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PULMONARY RESPONSES TO SINGLE VERSUS MULTIPLE INTRATRACHEAL INSTILLATIONS OF SILICA IN RATS

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The pulmonary toxicity of particles is often studied using a single intratracheal instillation of the material. It was hypothesized that smaller multiple intratracheal administrations of silica would result in differences in pulmonary responses as compared to a single large intratracheal administration. In the first of a series of experiments, the pulmonary responses in male F344 rats to a single intratracheal instillation of crystalline silica (5 mg/100 g body weight) given on d 0 were compared with those resulting from 5 consecutive daily intratracheal administrations of the dust (1 mg/100 g body weight/d) with the initial dose given on d 0. Controls received saline intratracheally. In the second experiment, the dose was reduced to 1 mg/100 g body weight for the single-dose protocol and 0.2 mg/100 g body weight/d for 5 consecutive days for the multiple-dose protocol. In both experiments, responses were assessed on d 14. In the third experiment, the doses were the same as the first experiment, but the responses were assessed on d 28. The indices of toxicity were cellular differentials recovered by bronchoalveolar lavage, which is an index of inflammation, and the level of albumin in the bronchoalveolar lavage fluid, a measure of damage to the capillary–epithelial barrier. At the higher dose of silica, similar levels of inflammation and lung damage were evident in both dosing protocols. Less severe responses occurred at the lower dose. The comparative pattern between the single and multiple dosing protocols was similar in all three experiments. Since only minor differences were noted in the pulmonary responses when the responses to the single- and multiple-dose protocols were compared, data indicate that the multiple-dose protocol does not offer any advantages over the single-dose protocol.

The inhalation of silica particles results in activation of alveolar macrophages, which is followed by a dramatic inflammatory response, damage to the respiratory epithelium and interstitial matrix, with the possible development of fibrosis (Bowden, 1987). Both inhalation (Vuorio et al., 1989; Driscoll et al., 1991; Warheit et al., 1991a) and intratracheal instillation (Lindenschmidt et al., 1990; Antonini et al., 1994; Blake et al., 1996)

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of silica in laboratory animals result in pulmonary inflammation and fibrosis. In animal studies in the laboratory setting, it is generally not possible to expose animals by inhalation; therefore, the technique of intratracheal instillation has been developed to administer specific doses of the particles to the lungs (Brain et al., 1976). Even though intratracheal instillation is less physiological than the inhalation of particles, there are advantages to this method (Brain et al., 1976). The actual dose delivered to the lungs of each animals is very uniform and can be measured accurately. The procedure is easy to perform and far less expensive when compared to the complex technology needed for aerosol generation and inhalation chamber construction.

While there is evidence that intratracheal instillation is a generally acceptable alternative to inhalation (Henderson et al., 1995; Oberdorster et al., 1997), at least to study the toxic potential of particles, it is obvious that there are limitations in using instillation as a surrogate for inhalation. Since most laboratories do not have inhalation facilities, intratracheal instillation will continue to be used as the means of exposure in studies involving silica and other dusts. It is plausible that multiple intratracheal instillations of small quantities of silica may be more representative of inhalation exposure than a large single instillation, which is the method used generally in studies. It was hypothesized smaller multiple intratracheal administrations of silica would result in differences in pulmonary responses as compared to a single large intratracheal administration. The purpose of our study, therefore, was to compare the pulmonary responses in rats resulting from five daily doses of silica to those of a single dose containing the same mass of silica as the total of the multiple doses. Three experiments were performed to test the effects of dose and duration of exposure in comparing the single and multiple administration protocols.

METHODS

Test Material

Crystalline silica particles (Min-U-Sil, U.S. Silica Corp., Berkeley Springs, WV), was 99.5% alpha-quartz with a mean diameter of 3.5 μm. Particles were cleaned as described previously (DiMatteo & Reasor, 1997).

Animals

Male F344 rats (Hilltop Lab Animals, Scottdale, PA) weighing 250–300 g were used in this study. Animals were fed a conventional diet (Purina Chow pellets) and were allowed water ad libitum.

