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SUBCHRONIC PULMONARY INFLAMMATION AND FIBROSIS INDUCED BY SILICA IN RATS ARE ATTENUATED BY AMIODARONE

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□ *A previous study demonstrated that the acute phase of silica-induced lung injury in rats can be attenuated by concomitant administration of amiodarone, a cationic amphiphilic drug that inhibits phospholipase activity in the lungs. The purpose of the present study was to determine whether continued amiodarone administration could inhibit subchronic silica-induced lung injury and fibrosis. Male Fischer-344 rats were administered amiodarone (150 mg/kg, p.o., 5 days/week) for 14 days and were then instilled with silica (100 mg/kg) intratracheally. Amiodarone treatment then continued for 60 days. Injury was evaluated by parameters in bronchoalveolar lavage fluid and fibrosis was assessed by lung hydroxyproline content and trichrome staining of collagen. Within the bronchoalveolar lavage fluid, amiodarone treatment resulted in significant decreases in silica-induced elevations in albumin levels, lactate dehydrogenase activity, β-glucuronidase activity, and neutrophil influx. Amiodarone treatment resulted in significant reductions in silica-induced increases in lung weight and hydroxyproline levels; the diminution of fibrosis due to amiodarone treatment was confirmed histologically. These results indicate that subchronic pulmonary inflammation and fibrosis induced by silica in the rat can be attenuated by the concomitant administration of amiodarone.*

Keywords *silica, amiodarone, fibrosis, inflammation, rat lung*

Inhalation of silica by humans can lead to a condition known as silicosis, which is marked by pulmonary inflammation and fibrosis [1]. Animal models of this process have been developed. Both inhalation [2, 3] and intratracheal instillation [4, 5] of silica in animals produce an acute inflammatory response. This response is initiated when macrophages phagocytize silica particles and become damaged or activated. The lung damage is characterized by infiltration of leukocytes and proteins into airspaces as well as the accumulation of phospholipids. Damage to some

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lung cells occurs while others are further stimulated to release mediators of inflammation. This inflammatory response persists and fibrosis of the lung becomes apparent within a few months of exposure.

An earlier study in our laboratory demonstrated that the acute injury due to the intratracheal instillation of silica to rats can be attenuated by administration of amiodarone (AD) [6]. AD is a cationic amphiphilic drug which when given chronically induces a pulmonary phospholipidosis in human patients [7, 8] and laboratory animals [9, 10]. Both AD and a metabolite, desethylAD, have been shown to be potent inhibitors of phospholipases in several in vitro preparations of animal tissues and cells [11–13]; additionally, phospholipase inhibition in cells obtained by bronchoalveolar lavage has been demonstrated in rats that were treated orally with AD [14]. Phospholipase inhibition, then, is presumed to be the mechanism whereby phospholipidosis is induced.

The alveolar macrophage is especially prone to the induction of phospholipidosis by AD [11, 15, 16]. Phospholipidotic macrophages, often termed "foamy macrophages," are characterized by extensive lamellar inclusions of phospholipids [17]. In our previous study we examined the effect of concomitant AD treatment on the acute stage of silica toxicity in rats, testing the hypothesis that AD-induced phospholipidosis and inhibition of phospholipase activity would be protective. This was the case as AD-treated rats were partially protected against silica-induced pulmonary inflammation and injury up to 14 days after particle instillation. We concluded that this protection was probably due primarily to the inhibition of phospholipases by AD. The goal of the present study was to examine whether continued AD treatment would similarly attenuate subchronic inflammation and subsequent fibrosis induced by silica. The inhibition of phospholipases and elevation in acellular phospholipids associated with silica as a result of AD treatment were expected to protect these phagocytes from silica-induced injury, thereby mitigating tissue damage. Rats were treated orally with AD or water vehicle for 14 days and then instilled intratracheally with silica or saline vehicle. AD was administered for an additional 60 days. We then utilized bronchoalveolar lavage to assess lung damage and inflammation. Histopathology and measurement of lung hydroxyproline were employed to assess development of fibrosis.

MATERIALS AND METHODS

Materials

Amiodarone and desethylamiodarone were gifts from Wyeth–Ayerst Laboratories (Princeton, NJ, USA). L8040 was a gift from Clin-Midy

Groupe Sanofi (Montpellier, France). Crystalline Min-U-Sil silica (U.S. Silica Corp., Berkeley Springs, WV, USA) was a gift from Val Vallyathan, National Institute for Occupational Safety and Health. Purity of the silica was determined by automated X-ray diffractometry and was 99.5% alpha quartz. Size fraction of <5 μm diameter was made by a centrifugal airflow particle classifier (Accucut Particle Classifier, Donalson-Majal Division, St. Paul, MN, USA). Of this fraction, 98% was <5 μm, with a median area equivalent diameter of 3.5 μm as estimated by automated scanning electron microscopic image analysis. Unless otherwise specified, other reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Experimental Design

Figure 1 is a depiction of the experimental design of this study showing treatment groups and methods of analysis.

