USE OF TETRANDRINE TO DIFFERENTIATE BETWEEN MECHANISMS INVOLVED IN SILICA- VERSUS BLEOMYCIN-INDUCED FIBROSIS

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Animals exposed to silica or bleomycin (BLM) develop pulmonary fibrosis. Tetrandrine (TT) has been shown to inhibit stimulant-induced macrophage respiratory burst and effectively reduce silica-induced lung injury. The present study employed TT as a probe to assess the differences in mechanisms involved in silica- and BLM-induced pulmonary responses. Rats received a single intratracheal instillation of silica (40 mg/rat, sacrificed 4 wk postexposure) or BLM (1 mg/kg or ~0.25 mg/rat, sacrificed up to 2 wk postexposure). TT was administered orally at 18 mg/kg, 3 times/wk for desired time periods beginning 5 d before silica or BLM exposure. Both the silica and BLM exposures resulted in a significant increase in lung weight, total protein, lactate dehydrogenase (LDH), and phospholipids (PL) content in the acellular fluid from the first lavage, and hydroxyproline content in the lung tissue. Alveolar macrophages (AM) isolated from rats exposed to silica or BLM exhibited significant increases in secretion of interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and transforming growth factor β (TGF-β). TT treatment significantly lowered the silica- or BLM-induced increase in lung weight, while marginally reducing the release of IL-1 and TNF-α by AM. TT, however, markedly inhibited the silica-induced increase in the acellular protein, LDH and PL, hydroxyproline content, and the production of TGF-β by AM but had no marked effect on these same parameters in BLM-exposed rats. Histological examination of rats exposed to BLM for 14 d showed pulmonary inflammation and fibrosis. TT treatment had only a small effect on limiting the extent of these lesions and did not significantly affect their severity. In summary, data indicate that many inflammatory and fibrotic effects of in vivo silica exposure are substantially attenuated by TT, whereas the stimulation by BLM is only marginally affected by this drug. Since TT acts to attenuate AM-mediated reactions, these results suggest that AM may play a pivotal role in silica-induced fibrotic development and may be less involved in the pathogenesis of BLM-induced fibrosis.

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Chronic inhalation of crystalline silica dust is known to cause inflammation, granulomas, and pulmonary fibrosis (Ziskind et al., 1976). Alveolar macrophages (AM) appear to play an important role in the pathogenesis of silicosis, as silica activates AM to release reactive oxygen species that can cause lung damage (Brain, 1980; Borm & Henderson, 1990; Lapp & Castranova, 1993). Lung injury due to silica exposure can be readily demonstrated by elevated levels of lactate dehydrogenase (LDH) and total protein in the bronchoalveolar lavage fluid (BALF), and fibrosis in silica-exposed lungs (Driscoll et al., 1990a), suggesting that a correlation may exist between changes in BALF markers of lung injury and the development of lung fibrosis. Activated AM also secrete inflammatory and fibrogenic mediators such as interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and transforming growth factor β (TGF-β). Proinflammatory cytokines, IL-1 and TNF-α, can mediate the recruitment and activation of inflammatory cells. IL-1 and TNF-α also stimulate fibroblast proliferation and collagen production (Schmidt et al., 1982; Driscoll et al., 1990b; Piguet et al., 1993). TGF-β is a potent chemoattractant for monocytes (Assoian et al., 1987) and fibroblasts (Postlethwaite et al., 1987), and a positive modulator of fibroblast proliferation (Fine & Goldstein, 1987), and a stimulator for collagen synthesis in fibroblasts (Ignotz & Massague, 1986). After an intratracheal instillation of silica to rats, a time- and dose-dependent relationship has been reported between AM release of IL-1 and TNF-α and histological evidence of inflammation and fibrosis (Driscoll et al., 1990b). In addition, pretreatment of rats with anti-TNF antibody decreases the magnitude of pulmonary fibrosis in response to silica (Piguet et al., 1990).

Therefore, it has been proposed that macrophage release of oxidants and cytokines plays a key role in the development and progression of silicosis (Lapp & Castranova, 1993).

Bleomycin is an antineoplastic agent that is especially effective in the treatment of squamous-cell carcinomas (Umezawa, 1974). Bleomycin has been shown to produce dose-dependent pneumonitis and pulmonary fibrosis in humans and animals (Crystal et al., 1976; Giri et al., 1980). The bleomycin-induced lung injury in animal models is characterized by pulmonary edema, hemorrhage, cellular infiltration, and fibrosis. The measurable parameters for bleomycin-induced fibrosis are similar to those observed for silicosis, which include lung damage, inflammation, stimulation of fibroblast proliferation, and collagen synthesis (Phan et al., 1980). In response to bleomycin, IL-1 (Jordana et al., 1988) and TGF-β (Khalil et al., 1989, 1993) secretions from AM are elevated. An increase in whole-lung mRNA levels for TNF-α has also been reported (Piguet et al., 1989). TNF-α seems to play a key role in bleomycin-induced fibrosis, since pretreatment of rats with anti-TNF antibody decreases the pulmonary response to bleomycin (Piguet et al., 1989). Studies from our laboratory have shown that unlike silica, bleomycin is not a potent stimulant

of superoxide anion release, and bleomycin inhibits stimulant-induced secretion of oxidants by AM (Bhat et al., 1994). These data suggest that bleomycin-induced lung damage may not be due to enhanced oxidant production by AM. Studies have shown that intracellular bleomycin can interact with Fe^{2+} and this intracellular complex can catalytically reduce molecular oxygen to produce various reactive oxygen species (Caspary et al., 1982). Indeed, it was found that bleomycin can increase intracellular oxidant levels (Bhat et al., 1994). These data suggest that bleomycin can act as a direct oxidant. Since antioxidants decrease bleomycin-induced toxicity, it is possible that the direct-oxidant burden from bleomycin initiates the fibrotic process (Giri et al., 1994).

Tetrandrine, a bisbenzylisoquinoline alkaloid, has been reported to exhibit antifibrotic properties. In animals exposed to silica, tetrandrine treatment resulted in a decrease in the synthesis of collagen by pulmonary fibroblasts and a reduction in the formation of silicotic nodules (Huang et al., 1981; Mo et al., 1982; Yu et al., 1983). Studies carried out in our laboratory have demonstrated that tetrandrine does not affect direct silica-induced cytotoxicity in vitro and that it is not a strong antioxidant (Castranova et al., 1991); however, tetrandrine effectively inhibits respiratory burst activity (Ma et al., 1992) and the release of IL-1-like cytokines from silica-exposed AM (Kang et al., 1992). The inhibitory action of tetrandrine on AM activity was attributed to the interaction of tetrandrine with the cytoskeletal structure of intact AM (Ma et al., 1992). In an in vivo study in rats using a multiple emulsion system to facilitate drug delivery to the pulmonary region, it was further demonstrated by light microscopy that tetrandrine treatment markedly decreases lung lesions in silica-exposed lungs (Chao et al., 1996). The potential effect of tetrandrine on the pulmonary response to bleomycin exposure has not been examined. Tetrandrine may potentially be useful as a molecular probe to delineate differences in mechanisms of pulmonary fibrosis in silica versus bleomycin animal models. In the present study, a comparative study of the tetrandrine effect on inflammatory responses common to both fibrotic models is reported.