Experimental Design

Study Protocol Silica was suspended in 0.9% sterile saline and sonicated for 1 min with a Sonifier 450 cell disruptor (Branson Ultrasonics,

Danbury, CT). On d 0, rats were anesthetized by an intraperitoneal injection of 0.6 ml of a 1% solution of methohexital sodium (Jones Pharma, Inc., St. Louis, MO) and instilled intratracheally with the silica suspension or an equivalent volume of saline vehicle according to the method of Brain et al. (1976). The following experiments were used:

1. High-dose 14-d experiment: (a) The Silica-Mult. group received 5 daily instillations of silica each at a dose of 1 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. (b) The Silica-Single group received 1 instillation of silica at a dose of 5 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle.
2. Low-dose 14-d experiment: (a) The Silica-Mult. group received 5 daily instillations of silica each at a dose of 0.2 mg/100 g body weight (0.1 ml of vehicle/100 g body weight). The Control-Mult. group received only vehicle. (b) The Silica-Single group received 1 instillation of silica at a dose of 1 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle.

In both experiments 1 and 2, all rats were killed and underwent bronchoalveolar lavage on d 14.

3. 28-d experiment: (a) The Silica-Mult. group received 5 daily instillations of silica each at a dose of 1 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. (b) The Silica-Single group received 1 instillation of silica at a dose of 5 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle. On d 28, all rats were killed and underwent bronchoalveolar lavage.

Bronchoalveolar Lavage

Rats were anesthetized with Nembutal (pentobarbital sodium, Abbott Laboratories, N. Chicago, IL) and exsanguinated by severing the abdominal aorta. The trachea was cannulated with PE160 tubing and cells were washed from the lungs using bronchoalveolar lavage (BAL). While massaging the lungs gently, 2 ml/100 g body weight of warm phosphate-buffered saline (PBS), pH 7.4, was instilled into the lungs. For the first instillation, PBS remained in the lungs 30 s, was withdrawn, and then was reinstilled for another 30 s. After the second withdrawal, the PBS was saved in a separate tube. For additional lavages, 5-ml aliquots of PBS were instilled sequentially, withdrawn, and placed in a centrifuge tube until about 40 ml was obtained. Both tubes from each rat were centrifuged at 800 × g for 7 min. The supernatant phase from the first lavage was transferred into a separate tube and used for albumin analysis. Cell pellets were resuspended in 5 ml PBS and the cells from each animal

were combined. The cells were recentrifuged and the pellet resuspended in 1 ml PBS for cell counting and further analysis.

Indices of Lung Damage

To assess cellular inflammation, BAL cells were counted using a hemacytometer and cellular differentials determined following staining with Wright–Giemsa stain using a Hema-Tek 2000 slide stainer (Bayer Corporation, Elkhart, IN). Alveolar macrophages (AMs), polymorphonuclear neutrophils (PMNs), and lymphocytes were enumerated.

To assess damage to the capillary–epithelial barrier, the albumin concentration in the first aliquot of BAL fluid was measured by using the Sigma Diagnostics albumin reagent kit, which utilizes the binding of bromcresol to the albumin. Absorbance was measured at 628 nm on a spectrophotometer. Quantification of albumin was accomplished by comparison to a bovine serum albumin standard curve.

Statistics

In each study, data were analyzed by two-way analysis of variance (ANOVA) and individual groups were compared by a Tukey's protected *t*-test. Significance was set at $p < .05$.

RESULTS

High-Dose 14-Day Experiment

The single administration of silica resulted in a significant elevation in the total number of BAL cells recovered at d 14 but not with the multiple administration protocol (Figure 1). The cells recovered from the rats in dosing protocol were differentiated (Table 1). There were no differences in the recovery of AMs with either protocol. Both administration protocols resulted in significant elevations in the recovery of PMNs in the silica groups compared to their respective controls, but there was no difference between the two silica groups. The same result occurred when lymphocytes were evaluated. Both protocols resulted in similar significant silica-induced increases in the appearance of albumin in the BAL fluid (Figure 2).

Low-Dose 14-Day Experiment

There was a small but significant increase in the recovery of total cells by BAL with both administration protocols (Figure 3). In comparing the cell differentials (Table 2), the recoveries of AMs and lymphocytes did not differ with either protocol. The number of PMNs was significantly elevated following administration of the single dose of silica but not following multiple doses. As with the high-dose study, both protocols resulted in similar significant silica-induced increases in the appearance of albumin in the BAL fluid (Figure 4).

