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SUBCHRONIC SILICA EXPOSURE ENHANCES RESPIRATORY DEFENSE MECHANISMS AND THE PULMONARY CLEARANCE OF *LISTERIA MONOCYTOGENES* IN RATS

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Both Listeria monocytogenes *infection and silica exposure have been shown to significantly alter immune responses. In this study, we evaluated the effect of preexposure to silica on lung defense mechanisms using a rat pulmonary* L. monocytogenes *infection model. Male Sprague-Dawley rats were instilled intratracheally with saline (vehicle control) or silica using either an acute treatment regimen (5 mg/kg; 3 days) or a subchronic treatment protocol (80 mg/kg; 35 days). At 3 or 35 days after silica instillation, the rats were inoculated intratracheally with either ~5000 or 500,000* L. monocytogenes. *At 3, 5, and 7 days postinfection, the left lung was removed, homogenized, and cultured on brain heart infusion agar at 37°C. The numbers of viable* L. monocytogenes *were counted after an overnight incubation. Bronchoalveolar lavage (BAL) was performed on the right lungs, and BAL cell differentials, acellular lactate dehydrogenase (LDH) activity and albumin content were determined. Alveolar macrophage (AM) chemiluminescence (CL) and phagocytosis were assessed as a measure of macrophage function. Lung-associated lymph nodes were removed, and lymphocytes were recovered and differentiated. Preexposure to silica significantly increased the pulmonary clearance of* L. monocytogenes *as compared to saline controls. Exposure to silica caused significant increases in BAL neutrophils, LDH and albumin, and lymph-nodal T cells and natural killer (NK) cells in infected and noninfected rats. CL and phagocytosis were also elevated in silica-treated rats. In summary, the results demonstrated that exposure of rats to silica enhanced pulmonary immune responses, as evidenced by increases in neutrophils, NK cells, T lymphocytes, and macrophage activation. These elevations in pulmonary immune response are likely responsible for the increase in pulmonary clearance of* L. monocytogenes *observed with preexposure to silica.*

Repeated exposure to different workplace particulates may predispose some workers in certain industries to an increased prevalence of respiratory infections. Human epidemiologic data suggest that inhalation of air pollutants generated in the workplace and the environment may be associated with an increased incidence and severity of acute respiratory infections (Howden, 1988; Dockery & Pope, 1994; Schwartz, 1994). Animal

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models have been developed as a means to study the mechanisms by which preexisting disease enhances the pulmonary response to inhaled particles (Zelikoff et al., 1993; Jakab, 1993). In addition, inhalation exposure to certain agents by laboratory animals prior to bacterial challenge has resulted in increased severity of the ensuing infection (Van Loveren et al., 1988; Jakab, 1997).

The principal function of pulmonary host defense mechanisms is to clear inhaled particles from the lungs and keep the lungs sterile (Sibille & Reynolds, 1990). Alveolar macrophages (AMs) serve as the first line of cellular defense (Brain, 1986). They play a central role in maintaining normal lung structure and function through their capacity to phagocytize inhaled particles, remove macromolecular debris, kill microorganisms, function as an accessory cell in immune responses, maintain and repair the lung parenchyma, and modulate normal lung physiology (Crystal, 1991). There are situations in which AMs not only fail in these functions, but are themselves implicated in the pathogenesis of pulmonary diseases. The ingestion of certain particles, that is, silica, and of microbes, can cause the release of lysosomal enzymes and reactive oxygen species that may damage surrounding cells and other macrophages; then dead or dying macrophages release specific mediators that can attract fibroblasts and elicit fibrogenic responses (Brain, 1992).

The inhalation of silica particles results in activation of AMs, which is followed by a dramatic, acute inflammatory response, damage to the respiratory epithelium and interstitial matrix, and the eventual development of fibrosis (Bowden, 1987). Both inhalation (Driscoll et al., 1991; Warheit et al., 1991) and intratracheal instillation (Lindenschmidt et al., 1990; Antonini et al., 1994a; DiMatteo et al., 1996) of silica in laboratory animals result in an acute inflammatory response. This response is initiated when AMs phagocytize silica particles and become damaged or activated (Lapp & Castranova, 1993). It has been well established that silica is highly cytotoxic to AMs (Vallyathan et al., 1988; Antonini & Reasor, 1994). While some AMs die, others are continually stimulated to release reactive oxygen species (Antonini et al., 1994b; Castranova et al., 1996) and inflammatory mediators, such as tumor necrosis factor-α and interleukin-1β (Driscoll, 1996). Silica also has been reported to decrease phagocytosis and severely impair the bactericidal capability of AMs in vitro (Zimmerman et al., 1986).

Our objective was to examine the effect of silica on pulmonary defense mechanisms. It has been reported that silica inhalation predisposes workers to mycobacterial infection and impairments in immune function (Sherson & Lander, 1990; Wagner, 1997). In animal studies, silica has been shown to influence T-lymphocyte recruitment and activation (Hubbard, 1989; Li et al., 1992). Previously, Van Loveren et al. (1988) reported that inhalation of ozone suppresses AM function and alters T-cell-mediated immune responses, decreasing the pulmonary clearance of *Listeria mono-*

cytogenes. In this current study, rats were preexposed to silica by intratracheal instillation and then infected with the bacterial agent *L. monocytogenes.* The development of lung injury and inflammation, alterations in AM function and T-lymphocyte number, and the effects on the pulmonary clearance of *L. monocytogenes* after silica exposure were assessed.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Hilltop Laboratories, Scottdale, PA) weighing 200–250 g were used for all experiments. They were given a conventional laboratory diet and tap water ad libitum, housed in a clean-air, viral- and antigen-free room with restricted access, and allowed to acclimate in an AAALAC-approved animal facility for 1 wk before use.

Materials

Crystalline Min-U-Sil-5 silica (U.S. Silica, Berkeley Springs, WV) had a purity of 99.5% alpha-quartz and was of respirable size with a count mean diameter of 1.362 μm. *Listeria monocytogenes* (strain 10403S, serotype 1) was obtained as a gift from Rosana Schafer of the Department of Microbiology and Immunology at West Virginia University.