METHODS

Materials and Treatment of Animals

Male Sprague-Dawley rats (~250 g) were obtained from Hilltop Laboratories (Scottdale, PA). After a 1-wk acclimation period in an AAALAC-approved animal facility, they were lightly anesthetized with sodium methohexital and received a single intratracheal (IT) instillation of 0.3 ml saline, 40 mg silica/0.3 ml saline, or 0.25 mg bleomycin/0.3 ml saline. For animals in drug treatment groups, tetrandrine was administered orally at 18 mg/kg body weight. Animals were pretreated with tetrandrine twice

at 5 and 3 d prior to silica or bleomycin instillation, then treated at the day of exposure, and 3 times/wk after exposure for the desired time period.

Tetrandrine was obtained from the Institute of Occupational Medicine, Chinese Academy of Preventive Medicine (Bejing, China). Crystalline silica (Min-U-Sil, particle size 5 μm) was obtained from U.S. Silica Corporation (Berkeley Springs, WV). Silica was sterilized by heating at 160°C for 90 min in the dry oven before use. Bleomycin was kindly provided by Bristol-Myers Squibb Company (Evansville, IN).

Isolation of Alveolar Macrophages

Animals were anesthetized with sodium pentobarbital (0.2 g/kg body weight) and exsanguinated by cutting the renal artery. Alveolar macrophages were obtained by pulmonary lavage with a Ca^{2+},Mg^{2+}-free phosphate-buffered medium (145 m*M* NaCl, 5 m*M* KCl, 1.9 m*M* NaH_2PO_4, 9.35 m*M* Na_2HPO_4, and 5.5 m*M* glucose; pH 7.4), 6 ml for the first lavage and 8 ml for the subsequent lavages. Cells were centrifuged at 500 × g for 5 min, washed, and resuspended in a HEPES-buffered medium (145 m*M* NaCl, 5 m*M* KCl, 10 m*M* HEPES, 5.5 m*M* glucose, and 1.0 m*M* $CaCl_2$; pH 7.4). In some experiments, the first lavage was saved separately from the following lavages for further analysis. Cell counts and purity were measured using an electronic cell counter equipped with a cell sizing attachment.

Alveolar Macrophage Cultures

Lavage cells were resuspended in RPMI-1640 media containing 2 m*M* glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% heat-inactivated calf serum. Aliquots of 1 ml containing 1×10^6 AM were added to 24-well tissue culture plate and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 2 h. The nonadherent cells were then removed with three washes of RPMI-media. The AM-enriched adherent cells were incubated in fresh RPMI media for an additional 24 h. The macrophage-conditioned media were collected, centrifuged, and the supernatants were stored in aliquots at –80°C for cytokine analysis.

Chemiluminescence Determination

Chemiluminescence (CL) generated by AM was determined using an automated luminometer (Berthold Autolumat LB 953, Wallac, Inc., Gaithersburg, MD). Rat AM (7.5×10^5/0.75 ml) were preincubated at 37°C for 5 min in HEPES-buffered medium containing 0.08 μg/ml luminol. Zymosan (2 mg/ml) was added to the preincubated sample and CL production was immediately measured for 10 min. Nitric oxide-dependent CL, that is, the *N*-nitro-L-arginine methyl ester (L-NAME) inhibitable portion of zymosan-stimulated CL, was determined by preincubation of AM with 1 m*M* L-NAME, an inhibitor of nitric oxide synthase, for 10 min before the addition of zymosan. Data were plotted as CL versus time, and the area under the curve was integrated to give the total CL in cpm/7.5×10^5 AM/10 min.

Measurement of Protein, Phospholipids, Lactate Dehydrogenase, and Hydroxyproline

Total acellular protein in the first alveolar lavage fluid sample was determined by the method of Lowry et al. (1951). The amount of phospholipid was measured as phosphorus present in lipid extracts of the lavage fluid (Bartlett, 1959). In order to obtain phospholipid content, lipid phosphorus values were multiplied by 25 (Oyarzun & Clements, 1978). Lactate dehydrogenase (LDH) was measured by monitoring the reduction of pyruvate coupled with the oxidation of NADH, which was monitored spectrophotometrically at 340 nm according to the method of Wroblewski and LaDue (1955).

The formation of collagen in rat lungs was analyzed by measurement of the hydroxyproline content in lung tissues. Rat lungs were chopped and hydrolyzed in 6 *N* HCl for 48–72 h at 110°C. Hydroxyproline was determined according to the method of Kivirikko et al. (1967).

IL-1 Assay

The IL-1 activity was determined according to the method described previously (Kang et al., 1992). Briefly, thymocytes were obtained from male CD-1 mice (~20 g; 6–10 wk of age) and suspended in RPMI-1640 media with 2 m*M* glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal calf serum (FCS), and 2×10^{-5} *M* mercaptoethanol. Cells (1×10^6/0.1 ml/well) and aliquots (0.1 ml) of macrophage-conditioned supernatant were added in quadruplicate to the 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 [^{3}H]thymidine (1 μCi/well; activity 11.3 Ci/mmol; Dupont NEN Products, Boston), and harvested using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, ME). The radioactivity in the collecting glass filter disks was measured using a liquid scintillation counter (1214 Rackbeta, Wallac, Finland). The amount of IL-1-like activity in the tested macrophage supernates was expressed as counts per minute and then calculated as percentage of the unstimulated control. Antibodies of anti-rat IL-1α and anti-rat IL-1β (Endogen, Cambridge, MA) were used to confirm IL-1 activity measured by the thymocyte proliferation assay. These antibodies completely neutralized the activity in the macrophage-conditioned supernate, thus indicating that the increase in thymocyte proliferation was due to the presence of IL-1 in the culture media.

TNF-α Assay

TNF-α activity was determined using the L929 cytotoxicity assay reported by Shahan et al. (1994). Briefly, the murine fibroblast cell line NCTC-clone L929 was obtained from American Type Culture Collection (Rockville, MD). Cells were plated in 96-well microculture plate at 2×10^4 cells/0.1 ml Isocove's modified Dulbecco's medium with 30% heat-inactivated horse serum (IMDM) and cultured at 37°C for 24 h. The medium

was removed and replaced with 0.1 ml of fresh IMDM with 0.5 μg/ml actinomycin D and 50 μl of TNF-α standards or conditioned medium and incubated at 37°C for additional 24 h. Medium and samples were removed and replaced with 150 μl of IMDM solution containing 10% Alamar blue (Alamar Biosciences, Sacramento, CA) and the cell culture was incubated for 18 h. The plates were read on a fluorescence 96-well plate reader fluorescence concentration analyzer (model 940-00050, IDEX Laboratories, Westbrook, ME) using a 545–575 nm bandpass filter. Each plate was run with negative and positive controls and a complete set of standards (recombinant murine TNF-α from R&D Systems, Minneapolis, MN). TNF-α concentration was determined from the respective standard curve.