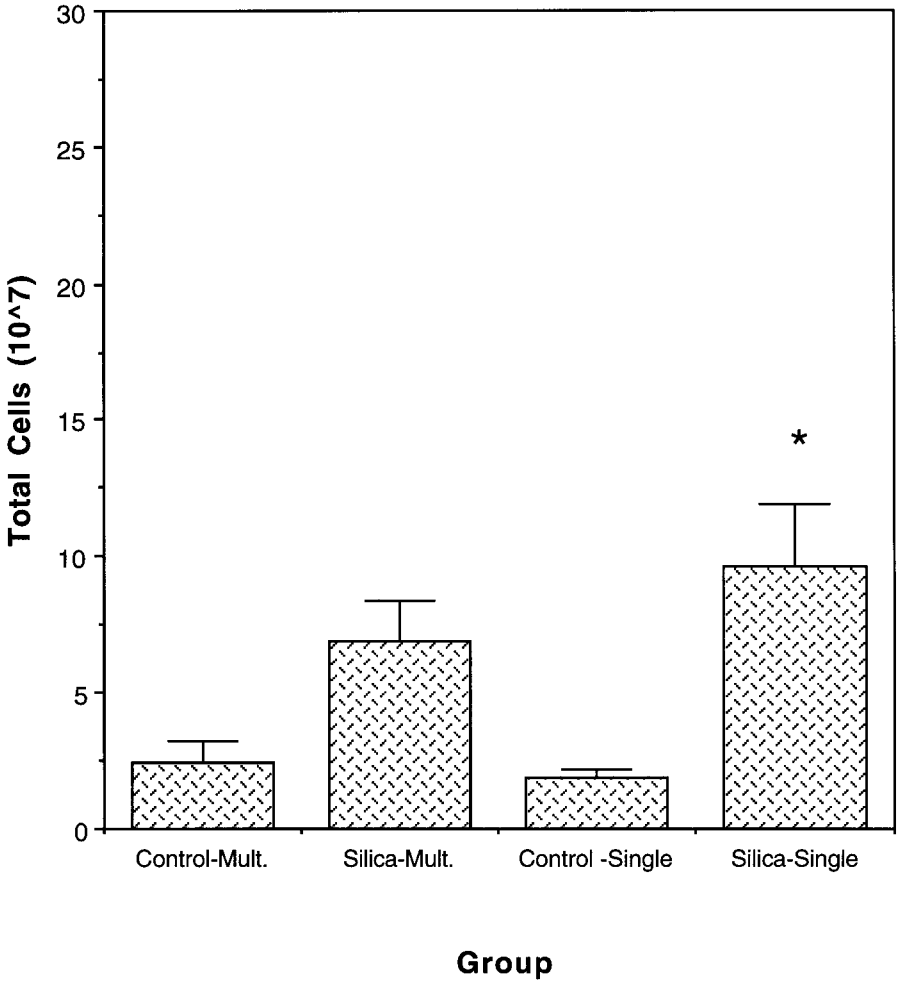

FIGURE 1. Effect of the intratracheal instillation of silica on the recovery of total cells by bronchoalveolar lavage. The high-dose 14-d experimental protocol was followed as described in the Methods. The Silica-Mult. group received 5 daily instillations of silica each at a dose of 1 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. The Silica-Single group received 1 instillation of silica at a dose of 5 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle. Values are means ± SEM (n = 4–6). Asterisk indicates significant at $p < .05$ compared to Control-Single.

28-Day Experiment

Examining biomarkers at d 28 revealed pronounced effects of silica. There were significant and equivalent increases in total BAL cells (Figure 5), PMNs and lymphocytes (Table 3), and albumin (Figure 6) recovered as a result of silica administration with both protocols. Macrophages were significantly elevated as well, with the response to the multiple administration significantly greater than to the single administration (Table 3).

TABLE 1. Cellular Differentials in Bronchoalveolar Lavage Fluid Following High-Dose Intratracheal Instillation of Silica: 14-Day Study

Protocol	Alveolar marcophages	Polymorphonuclear neutrophils	Lymphocytes
Control multiple	1.910 ± 0.505	0.470 ± 0.300	0.069 ± 0.010
Silica multiple	3.080 ± 0.560	3.280 ± 0.760[a]	0.550 ± 0.180[a]
Control single	1.720 ± 0.390	0.070 ± 0.040	0.064 ± 0.027
Silica single	3.820 ± 1.110	5.020 ± 1.150[a]	0.770 ± 0.170[a]

Note. Values are cell numbers ($\times 10^7$) and are displayed as means ± SEM (n = 4–6).
[a]Significant at $p < .05$ compared to respective controls.