Experimental Design

Rats were preexposed by intratracheal instillation of saline (vehicle control) or silica using either an acute treatment regimen (5 mg/kg; 3 days) or a subchronic treatment protocol (80 mg/kg; 35 days). After silica instillation, the animals were divided into 3 groups and intratracheally inoculated with saline, 5000 *L. monocytogenes*, or 500,000 *L. monocytogenes.* At 3, 5, and 7 days after bacteria instillation, bronchoalveolar lavage (BAL) was performed on the right lungs. The cells recovered were differentiated, and chemiluminescence and phagocytosis, measures of macrophage function, were determined. Albumin and lactate dehydrogenase (LDH) activity, indices of lung injury, were measured in the BAL, and hydroxyproline content of right lungs, a determinant of fibrosis, was also assessed. At the same time points, the left lungs were removed, homogenized, and bacterial number was determined. At 7 days, lung-associated lymph nodes were removed, and lymphocytes were recovered and differentiated.

Silica Treatment

Silica was suspended in sterile saline and sonicated for 1 min with a Sonifier 450 cell disruptor (Branson Ultrasonics, Danbury, CT). Rats (n = 6–11/treatment group) were lightly anesthetized by an intraperitoneal injection of 0.6 ml of a 1% solution of sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN) and intratracheally instilled with either 5 or 80

mg/kg body weight of silica in 300 µl of saline, according to the method of Brain et al. (1976). Animals in the vehicle control group were intratracheally dosed with 300 µl of sterile saline.

Intratracheal Bacteria Inoculation

Listeria monocytogenes was cultured overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) at 37°C in a shaking incubator. Following incubation, the bacteria concentration was determined spectrophotometrically at an optical density of 600 nm and diluted with sterile saline to the desired concentrations.

At 3 and 35 days after silica instillation, the rats were inoculated intratracheally with either ~5000 or 500,000 *L. monocytogenes* in 500 µl of sterile saline, according to the instillation method described in the previous section. These two doses were found to give a uniform infection, did not significantly affect animal body weight, and did not kill the animals. The intratracheal instillation of 5000 *L. monocytogenes* was selected because it did not elicit an inflammatory response in the lungs, while the higher 500,000 *L. monocytogenes* was observed to induce a massive influx of neutrophils into lungs soon after instillation.

Bronchoalveolar Lavage

At 3, 5, and 7 days after bacteria instillation, the rats were deeply anesthetized with an overdose of sodium pentobarbital and then exsanguinated by severing the abdominal aorta. The left bronchus was clamped off, and BAL was performed on the right lungs of rats from each group. Their lungs were first lavaged with a 4-ml aliquot of calcium- and magnesium-free phosphate buffer solution (PBS), pH 7.4. This first BAL fluid samples were centrifuged at 500 × g for 10 min and filtered with 0.22-µm sterile filters, and the resultant cell-free supernatant was analyzed for various biochemical parameters. Then, the lungs were further lavaged with 6-ml aliquots of PBS until a total of 50 ml BAL fluid was collected. These samples were also centrifuged for 10 min at 500 × g and the cell-free BAL fluid was discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 ml PBS buffer and evaluated as described later. After BAL, the lavaged right lungs were frozen at –70°C for later determination of hydroxyproline, a biochemical index of fibrosis.

Cellular Evaluation

Total cell numbers were determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). Cells were differentiated using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England); 1×10^5 cells were spun for 5 min at 800 rpm and pelleted onto a slide. Cells (200/rat) were identified on cytocentrifuge-prepared slides after labeling with Leukostat stain (Fisher Scientific, Pittsburgh, PA).

Biochemical Parameters of Injury

Within the acellular BAL fluid from the first 3-ml lavage, albumin content, a measure to quantitate increased permeability of the bronchoalveolar-capillary barrier, and LDH activity, an indicator of general cytotoxicity, were measured. Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromcresol green using an albumin BCG diagnostic kit (Sigma Chemical Company, St. Louis, MO). Measurements were performed with a Cobas Fara II analyzer (Roche Diagnostic Systems, Montclair, NJ). LDH activity was determined by measuring the reduction of pyruvate coupled with the oxidation of NADH at 340 nm according to the method of Wroblewski and LaDue (1955). LDH enzyme reagents were purchased from Roche Diagnostic Systems (Indianapolis, IN).

Lung hydroxyproline was measured as an index of pulmonary fibrosis. Frozen right lung samples that had been previously lavaged were thawed and minced, then processed and analyzed according to the method of Witschi et al. (1985). 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 Ehrlich's reagent. Absorbance was read at 560 nm on a Gilford spectrophotometer. The number of micrograms of hydroxyproline per right lung was calculated from a standard curve.

Chemiluminescence

Chemiluminescence (CL) is a measure of light production by resting or stimulated cells and represents the release of reactive oxidant species. CL was measured in a total volume of 0.5 ml HEPES buffer. Resting CL was determined by incubating 0.5×10^6 BAL cells at 37°C for 10 min in 0.008 mg% (w/v) luminol (Sigma Chemical Company, St. Louis, MO) followed by the measurement of CL for 15 min. Luminol is used as an amplifier to enhance detection of the light and was first dissolved in a small amount of ethanol before being brought up in HEPES buffer at its final concentration. To determine zymosan-stimulated CL, the assay was modified to include 1 mg unopsonized zymosan (Sigma Chemical Company, St. Louis, MO), which was added to the assay immediately prior to measurement of CL. Measurement of CL was done using an automated Berthold Autolumat LB 953 luminometer (Wallace, Inc., Gaithersburg, MD) for 15 min, and the integral of counts per minute (cpm) versus time was calculated. Zymosan-stimulated CL was calculated as the cpm of zymosan-stimulated cells minus the cpm of the corresponding resting cells.

Alveolar Macrophage Phagocytosis

The AMs were recovered by bronchoalveolar lavage from rats 3 days after the intratracheal instillation of silica (5 mg/kg of body weight) or

saline. The recovered AMs were allowed to attach to glass coverslips in a 24-well plate in PBS with 1% fetal bovine serum (FBS) at a concentration of 5×10^5 cells/well at 37°C for 1 h. After the incubation period, nonadherent cells were washed out of the wells with PBS. The adherent AMs were treated with carboxylate-modified, 2.0-μm yellow-green FluoSpheres (Molecular Probes, Eugene, OR) and rocked at 37°C for 1 h at a concentration of 30 beads/cell. After the second incubation period, the AMs were washed twice with PBS to remove any free beads, fixed with 2% paraformaldehyde for 30 min, and then stained with the fluorochrome nile red (0.1 μg/ml; Molecular Probes, Eugene, OR) for 5 min. The glass coverslips were mounted on microscope slides, and images were recorded from a Sarastro 2000 laser scanning confocal microscope fitted with an argon-ion laser (Molecular Dynamics, Inc., Sunnyvale, CA) using 514-nm excitation light. The glass-adhered AMs ingesting the beads were counted to determine the phagocytic index. Two hundred AMs per rat for each of the treatment groups were evaluated and scored as having 0, 1–4, or >5 beads/AM. A weighted phagocytic index was calculated by multiplying the number of AMs in each group by 0 (for 0 beads/AM), 2 (for 1–4 beads/AM), or 4 (>5 beads/AM), respectively, and dividing the total score by 200.