CCL-64 Mink Lung Epithelial Growth Inhibition Assay for TGF-β

Alveolar macrophages obtained 7 d after silica or bloemycin administration were maintained in serum-free conditions for 24 h at 37°C, and the conditioned medium was collected. TGF-β in the conditioned medium was determined using the method described by Danielpour et al. (1989). Briefly, CCL-64 mink lung epithelial cells were maintained in minimum essential medium (MEM) with 10% FBS. Subconfluent cells were used in the TGF-β growth inhibition assay. Cells were trypsinized and washed with MEM, 0.2% fetal bovine serum (FBS), and 10 m*M* HEPES at pH 7.4, as well as penicillin (100 U/ml) and streptomycin (100 μg/ml), and cultured at 37°C as 1×10^5 cells/0.5 ml in 24-well culture plates. Macrophage-conditioned media or conditioned media that was acidified and subsequently neutralized in the absence or presence of anti-TGF-β_1 antibodies (R&D Systems, Minneapolis, MN) was added 1 h later. After 22 h, the cells were pulsed with 0.25 μCi of [^{3}H]thymidine for 24 h at 37°C. Cells were then fixed with 1 ml of methanol-acetic acid (3:1, v/v). After 1 h at room temperature the wells were washed twice with 2 ml of 80% methanol. The cells were lysed with 0.5 ml of 0.05% trypsin for 30 min at room temperature, followed by the addition of 0.5 ml of 1% sodium dodecyl sulfate. Cells were harvested and [^{3}H]thymidine was counted in a liquid scintillation counter. Standard curves of porcine TGF-β and TGF-β_1 (R&D Systems, Minneapolis, MN) were included in each assay. The concentrations of TGF-β or TGF-β_1 were determined from the standard curve.

Histological Examination

Lung tissues from bleomycin-exposed rats with or without tetrandrine treatment were processed at 2 wk postexposure. Lungs were fixed immediately after sacrifice by intratracheal instillation of 10% neutral buffered formalin at a pressure of 30 cm H_2O (at an altitude of 960 ft) if the whole lung was available. For studies where only the right middle lung lobe was designated for pathology, the lungs were perfused by the transpleural injection of 1 ml of 10% neutral buffered formalin using a 25-gauge needle.

Lungs were fixed, embedded in paraffin, and stained with hematoxylin and eosin for light microscopic examinations (Prophet et al., 1992). Pathological scoring of lung injury was performed by a veterinary pathologist using coded slides not identified by treatment.

Data Analysis

For all experiments, data are presented as means ± standard errors of at least five measurements from different animals. Statistical analysis of semiquantitative histopathology was conducted using a one-way analysis of variance (ANOVA) with a Student–Newman–Keuls pairwise multiple comparison procedure with significance set at $p < .05$. If conditions for validity of the ANOVA were not met, a Kruskal–Wallis one-way analysis of variance on ranks with Dunn's pairwise multiple comparison procedure was performed.

RESULTS

Exposure of rats to silica or bleomycin with or without tetrandrine treatment did not markedly alter the body weight (data not shown), but these exposures had significant effects on lung weights. Figure 1 shows the gross effect of silica, tetrandrine, and their combination on lung weights. There was a threefold increase in lung weights in silica-exposed animals compared to the controls. Tetrandrine significantly ameliorated (by 60%) the sil-

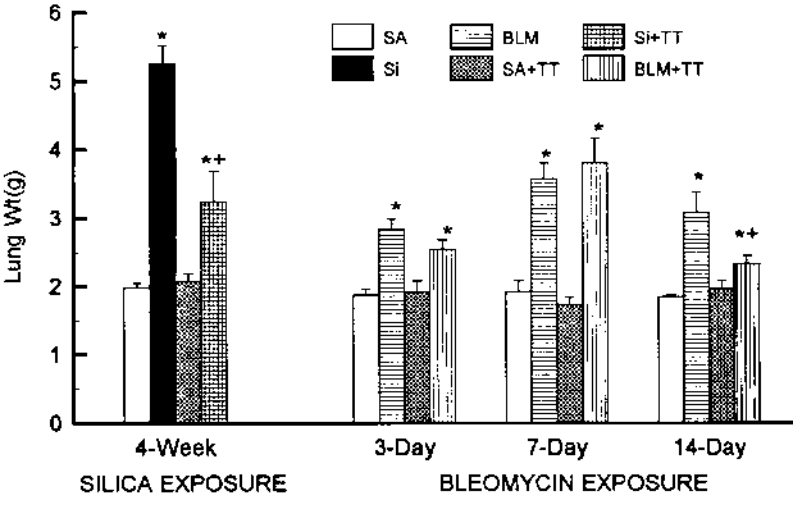

FIGURE 1. Effect of tetrandrine treatment on the lung weight 4 wk after a single intratracheal instillation of silica (40 mg) or 3, 7, and 14 d after IT exposure to bleomycin (1 mg/kg). Legend: SA, saline-exposed; Si, silica-exposed; BLM, bleomycin-exposed; SA + TT, saline-exposed and tetrandrine-treated; Si + TT, silica-exposed and tetrandrine-treated; BLM + TT, bleomycin-exposed and tetrandrine-treated. Asterisk indicates statistically significant difference from the saline group, $p < .05$. Plus sign indicates statistically significant difference from the silica- or bleomycin-exposed group, $p < .05$.

ica-mediated increase in lung weight. Figure 1 also shows that the wet lung weights of bleomycin-exposed rats were significantly higher than those of the controls at 3, 7, and 14 d postexposure. Tetrandrine treatment did not have a protective effect on the bleomycin-induced increase in lung weight at 3 and 7 d postexposure. However, tetrandrine significantly reduced the bleomycin-induced increase in lung weight by 58% when measured at 14 d after bleomycin exposure.

Figure 2 shows the differential cell counts in BALF from bleomycin-exposed rats. In comparison to the control, cell count for AM was significantly reduced at 3 d after bleomycin exposure and showed recovery at 7 and 14 d postexposure. Bleomycin-induced lung inflammation was indicated by a significant increase in red blood cells, lymphocytes, and polymorphonuclear leukocytes (PMN). Although these cellular changes were significantly elevated at all time points, the values at 14 d postexposure were substantially lower than at 7 d postexposure. Tetrandrine did not markedly alter these cellular responses to bleomycin exposure. Previous studies from this laboratory have shown that there is a significant infiltration of red blood cells, lymphocytes, and PMN into the alveolar space of rats exposed to silica (Shoemaker et al., 1995). At 2 wk post silica exposure, tetrandrine was shown to reduce the PMN in the lavage fluid (Chao et al., 1996).

