive that blood neutrophils, the source of pulmonary PMNs, increased significantly by 10 days of exposure and exhibited a dramatic increase from this level at 79 to 116 days of exposure (Table 1). A previous study also reported increased blood neutrophils immediately after inhalation exposure to 100 mg/m^3 silica for 3 days (Gavett et al., 1992).

AMs play an important role in particle clearance from the lung, and thus as silica deposition in the lung increased it might be expected that the num-

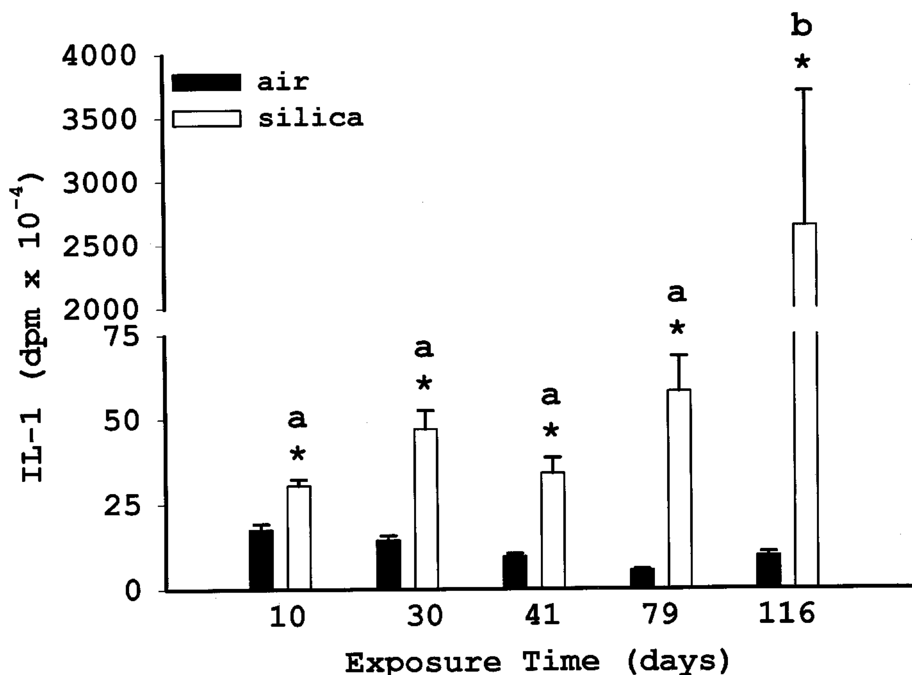

FIGURE 6. IL-1 production by BAL cells. Values represent means ± SE (n = 5 rats for each air-exposed and silica-exposed group at each exposure time). An asterisk indicates significant difference ($p \leq .05$) between air-exposed control and silica-exposed rats at a given exposure time. For silica-exposed rats, exposure times with different letters are significantly different from each other ($p \leq .05$).

ber of AMs isolated from silica-exposed rats would increase. To examine this hypothesis, AMs were determined in BAL cells isolated from both air-exposed and silica-exposed rats throughout the silica exposure (Table 2). These data indicate that by 41 days of exposure a significant increase in pulmonary AMs had occurred, and the magnitude of this increase was even greater at 116 days of exposure. Similar progressive increases in AMs after IT exposure to silica have been reported (Driscoll et al., 1990). Since blood monocytes are recruited to the lung and differentiate to form AMs, blood monocytes were measured from the same rats used to isolate AMs. Blood monocytes did increase significantly in silica-exposed rats, but this increase was later than that noted for AMs, occurring at 79 and 116 days of exposure (Table 2).

These BAL cell and blood cell data indicate that silica inhalation and deposition in the lung not only caused inflammation in the lung, but had systemic effects in regard to increasing the number of blood cells of the type that are recruited to the lung during an inflammatory response.