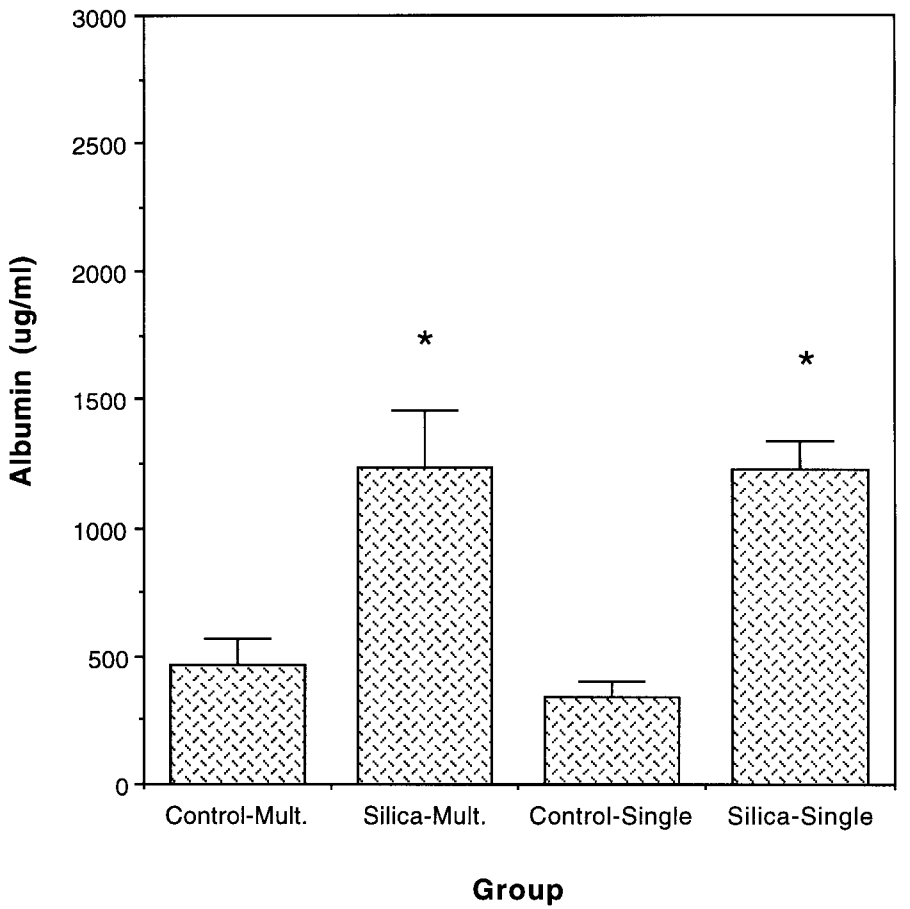

FIGURE 2. Effect of the intratracheal instillation of silica on the recovery of albumin in bronchoalveolar lavage. The high-dose 14-d experimental protocol was followed as described in the Methods. The Silica-Mult. group received 5 daily instillations of silica each at a dose of 1 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. The Silica-Single group received 1 instillation of silica at a dose of 5 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle. Values are means ± SEM (n = 4–6). Asterisk indicates significant at $p < .05$ compared to Control-Multiple.