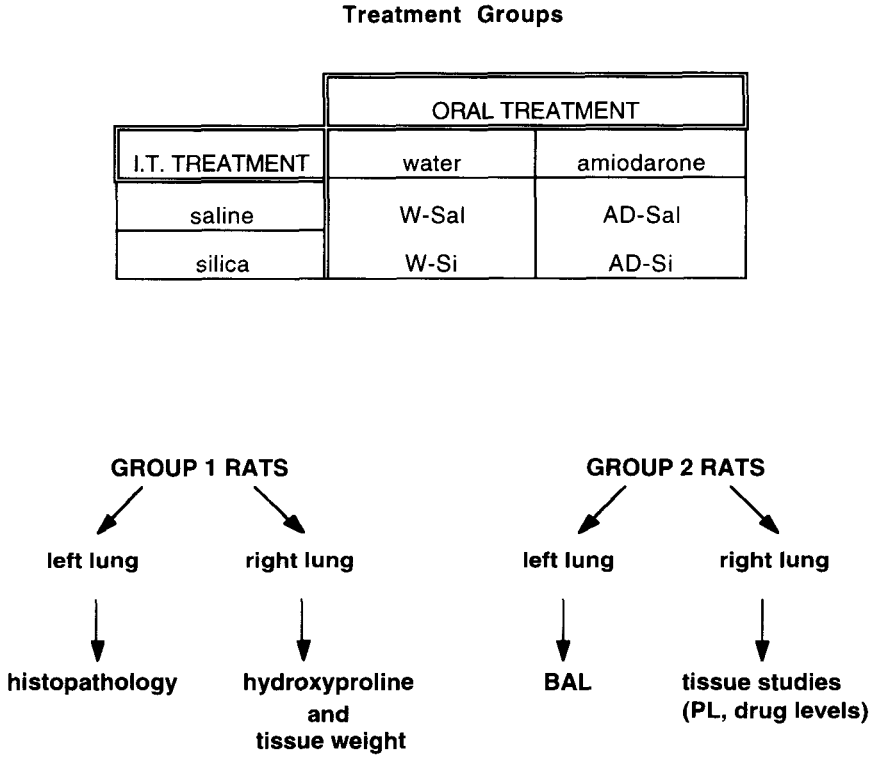

Figure 1. Experimental design.

Animal Treatment

Oral Treatment. Male Fischer 344 rats, age 57–67 days and weighing 200–250 g, were obtained from Hilltop Lab Animals (Scottdale, PA, USA) and were allowed 5 days for acclimation after arrival from the supplier. During this time, rats were given a conventional laboratory diet ad libitum. Rats were treated orally by gavage 5 days/week with AD (150 mg/kg) or water vehicle. Rats were treated orally for 14 days before intratracheal instillations were performed; oral treatment with AD or vehicle then continued on the same schedule after the instillations until the time of analysis. Since AD causes a decrease in weight gain, food was withheld from vehicle-treated animals to keep their weight changes similar to those of AD-treated animals (Figure 2).

Intratracheal (IT) Instillation. Silica was cleaned by boiling in 1.0 M HCl for 60 min, washed, and suspended in 0.9% sterile saline. Before instillation, the silica suspension was sonicated for 15 min. Silica (100

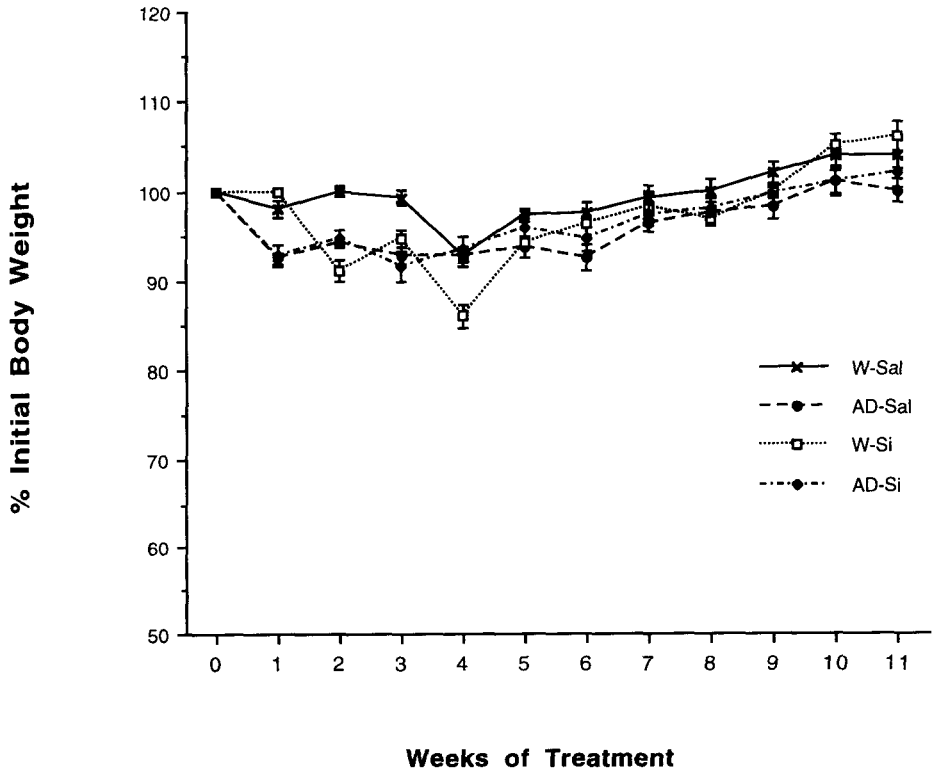

Figure 2. Body weight changes of rats in the four groups during the experimental period. Values are expressed as percentages of initial body weight, means ± SEM (n = 14).

mg/kg) in 0.5 mL saline or 0.5 mL saline alone was instilled in each rat. Instillations were performed by lightly anesthetizing the animals with sodium methohexital (40 mg/kg) and inserting a syringe with a ball-tipped needle into the trachea transorally. Instillations of silica or saline were performed after 14 days of oral treatment with AD or water, with oral treatment continuing after IT instillations. Animals were killed 60 days after the instillation of silica or saline and the lungs were analyzed for signs of inflammation and fibrosis.

The doses of silica and amiodarone were based on our earlier study which showed a protective effect of AD on acute silica-induced lung damage [6]. We chose 60 days after silica instillation to assess the effects of AD treatment on the response to silica because in preliminary studies, we observed nearly a twofold increase in hydroxyproline content in the lungs at that time.

Assessment of Fibrosis

Animals designated for fibrosis assessment were given an overdose of sodium pentobarbital and exsanguinated. After the trachea was cannulated and removed along with the lungs, the right lung lobes were ligated, removed, weighed, and frozen immediately at −80°C for later determination of hydroxyproline, a biochemical index of fibrosis. The left lung was infused with 10% phosphate-buffered formalin through the cannula. The trachea was ligated so the cannula could be cut away and the lung samples were stored in formalin solution until processed for histopathology.

Histopathology

Microscopic evaluation of disease development was accomplished by sectioning fixed lung lobes and then staining with hematoxylin and eosin and a trichrome stain. This evaluation of the samples was performed by a veterinary pathologist.