Pulmonary Clearance of *L. monocytogenes*

At 3, 5, and 7 days after bacteria instillation, left lungs were removed from all rats in each treatment group. The excised tissues were suspended in 10 ml of sterile water, homogenized using a Polytron 2100 homogenizer (Brinkmann Instruments, Westbury, NY), and cultured quantitatively on brain heart infusion agar plates (Becton Dickinson and Co., Cockeysville, MD). The viable colony-forming units (CFUs) were counted after an overnight incubation at 37°C.

Lymphocyte Differentiation

At 7 days postinoculation with *L. monocytogenes,* lung-associated lymph nodes were excised and homogenized in PBS to count and differentiate B cells, T cells, T-cell subsets (CD4+ helper and CD8+ cytotoxic cells), and natural killer (NK) cells. The respective cell types were labeled with an appropriate monoclonal antibody, which was conjugated with a fluorescent probe for visualization according to the method of Luster et al. (1988).

Statistical Analysis

Results are expressed as means ± standard error of measurement (SE). Statistical analyses were carried out with the JMP IN statistical program (SAS, Inc., Belmont, CA). The significance of the interaction among the different treatment groups for the different parameters at each time point was assessed using an analysis of variance (ANOVA). The significance of

difference between individual groups was analyzed using the Tukey–Kramer post hoc test. For all analyses, the criterion of significance was set at $p < .05$.

RESULTS

Lung Inflammation/Injury

A significant increase was observed in the total number of neutrophils recovered from the lungs of rats at each time point after intratracheal instillation with either dose of silica (5 mg/kg, 3 days; and 80 mg/kg, 35 days) as compared to the saline group (Figure 1). Intratracheal inoculation of 5000 *L. monocytogenes* did not change the number of neutrophils recovered at any of the time points for either the saline or silica groups (Figure 1A). Thus, increases observed in neutrophil number appeared to be related to the silica treatment and not to the pulmonary inoculation with the low dose of *L. monocytogenes.* On the other hand, intratracheal inoculation of 500,000 *L. monocytogenes* caused a significant increase in the number of neutrophils recovered at each time point as compared to saline controls (Figure 1B). Significant increases in total cells recovered were observed for the Si80 + 500,000 *L. monocytogenes* as compared to the saline + 500,000 *L. monocytogenes* group.

As indices of lung injury, LDH activity and albumin were measured in the acellular BAL fluid (Figure 2). The two silica treatments caused a significant increase in BAL fluid LDH and albumin as compared to saline control values (Figure 2, A and B). This elevation in lung injury persisted for the entire 7-day postbacteria inoculation time course (data not shown). Neither dose of *L. monocytogenes* caused any significant change in BAL LDH and albumin (data not shown).

As a determinate of pulmonary fibrosis, the hydroxyproline levels of the lavaged right lungs were measured (Figure 3). A significant elevation in right lung hydroxyproline levels was observed 35 days after intratracheal instillation of 80 mg/kg of silica when compared with control values. Neither treatment with 5 mg/kg of silica for 3 days nor *L. monocytogenes* inoculation caused an increase in pulmonary fibrosis (data not shown).

Macrophage Function

Intratracheal instillation of the different doses of silica alone (without *L. monocytogenes*) caused a significant increase in zymosan-stimulated chemiluminescence at the three time points as compared to the saline controls (Figure 4). The smaller 5000 *L. monocytogenes* dose had no effect on chemiluminescence (Figure 4A), while the higher 500,000 bacteria dose caused a significant elevation in chemiluminescence at 3 and 5 days as compared to the saline controls with the response subsiding over the 7-day time course (Figure 4B). No significant differences were observed