Table 1 shows acellular protein, LDH, and phospholipid contents in the first lavage from silica- and bleomycin-exposed rats with or without

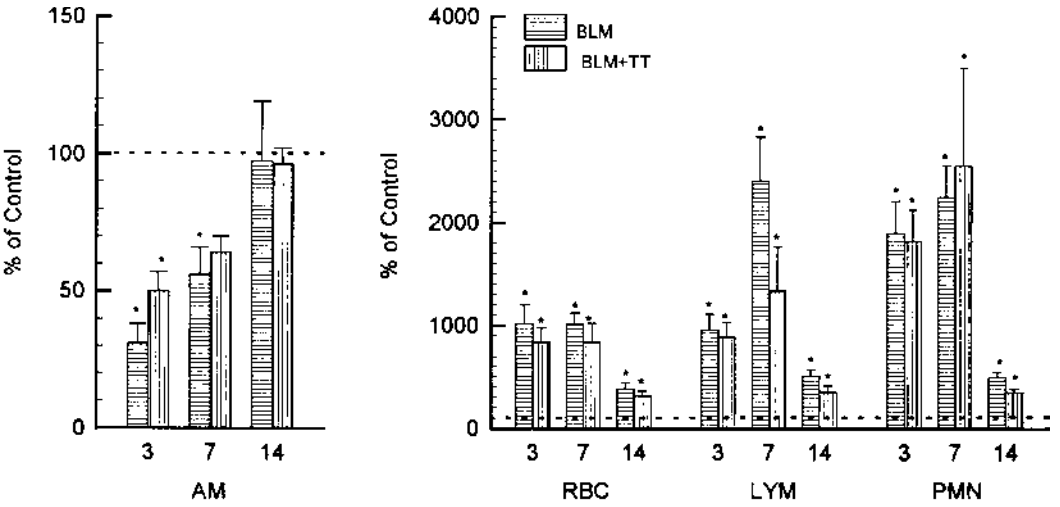

FIGURE 2. Effect of tetrandrine treatment on bronchoalveolar lavage cell yield at 3, 7, and 14 d postbleomycin exposure (1 mg/kg, IT). The saline group served as the control. The dotted line indicates the control value (100%). Tetrandrine treatment had no significant effect on cell differentials or yields in the saline group (data not shown). Legend: BLM, bleomycin-exposed; BLM + TT, bleomycin-exposed and tetrandrin-treated. Asterisk indicates statistically significant difference from the saline group, $p < .05$.

TABLE 1. Effects of Tetrandrine Treatment on Lavage Fluid Parameters of Silica- or Bleomycin-Exposed Animals

Treatment[a]	Protein	LDH	Phospholipids
Saline	100 ± 24%	100 ± 6%	100 ± 20%
Saline + TT	158 ± 29%	93 ± 5%	92 ± 13%
Silica (4 wk)	1959 ± 503%[b]	1507 ± 402%[b]	4597 ± 1272%[b]
Silica + TT (4 wk)	813 ± 223%[b,c]	370 ± 104%[b,c]	628 ± 241%[b,c]
BLM (14 d)	422 ± 50%[b]	190 ± 29%[b]	184 ± 75%[b]
BLM + TT (14 d)	450 ± 90%[b]	158 ± 14%[b]	210 ± 38%[b]

[a]Values within parentheses are times after an IT exposure to silica or bleomycin.
[b]Statistically greater than control (saline) group ($p < .05$).
[c]Statistically less than silica-exposed group ($p < .05$).

tetrandrine treatment. Silica exposure (4 wk postexposure) was associated with a 19-fold increase in total protein, a 15-fold increase in LDH, and a 45-fold rise of phospholipids in the acellular fluid collected from the initial lung lavage as compared to saline controls. Tetrandrine treatment of silica-exposed rats resulted in a significant reduction of each of these parameters. In comparison to silica exposure, a relatively smaller but significant increase in the total protein content (fourfold), LDH (twofold), and phospholipids (twofold) in the first lavagate was also observed from bleomycin-exposed rats (14 d postexposure). Tetrandrine treatment had no marked effect on the bleomycin-induced increases in lung lavage materials.

Table 2 shows the effect of tetrandrine on the tissue content of hydroxyproline, a major component of collagen, in control, silica-, or bleomycin-exposed lungs. In comparison to the controls, there was a 2-fold increase in

TABLE 2. Effects of Tetrandrine Treatment on Hydroxyproline Content in Silica- or Bleomycin-Exposed Rat Lungs

Treatment[a]	Hydroxyproline (mg/lung)
Saline	2.11 ± 0.21
Saline + TT	2.15 ± 0.05
Silica	4.57 ± 0.23[b]
Silica + TT	3.21 ± 0.19[b,c]
Bleomycin	3.40 ± 0.21[b]
Bleomycin + TT	3.60 ± 0.29[b]

[a]Measurements made 4 wk postexposure for silica and 14 d postexposure for bleomycin.
[b]Satistically greater than control (saline) group ($p < .05$).
[c]Statistically less than silica-exposed group ($p < .05$).

hydroxyproline content in the silica-exposed lungs (at 4 wk, postexposure) and a 1.5-fold increase in hydroxyproline content in the bleomycin-exposed lung tissue (measured at 14 d after exposure). Treatment with tetrandrine reduced the silica-induced increase of lung collagen by 56%, but had no effect on the bleomycin-induced increase in lung collagen.

The effect of in vivo silica or bleomycin exposure on oxidant production by AM was investigated by measuring the cellular generation of chemiluminescence (CL). As shown in Table 3, silica exposure and/or tetrandrine treatment had no marked effect on the level of CL generated by resting AM. However, when challenged by zymosan, AM from silica-exposed rats exhibited an eightfold increase in both total and nitric oxide-induced CL production over the control. This silica effect was not altered by in vivo treatment with tetrandrine. Table 4 shows the total and nitric oxide-induced CL generated by AM from rats exposed to bleomycin for 3 and 14 d. Bleomycin exposure was associated with an increase in both resting and zymosan-stimulated CL from AM obtained at 3 d postexposure; however, the bleomycin-induced effect is weaker than that of silica. This increase was diminished to the control level at 2 wk postexposure. The nitric oxide-dependent CL also exhibited a similar time-dependent response to bleomycin exposure. Tetrandrine treatment did not markedly affect the production of CL from AM of bleomycin-exposed rats.