Hydroxyproline

Lung hydroxyproline was measured as an index of pulmonary fibrosis. Frozen lung samples were thawed and minced then processed and analyzed according to the method of Witschi et al. [18]. Briefly, chopped lung samples were hydrolyzed in 6 M HCl for 72 h, then neutralized with NaOH. Aliquots of the hydrosylate were diluted in borate–alanine buffer and oxidized with chloramine T. The reaction was stopped with sodium

thiosulfate, toluene was added, and the samples were boiled. After centrifugation, an aliquot of the organic phase was added to Erlich's reagent. Absorbance was read at 560 nm on a Gilford spectrophotometer. Micrograms of hydroxyproline per right lung were calculated from a standard curve.

Lung Tissue Analyses

A separate group of rats was treated with AD or water and silica or saline. These animals were overdosed with sodium pentobarbital and exsanguinated. The right lung lobes were ligated and removed then frozen in saline for later analysis of drug and phospholipid levels.

Drug Levels

Right lung samples from rats treated with AD were homogenized in isotonic saline for the determination of tissue drug levels by high-performance liquid chromatography (HPLC). Tissue levels of AD and one of its metabolites, desethylAD, were determined by the method of Reasor et al. [16]. A Waters HPLC system with a C_{18} bond-a-pak reverse-phase column was utilized with a methanol/water/ammonium hydroxide buffer as the eluant. The effluent was monitored at a wavelength of 244 nm and drug levels were quantified by comparison of sample peak heights to those of drug standards. L8040, an AD analog [21], was used as an internal standard. Drug levels are reported as mg/g tissue. AD and desethylAD standards were run above and below the range of experimental values to construct the standard curve.

Phospholipid

The phospholipid content of homogenized lung tissue was assayed after chloroform/methanol extraction of phosphate by the method of Folch et al. [19]. Phosphate was quantified by the method of Ames and Dubin [20], which includes ashing to liberate inorganic phosphorus followed by ascorbic acid/ammonium molybdate determination of phosphorus; absorbance was read on a Gilford spectrophotometer at 820 nm. Tissue phospholipid was determined from a standard curve which was constructed using KH_2PO_4 as a standard.

Bronchoalveolar Lavage (BAL)

The left lungs of animals that had the right lobes removed for phospholipid and drug analysis were lavaged as follows. An aliquot of room-

temperature Hanks' Balanced Salt Solution (HBSS), pH 7.4, was instilled, left for 30 s, withdrawn, re-installed for another 30 s, and then withdrawn and reserved. The volume instilled was 15 mL/kg for this initial lavage. The lung lobes were further lavaged with 5-mL aliquots of HBSS until 40 mL of fluid had been obtained from each rat. The lavage effluents were centrifuged at 500 *g* for 7 min. The cell-free fluid obtained in the initial lavage was reserved for biochemical analysis. The two cell pellets for each rat were combined, washed with HBSS, and resuspended in HBSS. An aliquot of cells from each rat was removed for counting and differentials.

Cell Counting and Differentiation

Cells obtained by BAL and resuspended in HBSS were counted on a hemacytometer. Concurrently, cell viability was assessed by trypan blue exclusion. Intact cells were greater than 90% viable. A large amount of cell debris was present in the sample from the W/Si group, presumably because of the damage induced by silica. In addition, cells were affixed to microscope slides by cytospin. Slides were stained with Wright–Geimsa (SureStain, Fisher Scientific, Pittsburgh, PA, USA); cells were then differentiated under a light microscope by identifying at least 400 cells per slide. Data are presented for only neutrophils because of their value in assessing the inflammatory response.

BAL Fluid Albumin

The albumin content of acellular fluid obtained by BAL was assayed as an index of alveolar–capillary barrier permeability. The determination was made according to a Sigma Diagnostics method utilizing the reaction of albumin with bromcresol green. Albumin was quantified by comparison to a bovine serum albumin standard curve.

Beta-Glucuronidase

Beta-glucuronidase is a lysosomal enzyme assayed as an index of phagocytic cell activation or damage. The activity of β-glucuronidase in the acellular lavage fluid was analyzed according to the method of Lockard and Kennedy [22]. Briefly, lavage fluid to be assayed was warmed to 37°C. The substrate, *p*-nitrophenyl-β-D-glucuronide, was added and allowed to incubate for 10 min; the reaction was stopped by the addition of glycine-NaOH (pH 10.5). Absorbance was determined at 400 nm and activity of the enzyme was expressed as nmol/min mL^{-1}.

Lactate Dehydrogenase (LDH)

LDH is a cytosolic enzyme and was assayed as an index of cytotoxicity. The activity of LDH in the acellular lavage fluid was analyzed according to a Boehringer Mannheim LDH kit procedure. LDH activity in this method is assayed as NADH formed by the reaction of L-lactate and NAD^+ catalyzed by LDH. Absorbance was measured using a dual-beam scanning spectrophotometer and enzyme activity expressed as μmol/min mL^{-1}.

Statistics

Data were subjected to two-way analysis of variance and Tukey's protected t test. The level of significance was set at $p < .05$. Data are expressed as means ± SEM.

RESULTS

As a gross measure of lung damage, the ratio of right lung weight to body weight was calculated. W-Si animals had a significant increase in this parameter (Figure 3). The AD-Si animals had a 57% reduction in this ratio when compared to the W-Si group, but this decrease was not great enough to return the values to those of the control groups.

Silica treatment induced a pronounced fibrotic response as W-Si animals displayed a significant increase in lung hydroxyproline content (Figure 4). The subchronic oral administration of AD to rats significantly diminished (68% decrease) but did not eliminate the increase in lung hydroxyproline content occurring 60 days after the IT instillation of silica. Histopathological evaluation confirmed the diminution of the response to silica associated with AD administration (Figure 5). In the W-Si group, the response was characterized by marked granulomatous interstitial pneumonitis and a moderate alveolar histocytosis. Compared to controls, there was a moderate increase in the amount of collagen present as assessed by trichrome staining. In the AD-Si group, the histocytosis was present, but the granulomatous response was essentially absent; the trichrome staining was reduced compared to the W-Si group but still somewhat more prominent than controls. It was also noted by viewing slides under polarized light that AD-Si tissues appeared to have most of the silica particles retained in macrophages, whereas in W-Si tissues there was a greater amount of silica free in the interstitium and airspaces.