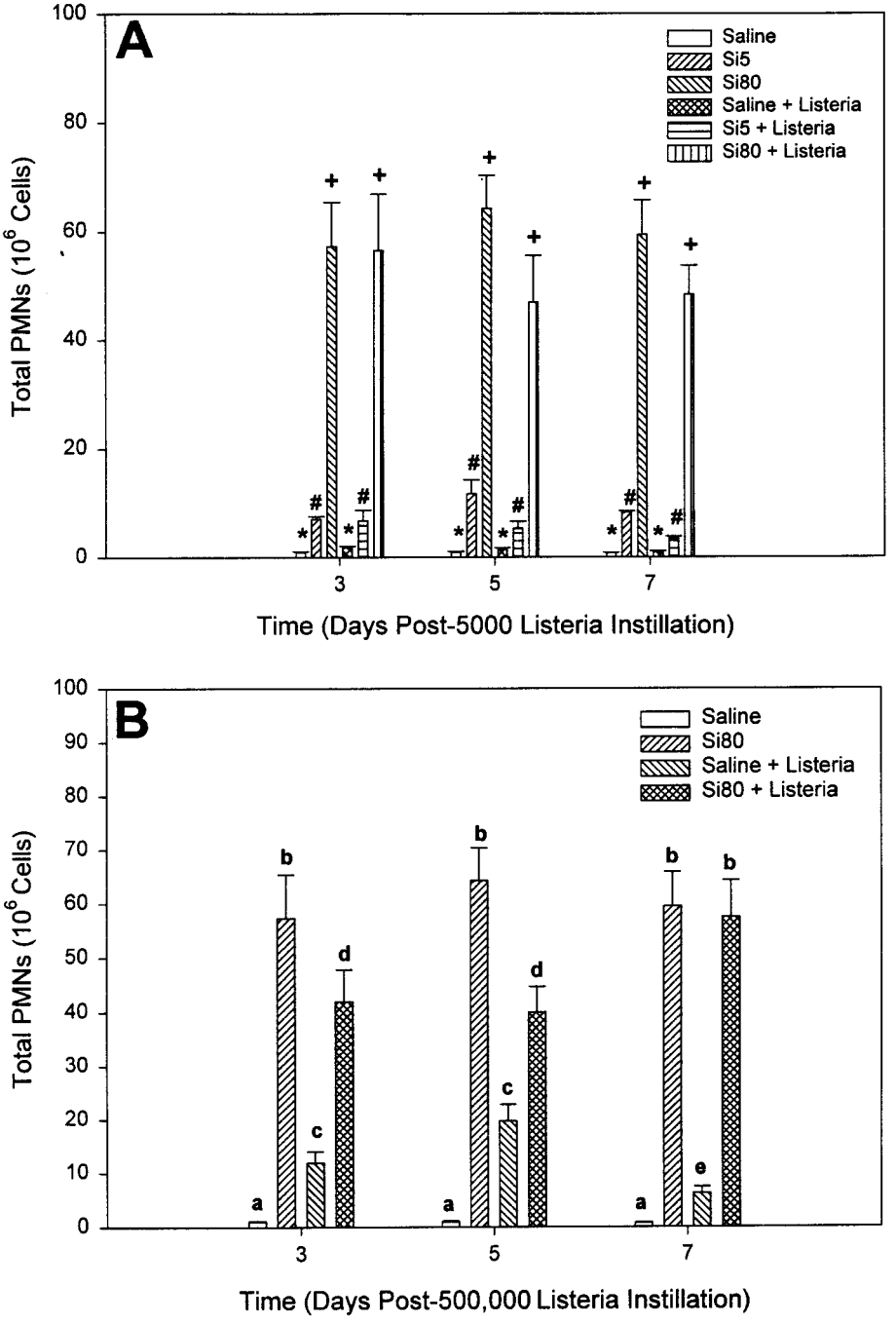

FIGURE 1. Total number of neutrophils (PMNs) recovered from rats preexposed to silica or saline (vehicle control) 3, 5, and 7 days after intratracheal inoculation with (A) 5000 or (B) 500,000 *L. monocytogenes*. Rats were preexposed to silica by intratracheal instillation 3 days (5 mg/kg silica) or 35 days (80 mg/kg silica) prior to bacterial inoculation. Values are means ± SE (n = 6–10); treatment groups with the same symbols are not statistically different from each other ($p < .05$).

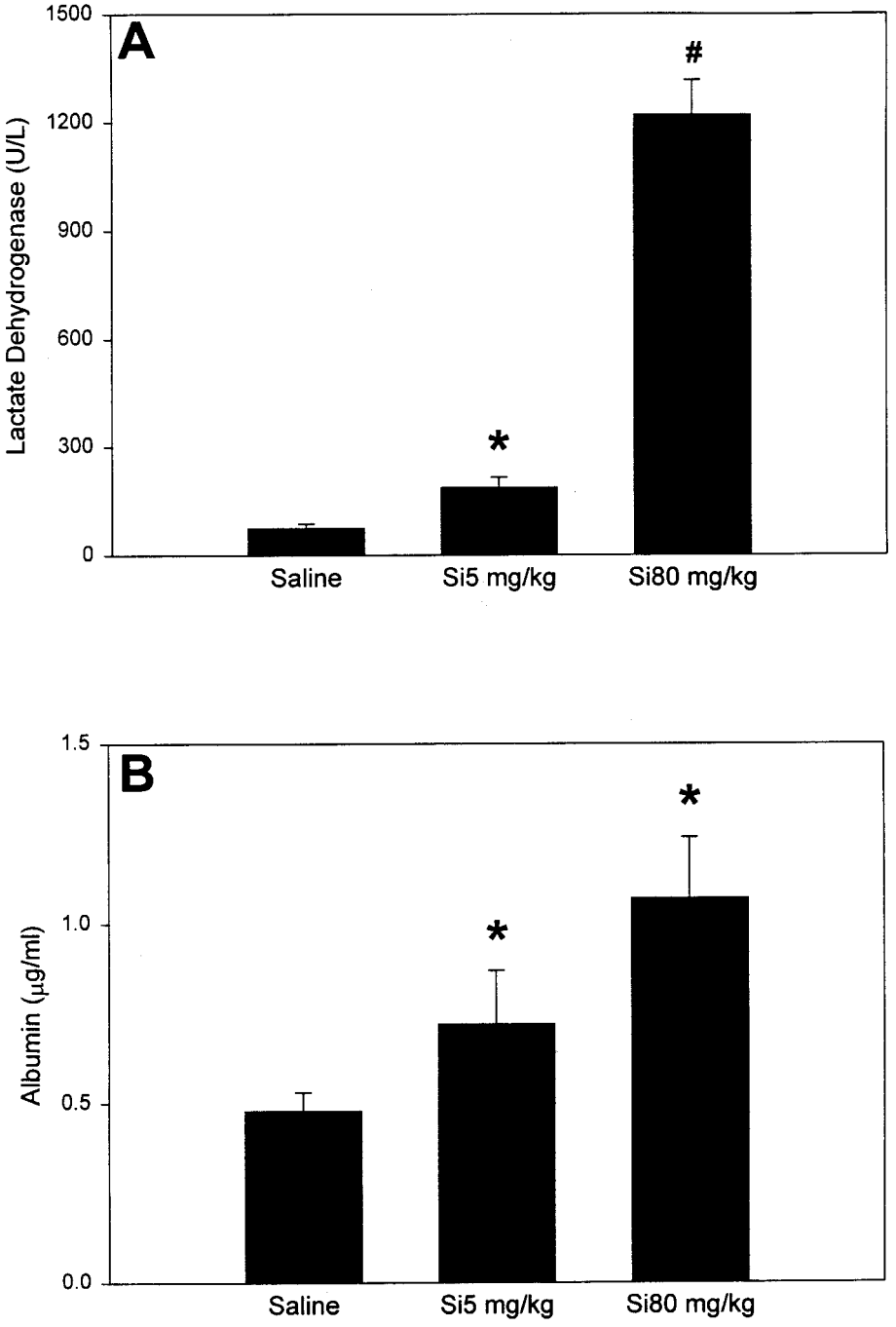

FIGURE 2. (A) Lactate dehydrogenase activity and (B) total albumin in acellular BAL fluid recovered from rats preexposed to saline (vehicle control) or silica by intratracheal instillation 3 days (5 mg/kg silica) or 35 days (80 mg/kg silica). Values are means ± SE (n = 6–10); asterisk indicates mean value group was significantly greater than the value of the saline group, and #, mean value of the silica 80-mg/kg group was significantly greater than the other two groups ($p < .05$).