Silica deposition in the lung would also be expected to cause cytotoxic damage to the lung. This damage would in part be directly through silica-mediated radical production (Vallyathan et al., 1995) as well as cellular-mediated radical production (Blackford et al., 1994). LDH is an intracellular enzyme; thus, its presence in the acellular BAL fluid serves as a indicator of cytotoxicity. BAL fluid LDH levels were measured throughout the study and were significantly elevated above air-exposed rats from 10 to 30 days of exposure and increased progressively thereafter (Figure 1). The source of the LDH is unknown, but could be from damaged inflammatory cells as well as epithelial cells in the lung. BAL fluid serum albumin was measured and serves as a marker of damage to the blood–gas barrier of the lung. In silica-exposed rats, BAL fluid serum albumin exhibited a significant elevation at 5 days of exposure (Figure 2). This marker of damage increased further from 16 to 41 days of exposure and dramatically increased with further silica exposure (Figure 2). Measurements of BAL fluid total protein and phospholipids previously reported exhibited a similar pattern of increases in silica-exposed rats (Porter et al., 2001).

In this study, we established that in silica-exposed rats, NF-κB was activated in BAL cells (Figure 3A), and the degree of activation increased in a nearly linear fashion throughout the exposure (Figure 3B). We previously reported NF-κB activation in BAL cells isolated from rats exposed to silica by IT instillation at one hour post-exposure (Sacks et al., 1998). After IT instillation of LPS, NF-κB activation has been detected at 15 min postexposure (Blackwell et al., 1999). The data here are the first report to date of NF-κB activation in BAL cells isolated from rats exposed to silica by inhalation. In addition, in contrast to the rapid activation in NF-κB activation after IT instillation of silica (Sacks et al., 1998), in this study silica inhalation resulted in a slow but progressive increase in NF-κB activation during the exposure.

The production of TNF-α and IL-1 by BAL cells increased in silica-exposed rats at 30 and 10 days of exposure, respectively (Figures 5 and 6). Furthermore, TNF-α and IL-1 expression increased further at 116 days of exposure. Increased production of TNF-α and IL-1 by BAL cells isolated from silica-exposed rats has been reported as early as 1 day after IT silica exposure (Driscoll et al., 1990; Ma et al., 1999). The difference in the rapid increase in cytokine production reported after IT instillation versus that reported here may reflect the fact that an IT exposure produces a larger and much more rapid silica lung burden in comparison to the slow but progressive increase in silica lung burden reported in this inhalation study (Porter et al., 2001).

In vitro studies with the mouse monocyte-macrophage cell line RAW 264.7 have indicated that cytokine expression, after in vitro silica exposure, is dependent on NF-κB activation (Chen et al., 1995; Rojanasakul et al., 1999). In contrast, the data from this silica inhalation study indicate that NF-κB activation in BAL cells preceded increases in BAL cell cytokine production. Furthermore, there was only a mild enhancement in NF-κB activation in BAL cells when the explosive increase in BAL cell cytokine expression, from 79 to 116 days of exposure, occurred. Thus, the results from this in vivo study suggest that the regulation of cytokine expression in BAL cells may not be solely controlled by NF-κB activation and may involve the complex interplay of additional transcription factor regulatory mechanisms.

One possible additional regulator of NF-κB activation and cytokine expression is nitric oxide (NO). Studies with RAW 264.7 cells exposed to silica and LPS have suggested that NO may inhibit NF-κB activation (Chen et al., 1995), and exogenous NO can significantly reduce NF-κB activation (Raychaudhuri et al., 1999) and TNF-α secretion (Dinakar et al., 1999) from primary human AMs exposed to LPS in vitro. Immunohistochemistry studies conducted on lung samples obtained from this silica inhalation study have determined increased inducible NO synthase, the enzyme that produces NO during an inflammatory response (Millecchia et al., 1999). These determinations suggest that pulmonary NO is increased in the silica-exposed rats, and thus it is possible that increased pulmonary NO concentrations may also function in the regulation of BAL cell NF-κB activation and cytokine expression.