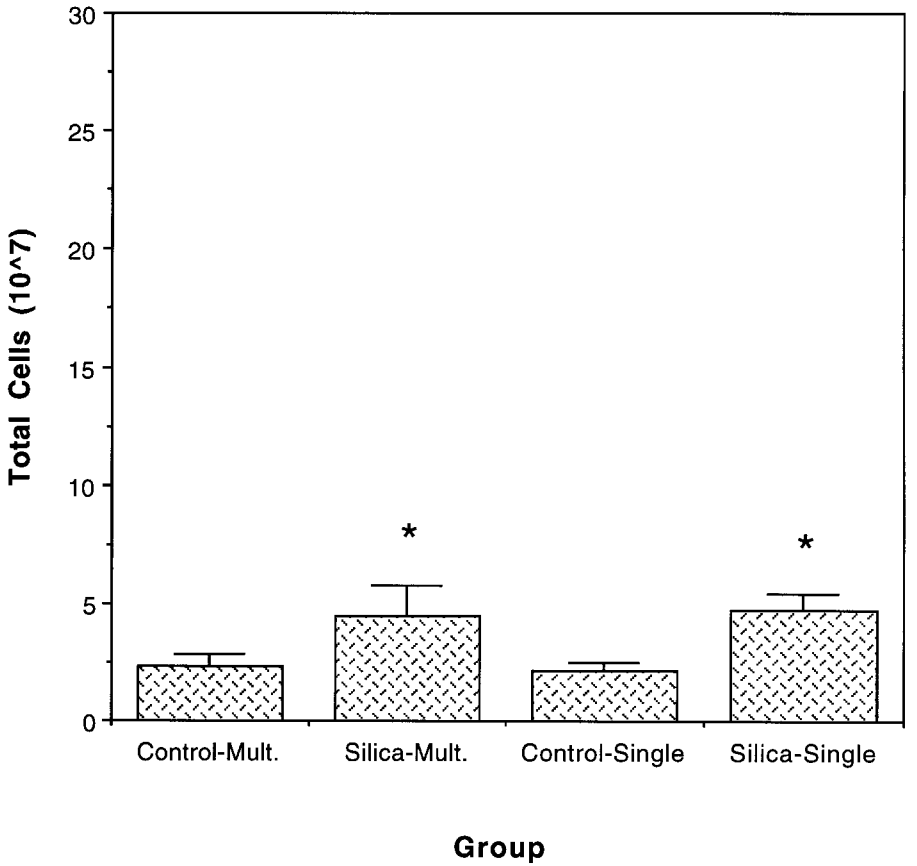

FIGURE 3. Effect of the intratracheal instillation of silica on the recovery of total cells by bronchoalveolar lavage. The low-dose 14-d experimental protocol was followed as described in the Methods. The Silica-Mult. group received 5 daily instillations of silica each at a dose of 0.2 mg/g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. The Silica-Single group received 1 instillation of silica at a dose of 1 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle. Values are means ± SEM (n = 4–6). Asterisk indicates significant at $p < .05$ compared to respective controls.

TABLE 2. Cellular Differentials in Bronchoalveolar Lavage Fluid Following Low-Dose Intratracheal Instillation of Silica: 14-Day Study

Protocol	Alveolar marcophages	Polymorphonuclear neutrophils	Lymphocytes
Control multiple	1.800 ± 0.250	0.440 ± 0.290	0.080 ± 0.040
Silica multiple	2.970 ± 0.930	1.380 ± 0.570	0.080 ± 0.030
Control single	1.630 ± 0.310	0.430 ± 0.160	0.080 ± 0.020
Silica single	2.590 ± 0.410	2.330 ± 0.640[a]	0.140 ± 0.030

Note. Values are cell numbers ($\times 10^7$) and are displayed as means ± SEM (n = 4–6).
[a]Significant at $p < .05$ compared to respective control.