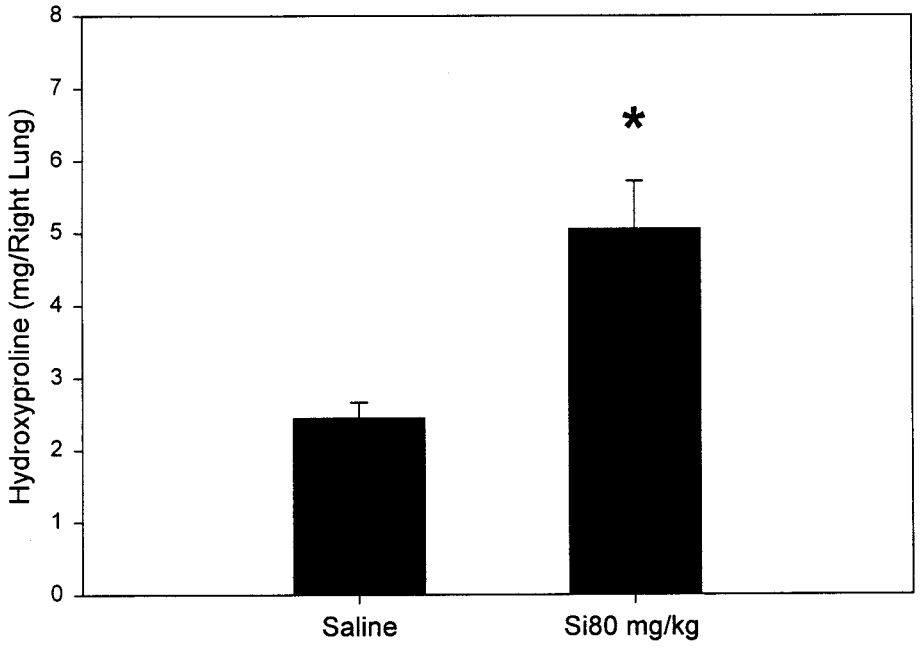

FIGURE 3. Total hydroxyproline of right lung at 35 days postinstillation of silica (80 mg/kg) or saline (vehicle control). Values are means ± SE (n = 8–9). Asterisk indicates that the mean value of the silica group was significantly greater than the value of the saline group, $p < .05$.

in chemiluminescence between the silica alone and silica + 500,000 *L. monocytogenes* groups at 3 and 5 days, but a significant decrease in the silica + 500,000 *L. monocytogenes* was seen at the 7-day time point when comparing these two groups (Figure 4B).

In comparing the phagocytic activity of AMs recovered from rats intratracheally instilled with saline (Figure 5A) or silica (Figure 5B), there appeared to be a greater uptake of the beads by the AMs from the silica group as analyzed by fluorescent confocal microscopy. Pseudopodia could even be visualized extending from some of the AMs recovered from the silica-treated rats in an attempt to phagocytize the beads (Figure 5B, arrows). A significant increase in the weighted phagocytic index was observed for the AMs from the silica group as compared to the AMs of the saline group (Figure 6).

Pulmonary Bacterial Number

Preexposure of silica inhibited replication of *L. monocytogenes* in the lungs when using either bacteria dose as compared to controls (Figure 7). A significant decrease in the number of *L. monocytogenes* (CFUs) present in the lungs of the different silica groups as compared to the saline groups was seen as early as 3 days after inoculation of 5000 or 500,000 bacteria (Figure 7, A and B). A significant difference in *L. monocytogenes* number in the lungs of the silica animals was also seen 5 days after inoculation as compared to saline controls when using the lower 5000-bacteria dose