Examination of the data from this study indicates that explosive increases in inflammatory cell influxes and BAL fluid markers of damage (LDH and serum albumin), as well as similar increases in lung edema and BAL fluid total protein and phospholipids previously reported (Porter et al., 2001), occur at or before 79 days of exposure, which is much earlier than similar increases in the expression of cytokines, which occur at 116 days of exposure. In fact, the explosive increase in cytokines at 116 days of exposure occurs later than the initial histological detection of fibrosis at 79 days of exposure reported previously (Porter et al., 2001), whereas the explosive increases of the other markers of lung inflammation and damage are tem-

porally associated with the development of fibrosis. This would suggest that cytokine production, at least from BAL cells in rats exposed to silica via inhalation, may not be the most prominent driver for the initiation of fibrosis. Further support for this hypothesis comes from another silica inhalation study, which reported that basal IL-1 release from AMs harvested from silica-exposed rats was not significantly increased from the control even though lipopolysaccharide (LPS)-simulated IL-1 release was increased in these silica-exposed AMs (Driscoll et al., 1991).

As discussed earlier, the explosive rise in TNF-α and IL-1 production by BAL cells occurs after the initial histological indicators of a fibrotic response, as previously reported (Porter et al., 2001). This suggests that cytokine production may not be the primary driver of fibrosis. However, the role of proinflammatory cytokine production by alveolar epithelial cells after exposure to silica is not yet understood. Driscoll and colleagues (Driscoll et al., 1997) reported that quartz induced chemokine production in a rat type II cell line, but no stimulation in TNF-α production was observed. However, other investigators have reported that type II cells can produce TNF-α in response to specific stimulation in vitro (Barrett et al., 1999). In a previous report from this inhalation study, histological evidence for type II cell hyperplasia and hypertrophy occurring as early as 20 days of exposure, progressively increasing with continued silica inhalation, was presented (Porter et al., 2001). This type II cell activation has been shown to result in enhanced phospholipid production that preceded the initiation of the fibrotic process (Porter et al., 2001). Whether cytokine production from alveolar epithelial cells is also increased after inhalation of silica warrants study.

It is important to note that the increase in NF-κB activation and in the markers of pulmonary inflammation and damage occurred in rat lungs that were not in silica dust overload, as defined by Oberdörster and colleagues (Oberdörster et al., 1992). Lung particle overload, according to Oberdörster and colleagues, begins when the volume of particulate in the lung is ≥10% of the lung AM volume. In this silica inhalation study, the silica dust burden was estimated to be 7% of the total AM volume after 116 days of exposure, and thus the lungs were not in overload as classically defined (Porter et al., 2001).

When the results of this study are considered in conjunction with an earlier report from this same inhalation study (Porter et al., 2001), it becomes apparent that in response to silica inhalation there is an initial period of controlled inflammation and damage without fibrosis, followed by a period of explosive increases in inflammation and damage during which fibrosis develops. NF-κB activation occurred after only 5 days of exposure and increased steadily throughout the rest of the exposure, and therefore preceded the initiation of fibrosis. The markers of cellular damage, including BAL fluid LDH, serum albumin, total protein, and phospholipid, were increased in silica-exposed rats at 5 days of exposure and remained relatively constant through 41 days of exposure, before exhibiting

an explosive increase thereafter. Inflammation, as measured by BAL PMNs, was elevated after 5 days of silica exposure and remained at this level through the first 41 days of exposure, before exhibiting an explosive increase thereafter. In contrast, AMs and BAL cell cytokine secretion did not increase explosively in silica-exposed rats until 116 days of exposure, when pulmonary fibrosis had developed.

In summary, the results of this study extend our understanding of the temporal relationship between silica-induced NF-κB activation, pulmonary inflammation, damage, and fibrosis. Specifically, NF-κB activation is induced in BAL cells after 5 days of silica inhalation and increases linearly with continued exposure. Furthermore, in response to silica inhalation, there is an initial period of controlled inflammation and damage without fibrosis, followed by a period of explosive increases in inflammation and damage during which fibrosis develops. This suggests that it is possible that NF-κB activation plays a role in the initiation and progression of silica-induced lung disease.

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