Another facet of this study involved analyzing BAL fluid on day 60 after silica or saline instillation from animals of each treatment group to examine parameters of inflammation and tissue injury. Silica produced a

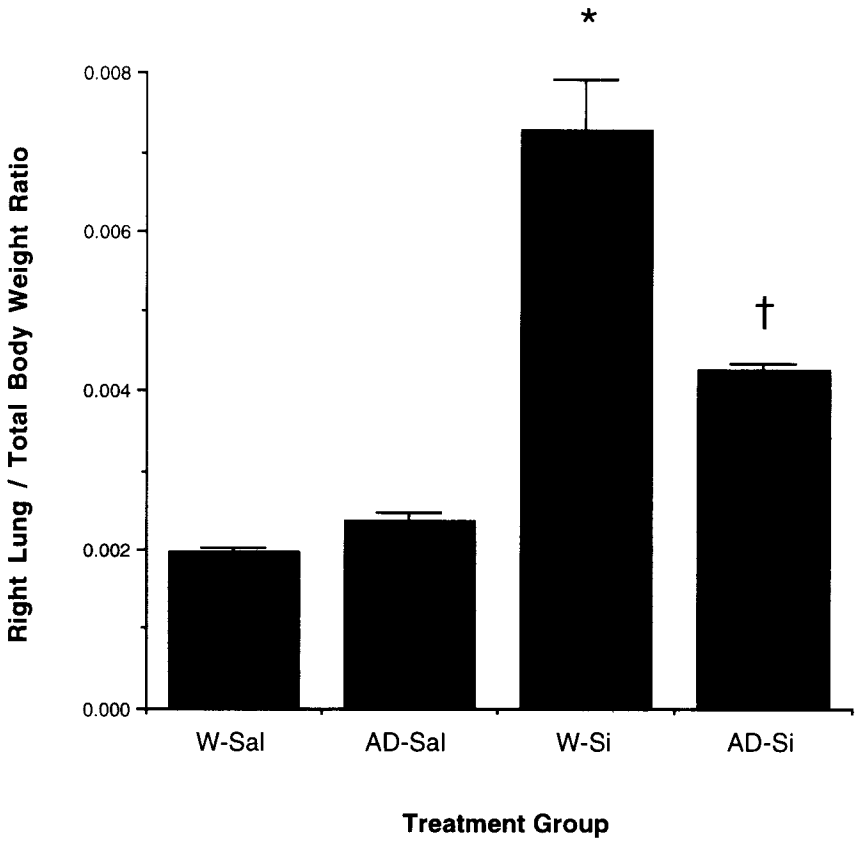

Figure 3. Right lung/total body weight ratio of animals treated orally with AD or water and intratracheally with silica or saline. Values are means ± SEM (n = 6). *, Significantly increased over all other groups. †, Significantly greater than W-Sal and AD-Sal but significantly lower than W-Si.

severe pulmonary inflammation which was reduced significantly by oral AD treatment. Figure 6 depicts neutrophils as a percentage of total cells found in the BAL fluid of rats from each of the four treatment groups. The W-Si group had a large increase in the proportion of neutrophils in BAL fluid. Animals of the AD-Si group also had a large percentage of neutrophils in BAL fluid, but the level was significantly less than that of the W-Si group.

The albumin content of acellular fluid obtained by the initial lavage of each rat was measured. Figure 7 shows a significant increase in albumin in the W-Si animals. The AD-Si group had intermediate albumin values—higher than controls (W-Sal and AD-Sal) but significantly lower (83%) than the W-Si group. W-Si treatment caused a dramatic increase in β-glucuronidase activity of acellular BAL fluid (Figure 8). The AD-Si

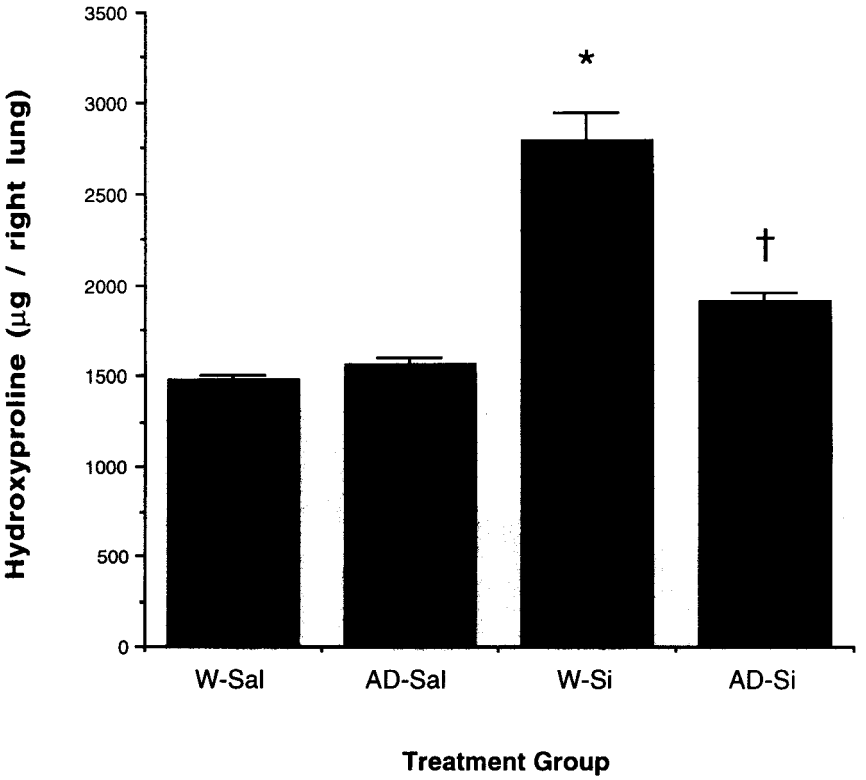

Figure 4. Right lung hydroxyproline content of rats treated orally with AD or water and intratracheally with silica or saline. Values are means ± SEM (n = 6). *, Significantly greater than all other groups. †, Significantly greater than W-Sal and AD-Sal but significantly lower than W-Si.

group had activity significantly lower (77%) than the W-Si group, but significantly greater than the W-Sal and AD-Sal groups. Similar results were noted for the analysis of LDH activity in BAL fluid. The W-Si group had a substantial increase in LDH activity while the AD-Si group had a mean value 78% lower than W-Si but significantly greater than controls (Figure 9). The lung tissue concentrations of AD and desethylAD, its principal metabolite, were measured in animals treated with AD orally (Figure 10). Animals instilled with silica accumulated significantly more AD and desethylAD than their saline-instilled counterparts.