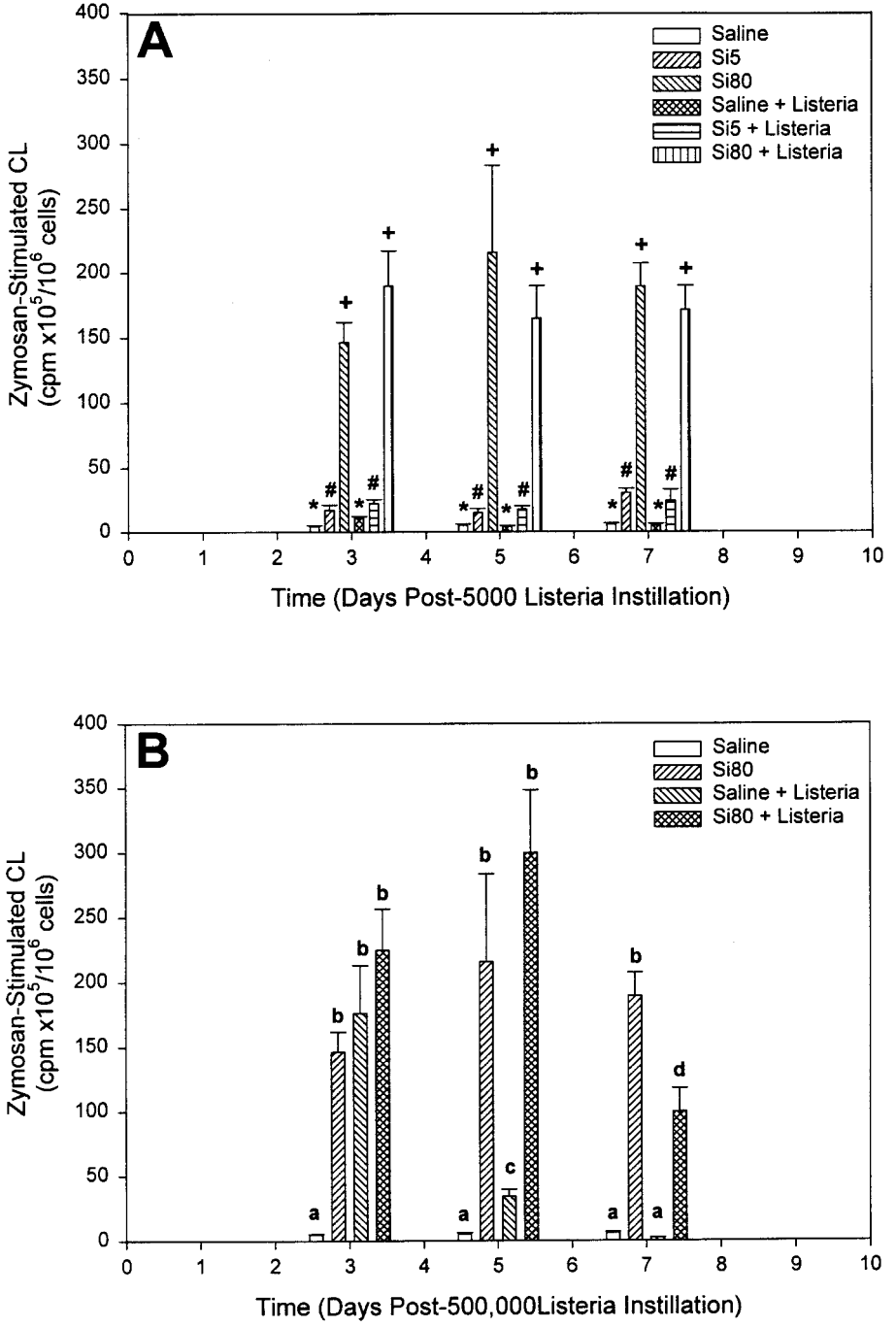

FIGURE 4. Zymosan-stimulated chemiluminescence (CL) of BAL cells recovered from rats preexposed to silica or saline (vehicle control) 3, 5, and 7 days after intratracheal inoculation with (A) 5000 or (B) 500,000 *L. monocytogenes.* Rats were preexposed to silica by intratracheal instillation 3 days (5 mg/kg silica) or 35 days (80 mg/kg silica) prior to bacterial inoculation. Values are means ± SE (n = 6–10); treatment groups with the same symbols are not statistically different from each other at $p < .05$.

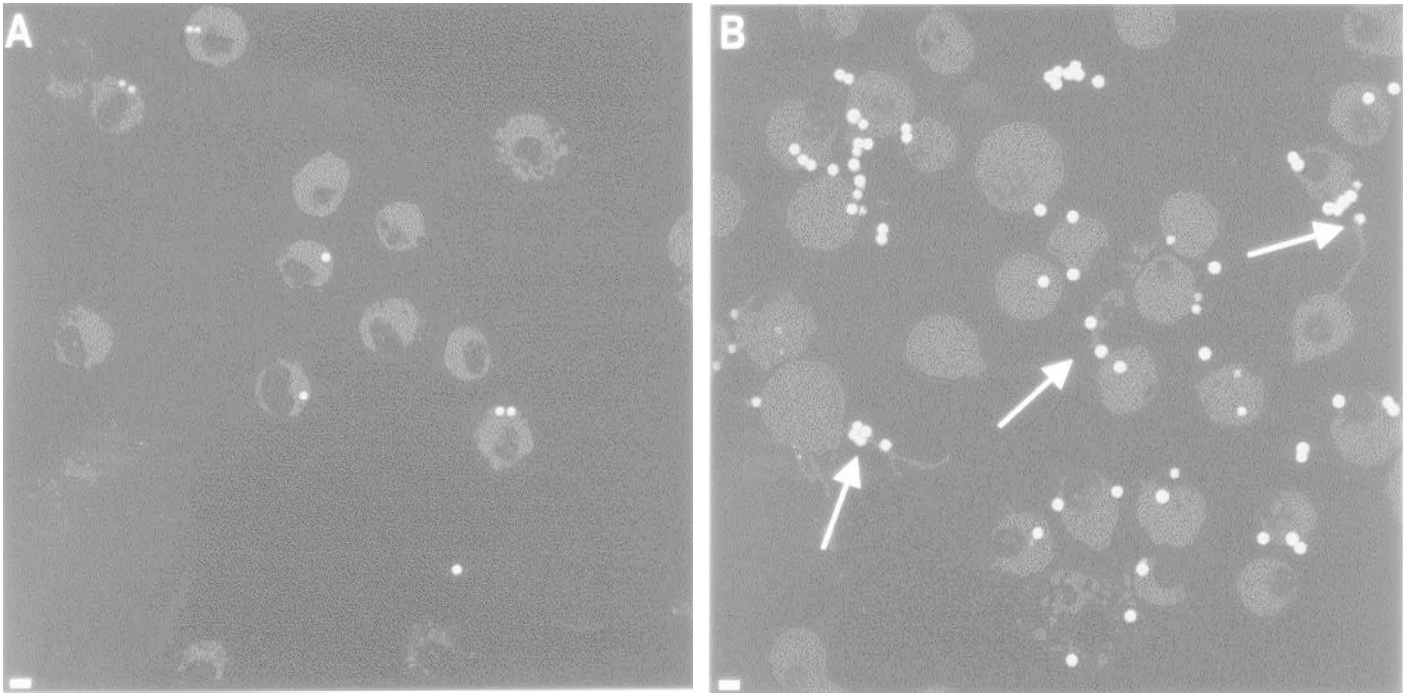

FIGURE 5. Confocal micrograph depicting phagocytosis of fluorescent beads by glass-adherent alveolar macrophages recovered from rats intratracheally treated with (A) saline or (B) silica, 5 mg/kg for 3 days. Cells are red; beads are yellow. Pseudopodia are observed extending from the AMs recovered from the silica-treated rats in the process of phagocytizing the beads (B, arrows). Bar is 5 μm.

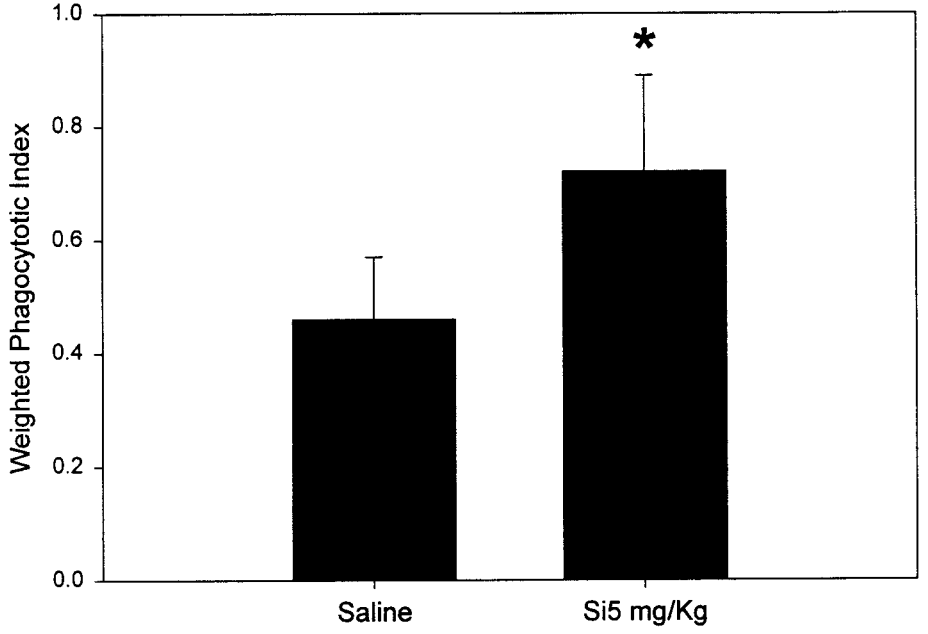

FIGURE 6. Weighted phagocytic index of glass-adherent alveolar macrophages recovered from rats intratracheally treated with (A) saline or (B) silica, 5 mg/kg for 3 days. Values are means ± SE ($n = 4$). Asterisk indicates that the mean value of the silica group was significantly greater than the value of the saline group ($p < .05$).