Only small amounts of IL-1 and TNF-α were detected in the conditioned media of control AM from saline-administered rats. The levels of IL-1 and TNF-α spontaneously released by AM from silica-exposed rats are shown in Figure 3, A and B, respectively. AM from silica-exposed rats consistently showed increased secretion of both IL-1 and TNF-α at 1, 7, 14, and 28 d postexposure. The corresponding measurements of cytokines secretion by AM from silica-exposed and tetrandrine-treated rats were consistently lower but not statistically different from those of silica-exposed, nontreated animals.

Intratracheal instillation of bleomycin stimulated AM release of IL-1 at 3, 7, and 14 d postexposure (Figure 4A). The bleomycin-stimulated IL-1

TABLE 3. Effects of Treatment with Tetrandrine on Chemiluminescence Generated from Silica-Exposed Alveolar Macrophages

Treatment (4 wk postexposure)	Resting (cpm × 10^{-4})	Zymosan-stimulated CL from AM (cpm × 10^{-4})	L-NAME-inhibitable CL from zymosan-stimulated AM (cpm × 10^{-4})
Saline	6.37 ± 0.65	26.51 ± 5.28	10.01 ± 3.05
Saline + TT	5.34 ± 0.16	26.81 ± 3.75	13.04 ± 2.44
Silica	6.67 ± 0.21	229.50 ± 19.10[a]	87.47 ± 7.09[a]
Silica + TT	6.47 ± 0.56	197.33 ± 36.84[a]	101.22 ± 13.19[a]

[a]Statistically greater than control (saline) group ($p < .05$).

TABLE 4. Effects of Treatment with Tetrandrine on Chemiluminescence Generated from Bleomycin-Exposed Alveolar Macrophages

	Resting CL (cpm × 10^{-4})		Zymosan-stimulated CL from AM (cpm × 10^{-4})		L-NAME-inhibitable CL from zymosan-stimulated AM (cpm × 10^{-4})	
Treatment	3 d	14 d	3 d	14 d	3 d	14 d
Saline	5.83 ± 0.43	4.57 ± 0.50	20.40 ± 2.62	19.74 ± 2.15	8.28 ± 1.27	6.71 ± 0.91
Saline + TT	6.93 ± 1.22	4.48 ± 0.37	29.77 ± 4.39	19.07 ± 2.65	11.45 ± 1.22	6.09 ± 1.46
BLM	10.23 ± 0.48[a]	5.01 ± 0.46	75.07 ± 11.02[a]	20.09 ± 1.92	18.53 ± 3.39[a]	6.24 ± 1.02
BLM + TT	8.83 ± 0.63[a]	5.00 ± 0.56	75.93 ± 29.74[a]	24.74 ± 5.83	17.06 ± 7.44	8.70 ± 1.57

Note. Data presented at 3 and 14 d postexposure.
[a]Statistically different from control (saline) group ($p < .05$).

production from AM was greatest at 3 d postexposure, gradually diminished at 7 and 14 d, and was absent at 21 d postexposure. At 3 and 7 d post bleomycin exposure, AM exhibited an increase in spontaneous secretion of TNF-α (Figure 4B), which is significantly higher than the peak level of TNF-α released from silica-exposed AM. Normal level of TNF-α was detected in the macrophage-cultured supernatant at 14 d postexposure. When bleomycin-exposed animals were treated with tetrandrine, the amount of IL-1 secreted from bleomycin-exposed AM was lower compared to AM from bleomycin-exposed, nontreated animals. The inhibitory effect, however, did not reach statistical significance. Tetrandrine was

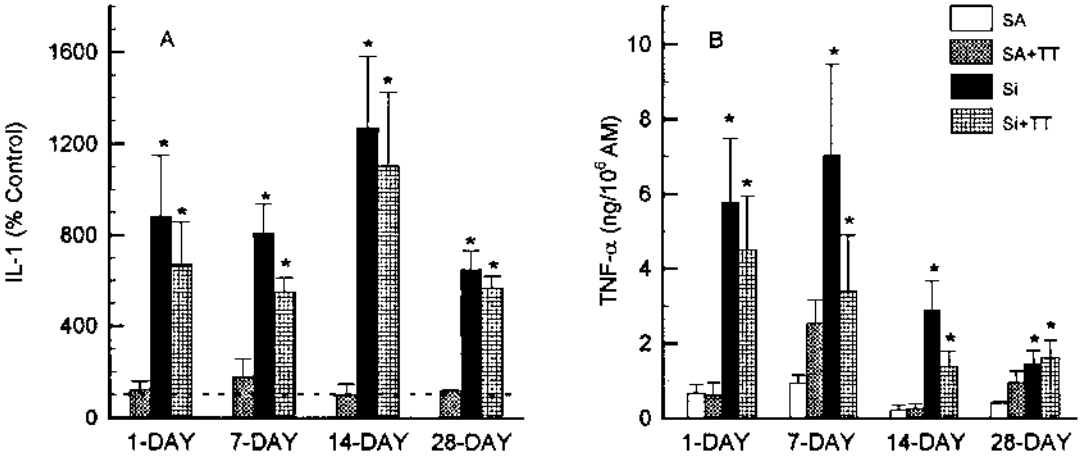

FIGURE 3. Effect of tetrandrine treatment on (A) IL-1 and (B) TNF-α production from macrophages in response to intratracheally instilled silica (40 mg) at 1, 7, 14, and 28 d postexposure. The dotted line indicates the control value (100%). Legend: SA, saline-exposed; Si, silica-exposed; SA + TT, saline-exposed and tetrandrin-treated; Si + TT, silica-exposed and tetrandrine-treated. Asterisk indicates statistically significant difference from the saline group, $p < .05$.