In an effort to explain why there was a greater degree of drug and metabolite accumulation in lungs of AD-Si animals than AD-Sal animals, tissue phospholipid content was measured. Calculated as µmoles phospholipid/g tissue ± SE, there was significantly more phospholipid in the lungs of the AD-Si group (87.5 ± 10.8) than the AD-Sal group (34.6 ± 3.32).

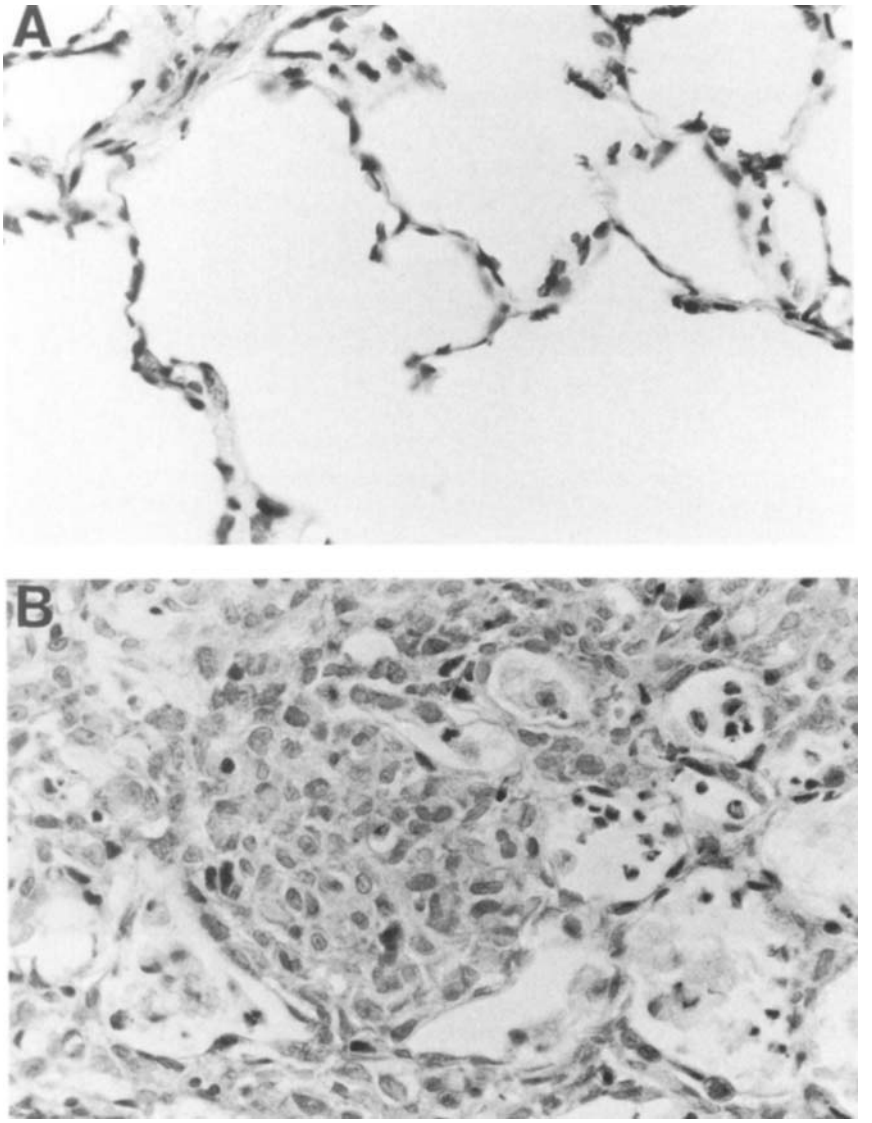

Figure 5. Representative light photomicrographs of sections of lung tissue from the following groups: (A) W-Sal, lung architecture is normal; (B) W-Si, there is a marked granulomatous interstitial pneumonitis and a moderate alveolar histocytosis (120×).

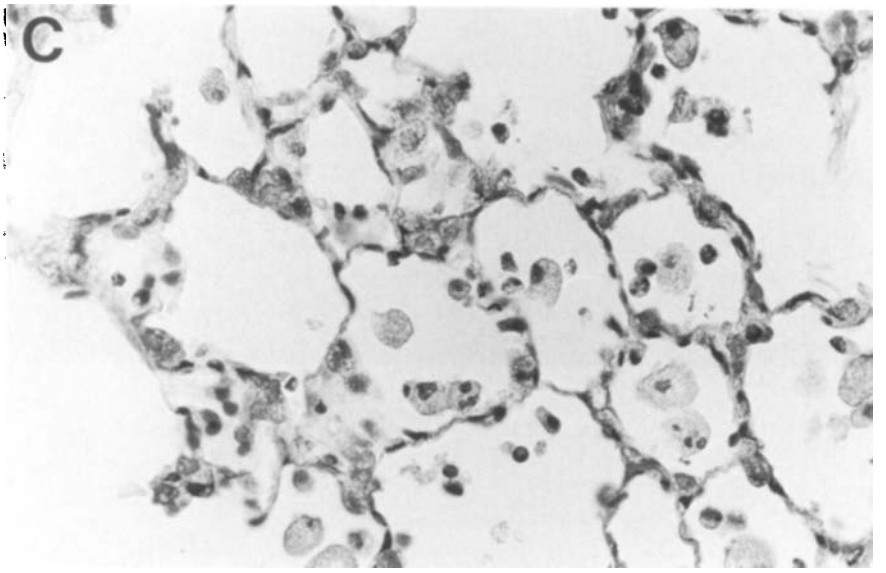

Figure 5. *(Continued).* Representative light photomicrographs of sections of lung tissue from the following groups: (C) AD-Si, there is an absence of a granulomatous response; however, an alveolar histiocytosis is present (120×).