(Figure 7A). Significantly fewer *L. monocytogenes* were present in the lungs of the silica group at 7 days as compared to the saline controls after inoculation with 500,000 bacteria (Figure 7B).

Lymphocyte Differentiation

The number of the different lymphocytes recovered from the lung-associated lymph nodes for each group 7 days after *L. monocytogenes* intratracheal inoculation was determined (Table 1). For each cell type except B lymphocytes, all treatments with silica and *L. monocytogenes* either alone or in combination caused a significant increase in cell number as compared to controls. A significantly greater number of each cell type was recovered from the silica and silica + *L. monocytogenes* groups as compared to the saline + *L. monocytogenes* groups. A significant decrease was observed in the number of each of the cell type, except NK cells, recovered from silica + 500,000 *L. monocytogenes* when compared with the silica + 5000 *L. monocytogenes.*

The percentage of the different lymphocytes recovered from the lung-associated lymph nodes for each group 7 days after *L. monocytogenes* intratracheal inoculation was determined (Table 2). No significant difference in the percentage of CD4+ cells was observed among the different

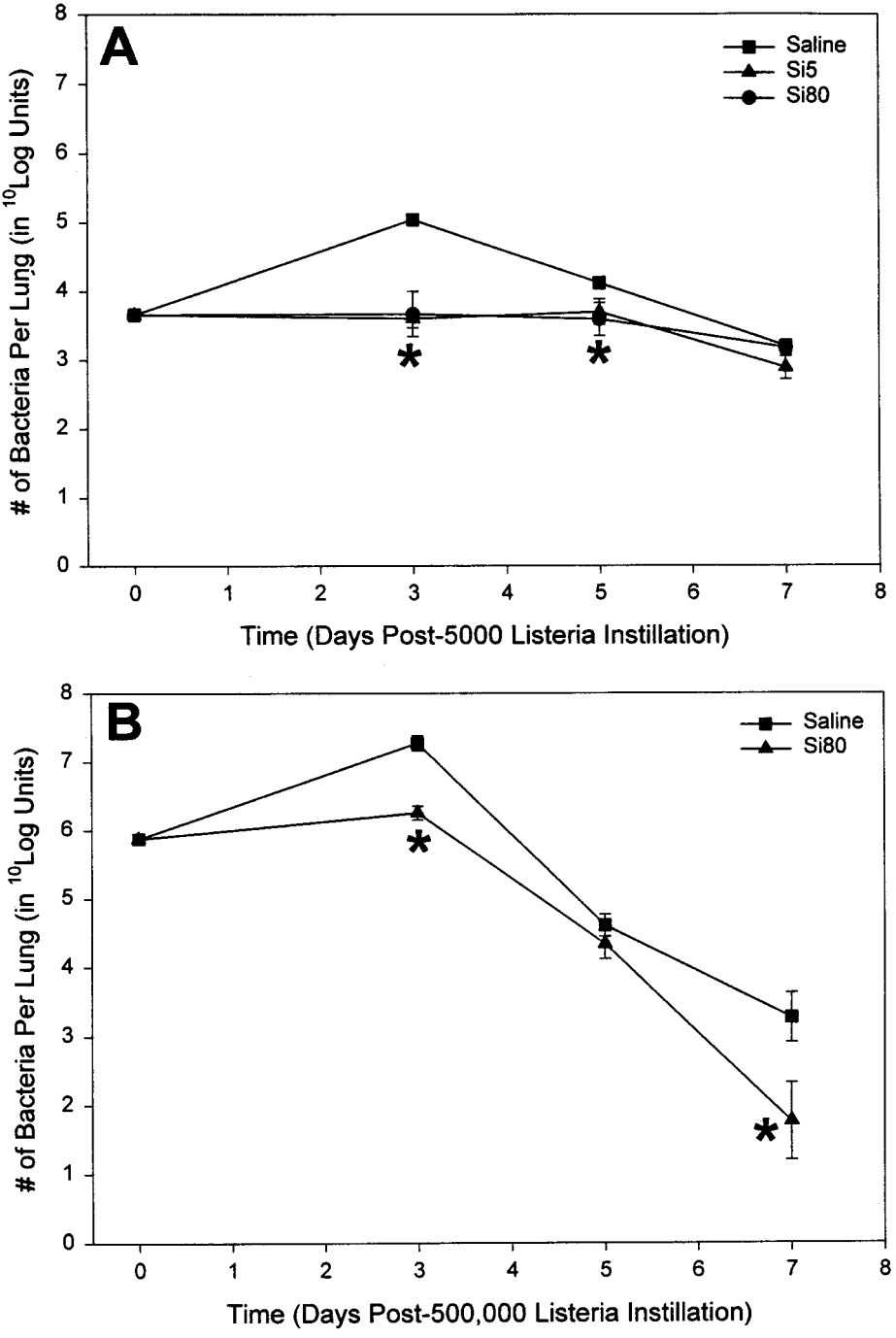

FIGURE 7. Number of bacteria counted in the lungs from rats preexposed to silica or saline (vehicle control) 3, 5, and 7 days after intratracheal inoculation with (A) 5000 or (B) 500,000 *L. monocytogenes*. Rats were preexposed to silica by intratracheal instillation 3 days (5 mg/kg silica) or 35 days (80 mg/kg silica) prior to bacterial inoculation. Values are means ± SE (n = 6–10). Asterisk indicates that the mean value of the saline group was significantly different from the value of the silica group ($p < .05$).