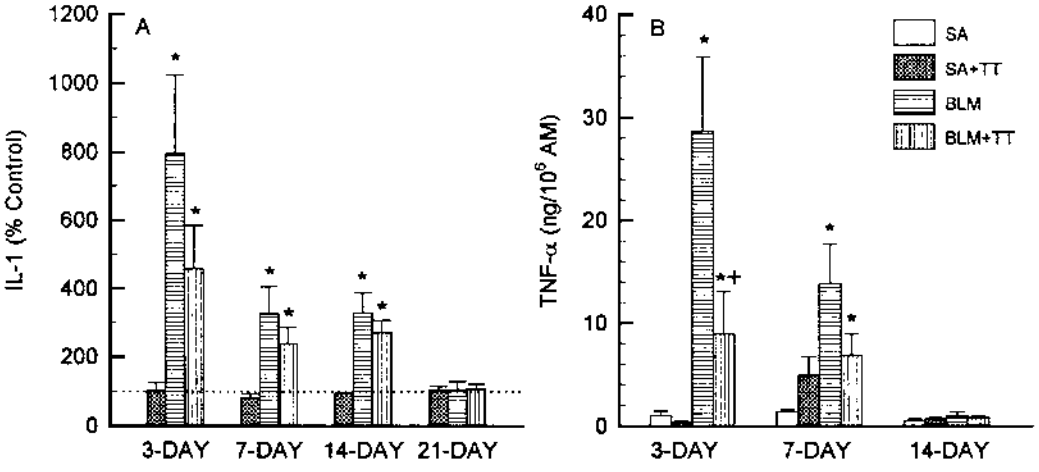

FIGURE 4. Effect of tetrandrine treatment on (A) IL-1 and (B) TNF-α production from macrophages of bleomycin-exposed animals. Macrophages were obtained from rats at 3, 7, 14, and 21 d after a single intratracheal instillation of bleomycin (1 mg). The dotted line indicates the control value (100%). Legend: SA, saline-exposed; BLM, bleomycin-exposed; SA + TT, saline-exposed and tetrandrine-treated; BLM + TT, bleomycin-exposed and tetrandrine-treated. Asterisk indicates statistically significant difference from the saline group, $p < .05$. Plus sign indicates statistically significant difference from the bleomycin-exposed group, $p < .05$.

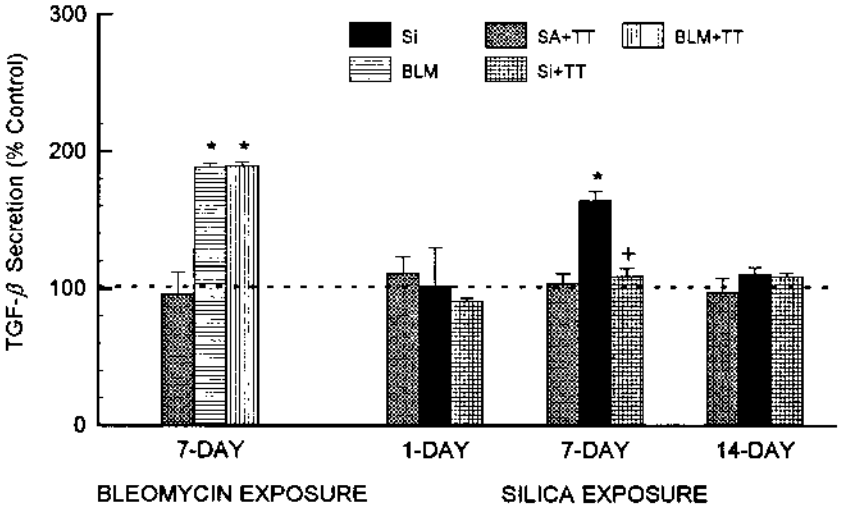

FIGURE 5. Effect of tetrandrine treatment on TGF-β secretion from macrophage of silica (40 mg) or bleomycin (1 mg/kg) exposed rats. The dotted line represents the control value (100%). Legend: Si, silica-exposed; BLM, bleomycin-exposed; SA + TT, saline-exposed and tetrandrine-treated; Si + TTS, silica-exposed and tetrandrine-treated; BLM + TT, bleomycin-exposed and tetrandrine-treated. Asterisk indicates statistically significant difference from the saline group, $p < .05$. Plus sign indicates statistically significant difference from the silica-exposed group, $p < .05$.

also shown to reduce bleomycin-induced macrophage production of TNF-α. The inhibitory effect of tetrandrine on TNF-α production at 3 d postexposure was statistically significant.

Figure 5 shows that TGF-β was significantly increased in the conditioned media obtained from AM harvested from rats 7 d after silica or bleomycin exposure. Neutralizing antibody to TGF-β_1 was used in the CCL-64 bioassay to quantitate the amount of TGF-β_1 in the AM-conditioned media. The results show that 21 and 56% of TGF-β is in the TGF-β_1 form in silica- and bleomycin-exposed macrophages, respectively. Tetrandrine treatment significantly reduced the silica-induced increase in TGF-β secretion, but did not affect bleomycin-induced TGF-β secretion from AM.

Histopathologic assessment of control, bleomycin-, and tetrandrine-treated bleomycin-exposed lungs at 2 wk postexposure showed that bleomycin exposure induced inflammatory and fibrotic changes (Figure 6). Tetrandrine treatment of bleomycin-exposed animals also caused a small but statistically significant reduction in the extent of fibrotic lesions and

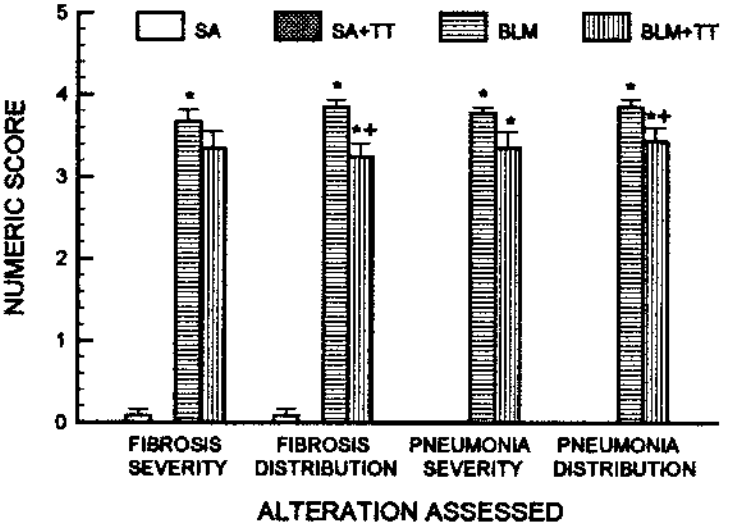

FIGURE 6. Semiquantitative histopathologic assessment of hematoxylin and eosin (H&E) sections of lung from rats 14 d after the following IT exposures/oral treatments: saline (SA), saline and tetrandrine (SA + TT), bleomycin (BLM), or bleomycin and tetrandrine (BLM + TT). Sections were scored for the severity (0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe) and distribution (1 = focal, 2 = locally extensive, 3 = multifocal, 4 = multifocal and coalescent, 5 = diffuse) of lesions. Pneumonia and fibrosis were associated lesions that followed bleomycin (1 mg) instillation. Tetrandrine treatment mildly reduced the distribution of pneumonia and fibrosis (asterisk indicates significantly different from saline-instilled rats, plus sign indicates significantly different from bleomycin-instilled rats). Severity of these lesions was not significantly reduced. A one-way analysis of variance with a Student–Newman–Keuls intragroup comparison was used for all analyses except pneumonia severity (which due to unequal variances was analyzed with a Kruskall–Wallis one-way analysis of variance on ranks).

inflammation (pneumonia) but not in their severity. Microscopically, bleomycin-induced lesions tended to be centered around terminal bronchioles and alveolar ducts. Adjacent alveoli were often replaced by loose fibrous connective tissue, which was infiltrated by a pleocellular population of inflammatory cells (Figure 7). Eosinophilic proteinaceous material, macrophages, and neutrophils sometimes extended into alveolar spaces. The alveolar ducts were often distorted, expanded, tortuous, and occasionally lined by cuboidal epithelial cells. Foci of tractional emphysema were observed near some areas of fibrosis. Although there was a small but statistically significant amelioration of these tissue alterations by tetrandrine treatment, these lesions remained an important finding under the current treatment regimen. In comparison, tetrandrine significantly inhibits silica-induced alveolar inflammation and fibrotic lung lesions (Chao et al., 1996).