DISCUSSION

Exposure to silica can lead to pulmonary inflammation and fibrosis [23]. In an earlier study, we reported that the presence of AD-induced phospholipidosis in the lungs of rats markedly inhibited inflammation and damage resulting during the first 2 weeks after the IT instillation of silica in rats [6]. Using alveolar macrophages in cell culture, we demonstrated that phospholipidotic and normal cells were equally susceptible to the cytotoxic effects of native silica. We concluded that the presence of elevated cellular phospholipid per se did not protect the cells against silica. In contrast, when the silica was coated with bovine pulmonary surfactant, which simulates the physiological conditions in the alveoli, the phospholipidotic cells were nearly completely resistant to the cytotoxic effects of silica compared to normal alveolar macrophages which were readily killed. From the results of that study, we hypothesized that the inhibition of cellular lysosomal phospholipase by AD and its metabolite desethylAD prevented digestion of the protective surfactant coating, thus preventing "toxification" of the silica particles. In the same study we observed an increased amount of phospholipid coating on the silica particles, resulting presumably from the elevated level of free phospholipid in the alveoli as a result of AD treatment. We suggested that the increased coating may be partially responsible for the protective action of the AD-

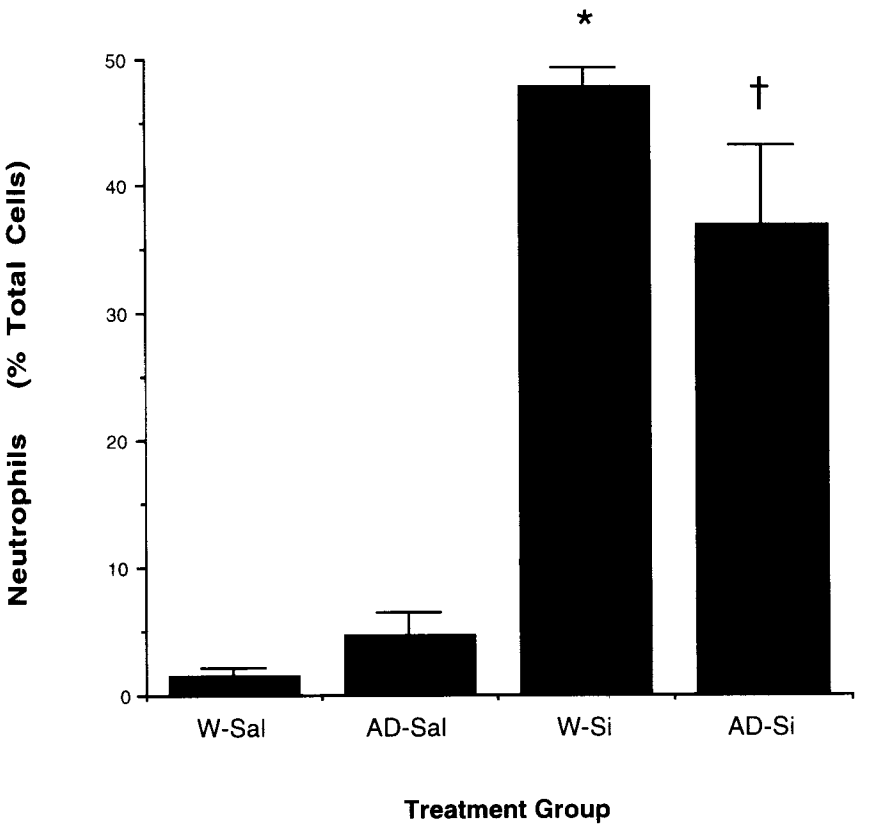

Figure 6. Neutrophils as percentage of total cells obtained by bronchoalveolar lavage from rats treated orally with AD or water and intratracheally with silica or saline. Values are means ± SEM (n = 5 or 6). *, Significantly greater than other groups. †, Significantly greater than W-Sal and AD-Sal but significantly lower than W-Si.

induced phospholipidosis. The objective of the present study was to examine the effect of continued AD treatment on the subchronic toxicity of silica, including inflammation, tissue damage, and the induction of pulmonary fibrosis.

Consistent with its actions on the acute response to silica, AD treatment provided significant protection against severe silica-induced inflammation when assessed 60 days after the instillation of silica. The values in the AD-Si group were intermediate between the control and W-Si values for all inflammation or damage parameters studied (% neutrophils, albumin, and β-glucuronidase and LDH activities).

Assessment of the effect of AD on the fibrogenicity of silica revealed that AD treatment inhibited but did not eliminate the fibrotic response when assessed biochemically and histopathologically. The present study,

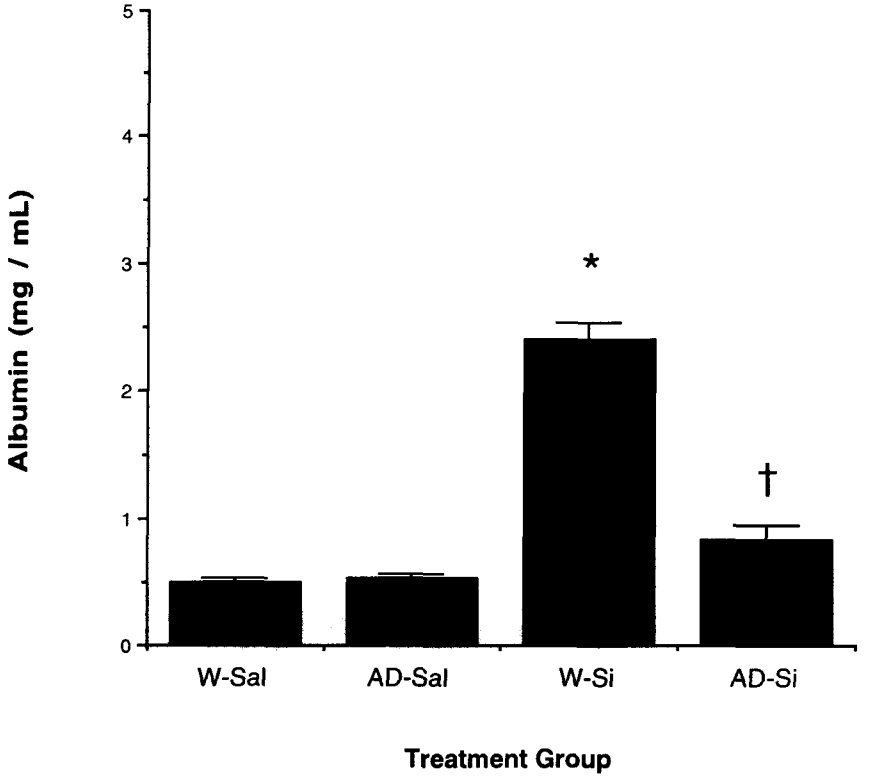

Figure 7. Bronchoalveolar lavage fluid albumin content of rats treated orally with AD or water and intratracheally with silica or saline. Values are means ± SEM (n = 5 or 6). *, Significantly greater than all other groups. †, Significantly greater than controls (W-Sal and AD-Sal) but significantly lower than W-Si.

therefore, supports the hypothesis that in rats the continued administration of AD offers continued protection against silica-induced inflammation and helps blunt the fibrotic response to silica.