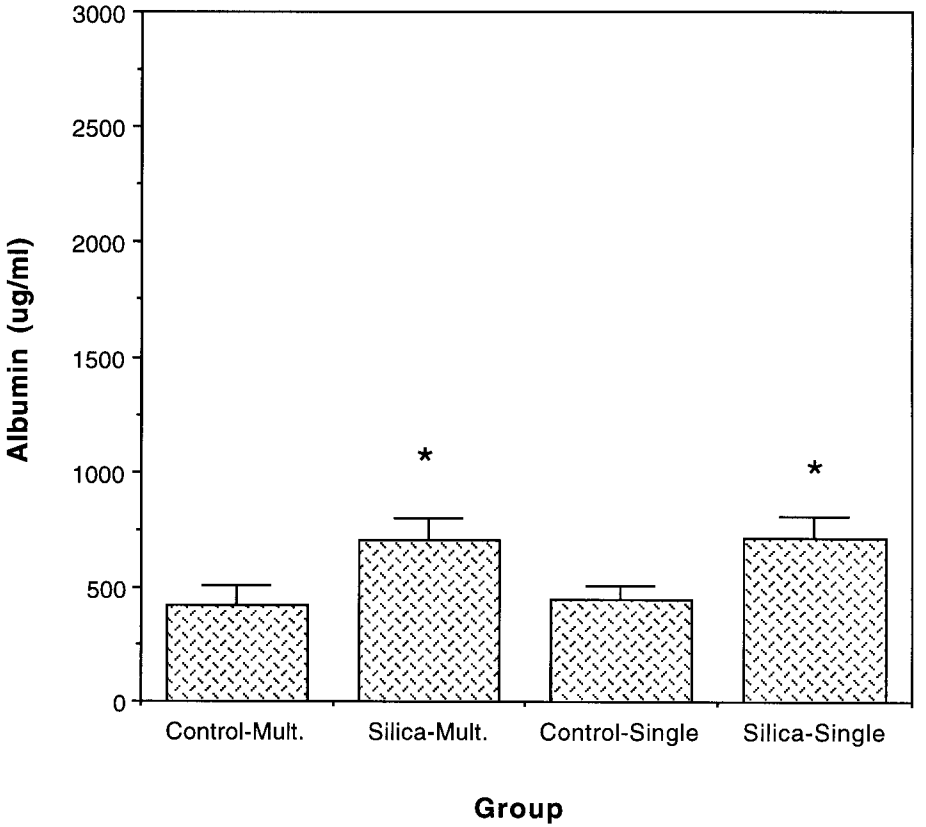

FIGURE 4. Effect of the intratracheal instillation of silica on the recovery of albumin in bronchoalveolar lavage. The low-dose 14-d experimental protocol was followed as described in the Methods. The Silica-Mult. group received 5 daily instillations of silica each at a dose of 0.2 mg/g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. The Silica-Single group received 1 instillation of silica at a dose of 1 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle. Values are means ± SEM (n = 4–6). Asterisk indicates significant at $p < .05$ compared to respective controls.

DISCUSSION

Intratracheal instillation is routinely used to study the pneumotoxic potential of respirable particles. While the inhalation route is more physiological, there are advantages to using the intratracheal instillation method (Brain et al., 1976). The actual dose delivered to the lungs can be measured accurately and administered more uniformly. The intratracheal instillation method is simpler and minimizes hazards to laboratory personnel when the materials are highly toxic. Large and therefore more effective doses of particles can be introduced by intratracheal instillation in a short period of time. The cost of setting up aerosol generation systems and proper exposure chambers may also be prohibitive compared to the negligible expense of using intratracheal instillations.

However, the use of the intratracheal instillation technique is open to criticism. The intratracheal instillation of a large bolus of particles into the lungs could cause an inflammatory response that would not be observed if the same amount of particles accumulated gradually during inhalation (Henderson et al., 1995). After intratracheal instillation, particles are increasingly deposited in the basal regions of the lungs (Brain et al., 1976) and have less homogeneous particle distribution than an inhalation exposure (Pritchard et al., 1985; Dorries & Valberg, 1992). The vehicle used to instill the particles might also affect the response of the lungs after administration (Henderson et al., 1995). New methods, such as intratracheal inhalation, are continually being developed to administer particles to the lungs to overcome these problems (Osier et al., 1997; Oberdorster et al., 1997).