DISCUSSION

The pulmonary responses to silica or bleomycin exposure were characterized by analysis of lavage fluid for markers of lung injury and inflammation, determination of proinflammatory cytokines released from macrophages, assessment of lung collagen content as an indication of fibrosis, and histopathologic evaluation of lung tissue. Intratracheal instillation of either silica or bleomycin induced lung injury and inflammation as evidenced by a significant increase in all lavage fluid parameters measured including total protein, phospholipids, and LDH, and by a substantial increase in macrophage production of TNF-α, IL-1, and TGF-β. Fibrosis in silica- or bleomycin-exposed animals was indicated by the significant increase in the hydroxyproline content of the lung tissue. Inflammatory and fibrotic lesions were evident microscopically after bleomycin exposure as well as in response to silica (Chao et al., 1996). Tetrandrine has been shown to be an effective antifibrotic agent in animal models of silicosis. Therefore, it was used as a probe in the present study in an effort to elucidate mechanisms leading to silica- versus bleomycin-induced pulmonary injury and fibrosis. The results of this and previous studies indicate that tetrandrine treatment was effective in protecting against silica-induced lung damage and fibrosis. That is, tetrandrine moderated the increase in lung weight, lavage protein, lavage LDH, lavage phospholipid, tissue hydroxyproline (this study), and mophological evidence of inflammation and fibrosis (Chao et al., 1996). In contrast, tetrandrine had little effect on most of the markers of bleomycin-induced pulmonary injury.

Studies have shown that the changes in cellular constituents and biochemical markers such as LDH, phospholipids, and total protein in the acellular lavage fluid provide important information on the pulmonary responses to mineral dust (Driscoll et al., 1990b; Henderson, 1984) and chemical agents (Giri et al., 1980). Proinflammatory cytokines, IL-1 and

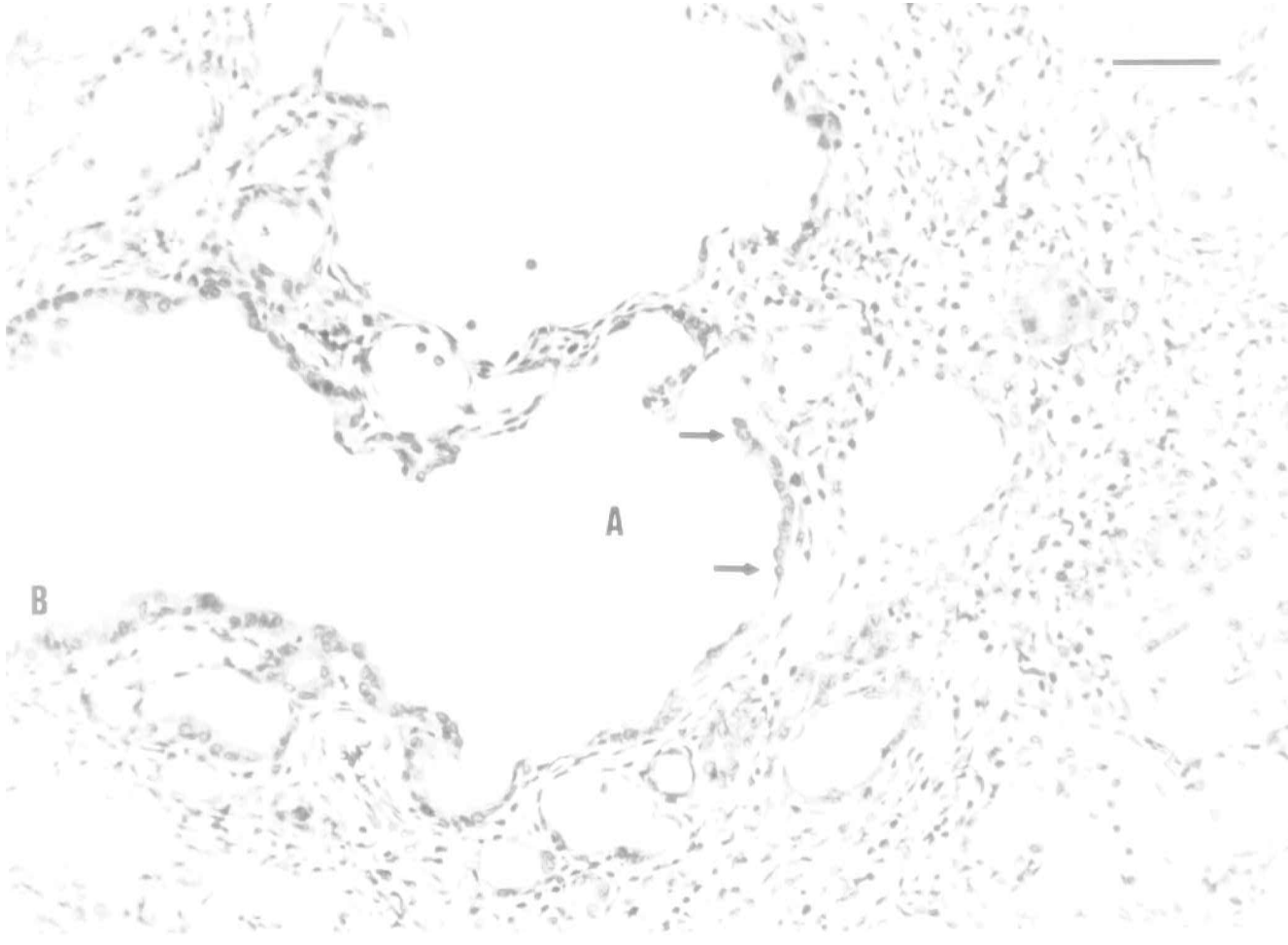

FIGURE 7. Hematoxylin and eosin-stained light micrograph of bleomycin-exposed rat lung sections at 2 wk postexposure. (A) The bronchiolar epithelium is adjacent to tortuous alveolar ducts lined by cuboidal epithelium (arrows). (B) The interstitium is fibrotic. Bar = 0.05 mm (magnification, 300×).