Kodavanti and Mehendale [14] reported that phospholipases A and C in sonicated lavage cells of rats were inhibited after AD treatment, demonstrating that inhibition of the enzymes can be detected following in vivo administration. While we did not measure phospholipase activity in this study, we measured lung concentrations of AD and desethylAD study for comparison to concentrations reported to inhibit phospholipases, in vitro. The mean concentration of AD in the lung tissue of AD-Si rats was 3.47 mg AD/g lung tissue. If we assume that 1 g of lung tissue is a volume of 1 mL, then the concentration of AD uniformly distributed throughout the lung can be calculated as 5.38 mM. This is far in excess of the reported IC_{50} of 8.7 μM for the in vitro AD inhibition of phospholipase A_1 from rat lung. DesethylAD was found to be an even more

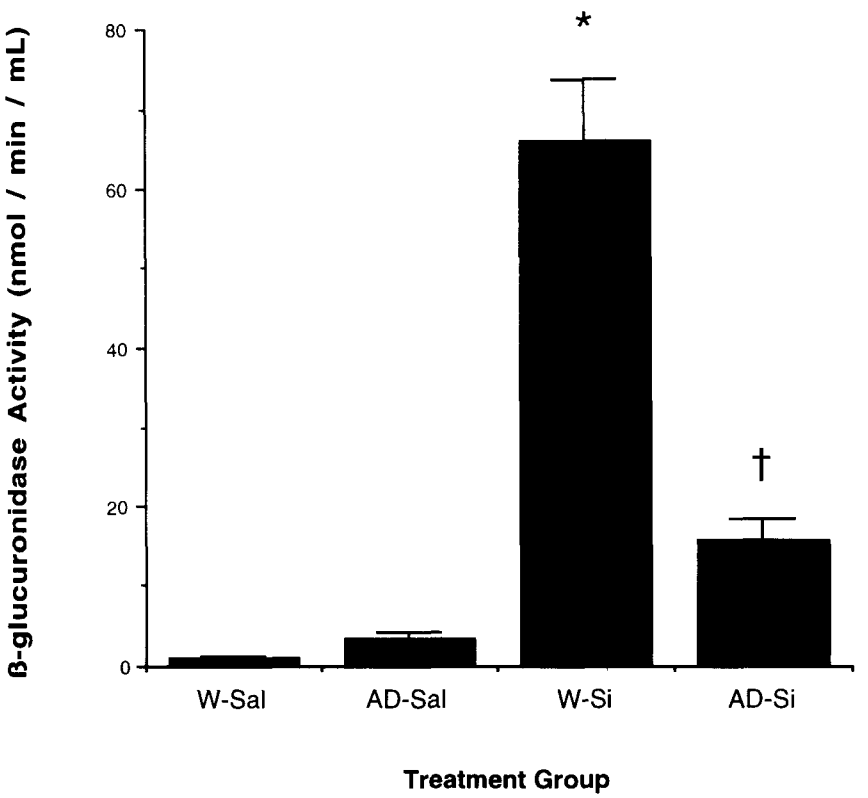

Figure 8. β-Glucuronidase activity in cell-free bronchoalveolar lavage fluid from rats treated orally with AD or water and intratracheally with silica or saline. Values are means ± SEM (n = 5 or 6). *, Significantly greater than all other groups. †, Significantly greater than W-Sal and AD-Sal but significantly lower than W-Si.

potent phospholipase inhibitor than AD [12]. Therefore, the lung drug concentrations achieved in our study were great enough to expect substantial inhibition of lung phospholipases, and to support the hypothesis presented earlier.

Another observation we made in this study was that rats instilled with silica and given AD orally accumulated AD and desethylAD in their lungs to a greater extent than rats given AD orally and instilled with the saline vehicle. We feel the most likely explanation for this is the increased phospholipid in the lungs of AD-Si animals favoring association with the amphiphilic drug and metabolite. Kodavanti and Mehendale [14] reported an increase in AD uptake in the phospholipidotic lungs of rats, and silica has been demonstrated to stimulate surfactant synthesis leading to pulmonary phospholipidosis [24, 25].