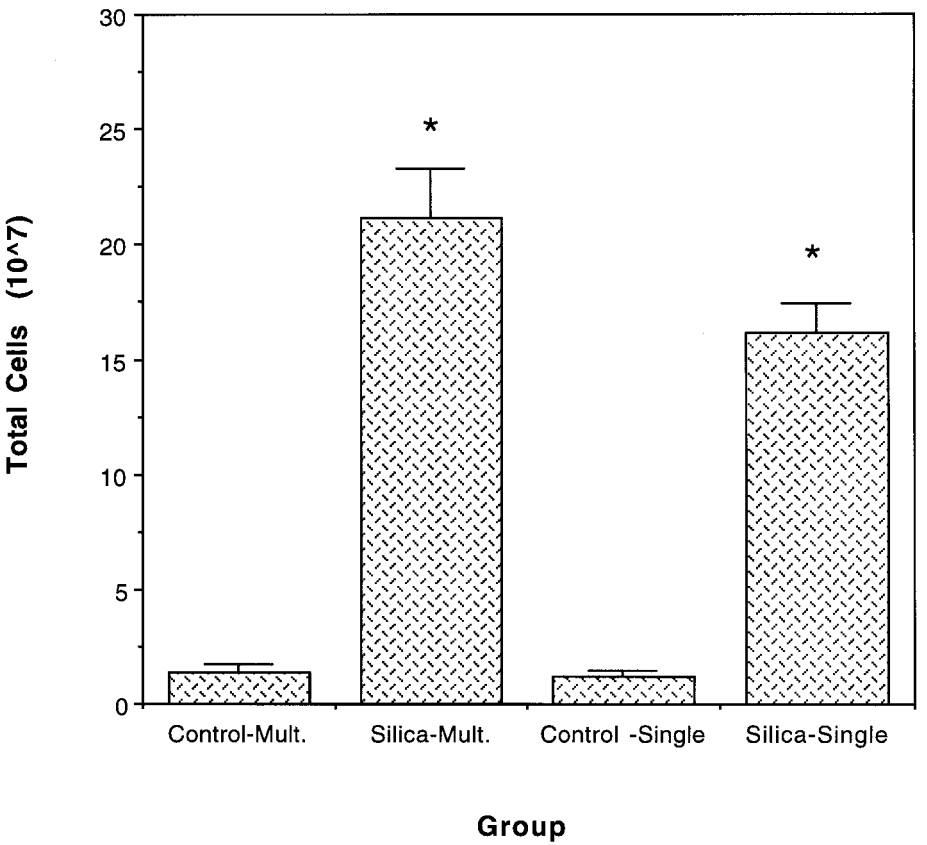

FIGURE 5. Effect of the intratracheal instillation of silica on the recovery of total cells by bronchoalveolar lavage. The 28-d experimental protocol was followed as described in the Methods. The Silica-Mult. group received 5 daily instillations of silica each at a dose of 1 mg/g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. The Silica-Single group received 1 instillation of silica at a dose of 5 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle. Values are means ± SEM (n = 4–9). Asterisk indicates significant at $p < .05$ compared to respective controls.

TABLE 3. Cellular Differentials in Bronchoalveolar Lavage Fluid Following Intratracheal Instillation of Silica: 28-Day Study

Protocol	Alveolar marcophages	Polymorphonuclear neutrophils	Lymphocytes
Control multiple	1.380 ± 0.360	0.001 ± 0.001	0.007 ± 0.003
Silica multiple	9.580 ± 1.540[a]	11.340 ± 1.260[a]	0.200 ± 0.050[a]
Control single	1.220 ± 0.220	0.001 ± 0.001	0.001 ± 0.001
Silica single	5.070 ± 0.480[a]	10.950 ± 1.480[a]	0.190 ± 0.036[a]

Note. Values are cell numbers ($\times 10^7$) and are displayed as means ± SEM (n = 4–9).
[a]Significant at $p < .05$ compared to respective controls.
[b]Significant at $p < .05$ compared to Silica single.