TNF-α, have been associated with the development of inflammation in both silica-exposed (Driscoll et al., 1990b; Piguet et al., 1990) and bleomycin-exposed lungs (Jordana et al., 1988; Piguet et al., 1989). In the present study, similar changes in biochemical markers, AM cytokines release, and hydroxyproline content in the lung tissue in both animal models suggest that silica and bleomycin exhibit a similar profile of pulmonary inflammation and fibrosis.

Macrophages of silica-exposed rats generated resting CL similar to control; however, significantly greater CL in response to zymosan stimulation suggests that macrophages were primed after silica exposure, presumably through the internalization of silica. The results of the present study show that the sensitization of AM to zymosan-induced CL production, resulting from in vivo silica exposure, was not affected by the tetrandrine treatment. However, in vivo inhibition of oxidant generation in AM by tetrandrine from silica-exposed rats has been reported in other studies (Castranova et al., 1990). This discrepancy may be due to the difference in type of exposure (exposure time and concentration of silica) and the dose of tetrandrine used. Similar priming was observed with macrophages isolated from bleomycin-exposed rats 3 d postexposure. However, this AM priming was no longer evident 14 d after bleomycin exposure. This difference in the duration of priming suggests that AM may play a more important role in the pulmonary response to silica than to bleomycin. Our results also show substantial elevation of proinflammatory cytokines, TNF-α and IL-1, secreted from AM of both silica- and bleomycin-exposed rats. However, the silica-induced effect is more persistent than that from bleomycin. Consistently increased TNF-α and IL-1 release from silica-exposed AM was observed through 28 d postexposure. In contrast, AM from bleomycin-exposed rats exhibited increased TNF-α production only through 7 d postexposure and elevation of IL-1 secretion through 14 d postexposure. These results show that the response of AM to bleomycin and silica differs in both magnitude and duration. The present study also shows that there is a significant increase of collagen content in lung tissue after either silica or bleomycin exposure. Orally administered tetrandrine significantly reduced hydroxyproline content in silica-exposed lung tissue. This is consistent with a previous study that showed marked reduction in histopathologic evidence of silica-induced granulomas and fibrosis after intravenous tetrandrine treatment (Chao et al., 1996). Our data also indicate the bleomycin exposure increases lung hydoxyproline. In agreement, Thrall et al. (1979) have reported that there is a biochemically detectable increase in lung collagen 2 wk after intratracheal instillation of bleomycin to rats. Oral tetrandrine treatment showed little effect against hydroxyproline/collagen production in bleomycin-exposed lungs. Light-microscopic studies showed that bleomycin exposure induced inflammation and bronchointerstitial pneumonia with the development of moderate to marked interstitial fibrosis in the lungs. Oral tetrandrine treatment

only mildly ameliorated these tissue alterations. These differences support a hypothesis that the pathogenesis of silica may be mechanistically different from that for bleomycin.

To differentiate between the mechanisms involved in silica- versus bleomycin-induced pulmonary injury, tetrandrine was used as a probe. Contrasting the two fibrotic stimulants, silica induced a more severe pulmonary response (15- to 45-fold increase of protein, phospholipids, and LDH) compared to bleomycin (less than 4-fold increase of these parameters). Similarily, silica induced a greater and more prolonged increase in wet lung weight and collagen in the lung tissue than bleomycin. In spite of the more pronounced damage in silica-exposed lungs, tetrandrine treatment was able to substantially reduce the silica-induced increase in lung weight, all the parameters measured in lavage fluid, and hydroxyproline content in the lung tissue (this study) as well as decrease morphological evidence of inflammation and fibrosis (Chao et al., 1996). However, tetrandrine treatment showed little protective effect against bleomycin-induced inflammation and fibrosis. These differences in response to tetrandrine treatment suggest that silica- and bleomycin-induced pulmonary fibrosis may be mediated through different pathways. Studies from Kobzik (1995) and Iyer et al. (1996) have shown that macrophages engulf particulates through the interaction of silica particles with scavenger receptors. Previously, in vitro studies in our laboratory have demonstrated that tetrandrine significantly inhibits silica-stimulated respiratory burst activity of AM (Ma et al., 1992) and silica-induced IL-1 (Kang et al., 1992) and TNF-α (Ma et al., 1996) production from AM. It was also shown that cytoskeletal modifiers significantly inhibit particle-induced activation of AM and reduce the binding of tetrandrine to macrophages (Ma et al., 1992). This suggests that the inhibitory effect of tetrandrine on particle-induced activation of AM is through the binding of tetrandrine to viable phagocytes at the cellular membrane cytoskeletal system. The ability of tetrandrine to significantly depress silica-induced markers of inflammation and fibrosis suggests that silica-induced lung damage and inflammation may be mediated through the initial interaction of silica to scavenger receptors of AM, modification of the cytokeletal system of the membrane, and release of oxidants and cytokines. Indeed, AM have been proposed as a key player in the initiation for progression of silicosis and fibrosis (Lapp & Castranova, 1993).

Tetrandrine was also used to probe the mechanisms leading to bleomycin-induced pulmonary inflammation and fibrosis. In contrast to its effect in silica-exposed lungs, tetrandrine had little effect on bleomycin-induced lung damage (lavage LDH, protein, and phospholipid), inflammation, and fibrosis (hydroxyproline and histology). Bleomycin is known to generate oxygen radicals through complexation with Fe^{2+} and reduction of oxygen to lead to DNA damage (Caspary et al., 1982). It was previously shown that bleomycin is a weak stimulant of superoxide release from AM

in comparison to silica, but bleomycin results in significant increase of intracellular oxidation in these phagocytes (Bhat et al., 1994). Giri et al. (1994) have demonstrated amelioration of bleomycin-induced intracellular oxidation and lung fibrosis by dietary supplementation with antioxidants, such as taurine and niacin, in hamsters. The results from these studies suggest that bleomycin-induced lung damage may result in large part from direct oxidant injury and that activation of AM may play a lesser role. Previously it was found that tetrandrine is not an antioxidant (Castranova et al., 1991), which may explain the nonprotective effect of tetrandrine on pulmonary inflammation and fibrosis induced by intratracheal instillation of bleomycin to rats.

In summary, the present study shows that both silica and bleomycin induce pulmonary fibrosis and exhibit similar pulmonary reactions involving damage, inflammation, and collagen deposition. The oral tetrandrine treatment is more effective in reducing the silica-induced inflammatory and fibrogenic response than bleomycin-induced reactions. The difference in response to tetrandrine treatment suggests that a different pathogenic mechanism may be involved in causing pulmonary inflammation and fibrosis with different stimuli. Since tetrandrine inhibits silica-induced activation of AM (Castranova et al., 1990) but is not an antioxidant (Castranova et al., 1991), it is proposed that AM play a vital role in the initiation of silica-induced lung damage and fibrosis, while the direct oxidant effects of bleomycin may be more important factors in the development of bleomycin-induced lung damage. The present study has shown that molecular probes such as tetrandrine may be useful in providing insight into the pathogenic process.

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