In the lung tissue sections studied by microscopy it appeared that in

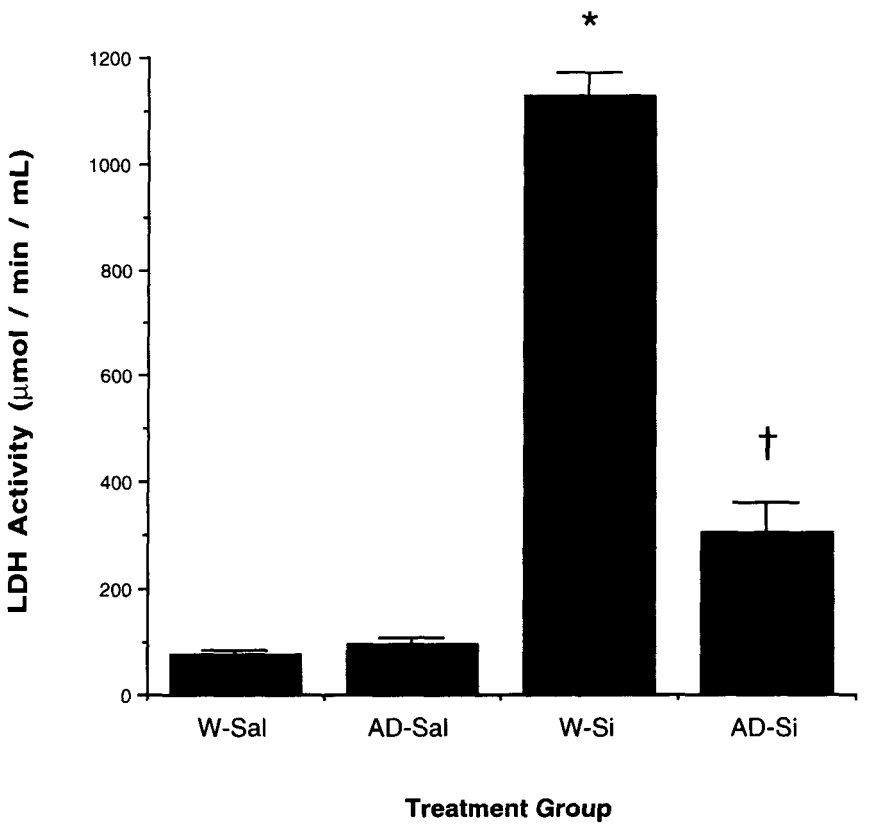

Figure 9. Lactate dehydrogenase activity in cell-free bronchoalveolar lavage fluid from rats treated orally with AD or water and intratracheally with silica or saline. Values are means ± SEM (n = 5 or 6). *, Significantly greater than other groups. †, Significantly greater than controls (W-Sal and AD-Sal) but significantly lower than W-Si.

the lungs of AD-treated rats silica particles were retained in macrophages, whereas in control (water vehicle) rats, much more free silica was present in the interstitium and airspaces. This would support the theory that alveolar macrophages take up the surfactant-coated silica particles, but inhibition of phospholipase activity by AD and desethylAD prevents digestion of the lipid. The particles are then retained in the cells rather than being "toxified" to kill the macrophages with release back into the alveoli. Taken together, the results of this study and our previous work support the theory that AD and desethylAD inhibit lysosomal phospholipase activity in alveolar macrophages, resulting in significant protection against silica-induced lung injury.

This is the first report of which we are aware describing the inhibition of pulmonary injury and fibrosis by the chronic administration of AD.

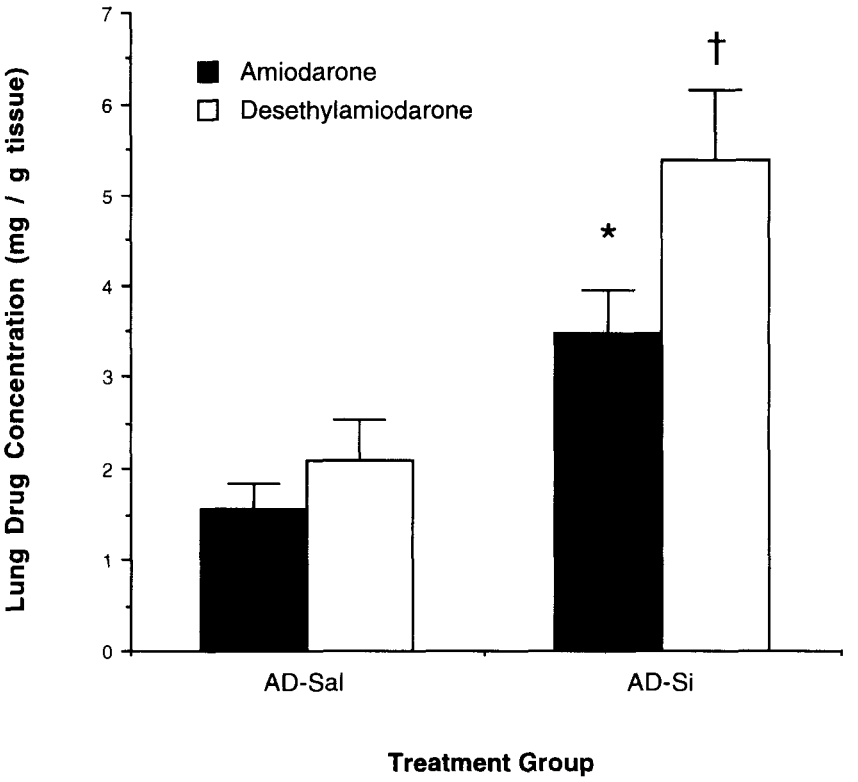

Figure 10. Lung tissue concentrations of amiodarone and desethylamiodarone in rats treated orally with AD and instilled intratracheally with saline or silica. Values are means ± SEM (n = 5 or 6). *, Significantly more AD present than in the AD-Sal group. †, Significantly more desethylAD present than in the AD-Sal group.

Using a variety of agents, other studies have demonstrated inhibition of pulmonary fibrosis induced by silica and other chemicals. Piguet et al. [26] reported that the continuous administration of the interleukin receptor antagonist IL-1ra inhibited the development of pulmonary fibrosis in mice resulting from the IT instillation of either silica or bleomycin. In fact, IL-1ra actually resulted in the reduction of fibrosis when given after fibrosis had developed. The authors speculated that IL-1 was involved in the fibrogenic response to both of these materials.

Wang et al. [27] reported that taurine and niacin administered before and daily following the IT administration of bleomycin were effective in preventing lung collagen accumulation in hamsters. The IT administration of AD to hamsters similarly results in the development of pulmonary fibrosis [28]. As with the bleomycin model, administration of taurine and niacin inhibited the development of AD-induced pulmonary fibrosis. An-

tioxidant activity of these agents may be among the ways they offer protection.

The herbal drug tetrandrine has been used in China to treat pulmonary fibrosis [29], and has been demonstrated to reduce silica-induced fibrosis in rats [30–32]. Reist et al. [33] provided evidence that the antifibrotic action of tetrandrine may be partially mediated by direct inhibition of fibroblast proliferation. Therefore, it appears that it is possible to modulate the development of chemically induced pulmonary fibrosis using different mechanistic approaches, including inhibition of pulmonary lysosomal phospholipase activity.

While the toxicities of AD may be serious enough to preclude its use in humans to treat or prevent silicosis, its continued study, and that of other cationic amphiphilic drugs, and the role of phospholipases in the modulation of silica-induced injury is certainly warranted. It may be possible to develop a less toxic phospholipase inhibitor that would be clinically useful in the prevention or attenuation of silicosis in humans.

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