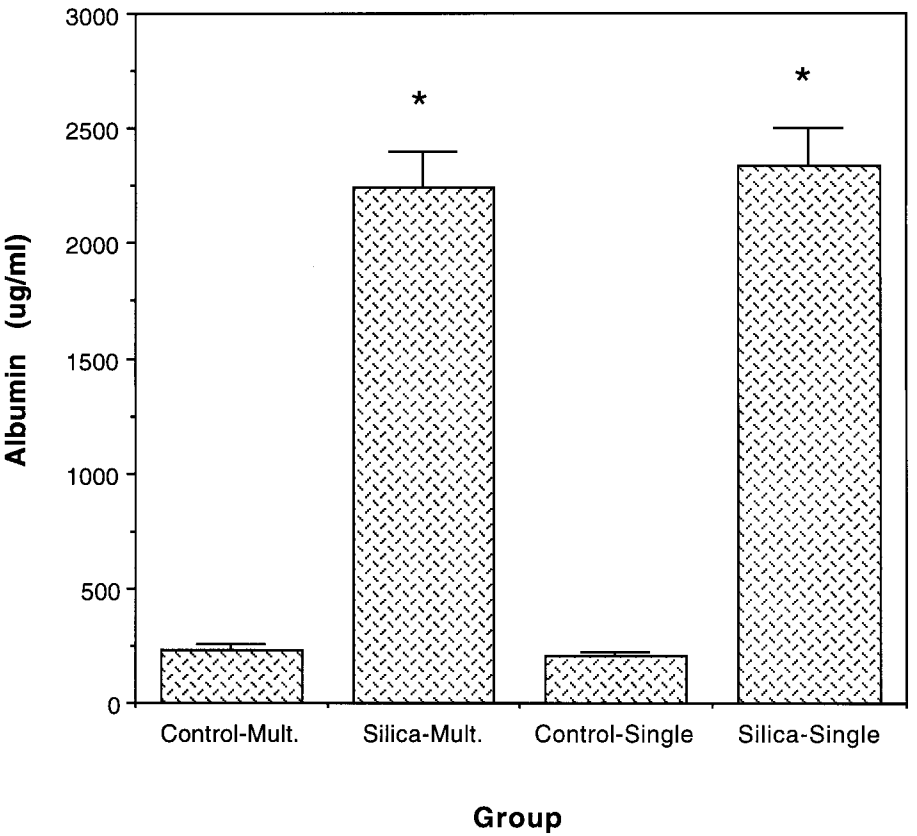

FIGURE 6. Effect of the intratracheal instillation of silica on the recovery of albumin in bronchoalveolar lavage. The 28-d experimental protocol was followed as described in the Methods. The Silica-Mult. group received 5 daily instillations of silica each at a dose of 1 mg/g body weight (0.1 ml vehicle/100 g body weight). The Control-Mult. group received only vehicle. The Silica-Single group received 1 instillation of silica at a dose of 5 mg/100 g body weight (0.1 ml vehicle/100 g body weight). The Control-Single group received only vehicle. Values are means ± SEM (n = 4–9). Asterisk indicates significant at $p < .05$ compared to respective controls.

For laboratories that are confined to using intratracheal instillation to study pulmonary responses to particles, it is desirable to develop approaches that minimize its limitations. It seemed reasonable to hypothesize that the responses to multiple instillations of smaller quantities of particles on consecutive days would result in a different pattern of pulmonary damage than would a large single dose and might serve as a surrogate for short-term inhalation. In the current study this hypothesis using the two protocols was tested.

While there were some statistically significant differences in the responses to silica when the single-dose and multiple-dose protocols were compared, they were relatively minor. Therefore, whether a given mass of silica was administered intratracheally as a single dose or in multiple doses, the inflammatory response and tissue damage, as assessed in this study, were not substantially different. As a result, our hypothesis was not supported, and subjecting rats to multiple exposures of silica did not offer any advantage over the single-dose protocol.

There are possible reasons why a slight difference was observed in pulmonary responses when the single- and multiple-instillation protocols were compared. First, we examined only a limited number of biomarkers, cellular differentials to assess the inflammatory response and the appearance of albumin the BAL fluid to assess tissue damage. It is possible that other biomarkers would not behave in the same manner as these did; however, we have found that biomarkers behave similarly for a given exposure protocol (Blake et al., 1996; DiMatteo & Reasor, 1997). In comparing different pulmonary exposure methods, Osier et al. (1997) observed that the levels of the chemokine macrophage inflammatory protein-2 (MIP-2) were greater in persistence and value after an instilled dose than after an intratracheal inhalation of a similar dose of titanium dioxide particles.

The selection of silica as the test particle for use in this current study may be another reason why little change was seen in the lung injury and inflammation between the two protocols. It is well established that silica persists in the lungs. Animal studies indicate that silica is more likely to be retained in the lungs than less toxic inert dusts such as titanium dioxide (Driscoll et al., 1990) and carbonyl iron (Warheit et al., 1991b) and that silica particles become concentrated at convergence points for clearance pathways (Brody et al., 1982). The cytotoxic activity of silica also appears related to the activation of alveolar macrophages and the production of reactive oxygen species, leading to cell injury and rupture of the macrophage lysosomal membrane and eventual release of damaging hydrolytic enzymes (Davis, 1986; Vallyathan et al., 1988). Following lysis of the macrophages, the free silica particles are once again released to be ingested by fresh macrophages, some of which in turn are also killed. Thus, unlike most other particles, silica is not completely detoxified and removed from the lungs by macrophage phagocytosis. Due then to the lungs' compromised ability to clear silica particles, the lung burden would not be different because

the silica would accumulate in the lungs regardless of whether it was instilled in one large bolus or given in five smaller doses. Studies are currently ongoing in the laboratory using nuisance dusts that have been shown to be easily removed by the normal lung clearance mechanisms.

In summary, pulmonary injury in rats, as assessed under the conditions of these experiments, was not significantly different whether the same mass of silica particles was instilled intratracheally as a single dose or as five smaller doses on consecutive days. The multiple-instillation protocol does not appear to offer any advantages to the single-dose protocol when evaluating the responses to silica particles.

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