TABLE 1. Total Lymphocyte Numbers of Lung-Associated Lymph Nodes ($\times 10^6$)

Treatment groups	CD4+ T-helper cells	CD8+ T-cytotoxic cells	Total B lymphocytes	Natural killer cells
Saline	8.58 ± 1.73^a	2.61 ± 1.34^e	4.76 ± 0.88^i	0.63 ± 0.03^m
Silica 80 mg	79.2 ± 7.32^b	55.7 ± 8.43^f	52.8 ± 11.8^j	11.3 ± 1.74^n
Saline + 5000 *Listeria*	39.7 ± 2.92^c	25.3 ± 1.62^g	22.0 ± 2.60^k	5.90 ± 0.55^o
Silica 80 mg + 5000 *Listeria*	121.1 ± 17.2^d	93.5 ± 14.4^h	96.5 ± 22.1^l	22.0 ± 2.81^n
Saline + 500,000 *Listeria*	18.5 ± 1.46^c	13.0 ± 1.97^g	8.5 ± 1.88^i	2.92 ± 0.54^o
Silica 80 mg + 500,000 *Listeria*	93.7 ± 4.15^b	61.0 ± 5.78^f	40.8 ± 4.26^j	14.4 ± 1.92^n

Note. Values are means ± SE of cell numbers 7 days after *L. monocytogenes* inoculation ($n = 5$–6).
[a–o]For each cell type, groups with different denotations are significantly different from each other ($p < .05$).

groups. The percentage of CD8+ and NK cells recovered was significantly elevated in all the groups as compared to the saline control. The saline + 500,000 *L. monocytogenes* and silica 80 mg + 500,000 *L. monocytogenes* had a significantly lower percentage of B lymphocytes as compared to the other groups.

DISCUSSION

Silica exposure is associated with increased rates of infection with mycobacterial infection (Wagner, 1997). It was the goal of this current study to evaluate whether the presence of silica-induced lung injury, inflammation, and alterations in pulmonary immune function would have an effect on how the lungs would respond to bacterial challenge with *L. monocytogenes.* Van Loveren et al. (1988) have shown that ozone slows the pulmonary clearance of *L. monocytogenes.* They demonstrated that ozone suppressed AM function and cellular immunity. Ozone decreased the number of *L. monocytogenes* ingested and killed by AMs. In our cur-

TABLE 2. Percentage of Lymphocyte Type from Lung-Associated Lymph Nodes

Treatment groups	CD4+ T-helper cells	CD8+ T-cytotoxic cells	Total B lymphocytes	Natural killer cells
Saline	34.9 ± 3.17	6.36 ± 1.75^a	20.3 ± 2.75	2.16 ± 0.12^a
Silica 80 mg	31.0 ± 3.17	19.7 ± 2.75	18.8 ± 2.90	4.24 ± 0.42
Saline + 5000 *Listeria*	30.5 ± 0.83	19.6 ± 1.37	16.8 ± 1.36	4.58 ± 0.46
Silica 80 mg + 5000 *Listeria*	24.5 ± 1.94	18.6 ± 0.67	19.6 ± 4.50	4.45 ± 0.24
Saline + 500,000 *Listeria*	26.7 ± 1.90	18.2 ± 1.23	11.1 ± 1.87^b	4.00 ± 0.41
Silica 80 mg + 500,000 *Listeria*	30.7 ± 1.83	19.4 ± 0.75	12.9 ± 0.68^b	4.53 ± 0.42

Note. Values are means ± SE 7 days after *L. monocytogenes* inoculation ($n = 5$–6).
[a]CD8+ and natural killer cell values for the saline group were significantly different from the values of the other groups ($p < .05$).
[b]B lymphocyte values for the saline + 500,000 *L. monocytogenes* and silica 80 mg + *L. monocytogenes* groups were significantly different from the values of the other groups ($p < .05$).

rent study, rats were intratracheally instilled with two doses of silica (5 and 80 mg/kg body weight). Lung responses were examined at 3 and 35 days postinstillation. The silica exposure resulted in inflammation and pulmonary injury that were more pronounced with the high dose as compared to the low dose, and the responses observed at 35 days postinstillation were greater than those at 3 days. The progressive silica-induced damage observed with dose and time was consistent with that reported in other studies (Lindenschmidt et al., 1990; Antonini et al., 1994a).

After exposure to silica, rats were intratracheally inoculated with two doses of *L. monocytogenes*: one dose (5000 bacteria) that did not elicit an inflammatory response in the lungs, and a higher dose (500,000 bacteria) that induced a massive influx of neutrophils into lungs and significantly altered lung lymph-nodal lymphocyte numbers soon after instillation. A number of animal studies have used *L. monocytogenes* to assess pulmonary host defense mechanisms (Jakab, 1993; Van Loveren et al., 1988; Dormans et al., 1990; Reasor et al., 1996). *Listeria monocytogenes* is a gram-positive, facultative intracellular bacterium that can live within a variety of host cells, including endothelial and epithelial cells, as well as some macrophages (Fleming & Campbell, 1997). The initial immune response of the host to *L. monocytogenes* is marked by a rapid recruitment of neutrophils and macrophages to the site of infection and activation of NK cells (Seaman et al., 1999). While the innate immune response is efficient at limiting the initial spread of infection, rapid clearance of *L. monocytogenes* depends on acquired T-cell-mediated immunity (Shen et al., 1998). Mackaness (1969) demonstrated the importance of activated macrophages in defense against *L. monocytogenes,* the requirement of lymphocytes for the development of specific resistance, and the interaction between the specific (lymphocyte) and nonspecific (macrophage) elements of the immune system.

We observed in this investigation that preexposure to silica significantly increased the rate at which the bacteria was cleared from the lungs as compared to the saline controls. Acute silica pretreatment (Si 5 mg/kg; 3 days) had the same effect in enhancing *L. monocytogenes* pulmonary clearance as did a subchronic silica preexposure (Si 80 mg/kg; 35 days) where fibrosis was present. The *L. monocytogenes* dose, whether it was inflammatory or noninflammatory, also didn't seem to matter. This enhancement in bacterial clearance could be attributed partially to an increase in the activation of AMs. Both phagocytosis and CL were elevated after silica exposure. Thus, the AMs recovered from the silica-exposed animals were more aggressive in engulfing the *L. monocytogenes* and produced more reactive oxygen species to aid in the killing of the bacteria.

Evidence clearly indicates that *in vivo* exposure of rats to silica potentiates the production of oxidants and enhances the surface activity of pulmonary phagocytes (Castranova et al., 1996). Rom et al. (1987) have reported that the resting release of reactive oxygen species is elevated in

the lungs of patients with chronic silicosis. Our laboratory has observed elevated basal CL in pulmonary phagocytes harvested from a rock driller with acute silicosis as compared to healthy volunteers (Goodman et al., 1992). It is important to note that Zimmerman et al. (1986) observed that silica caused a decrease in macrophage phagocytosis and an inhibition of their ability to kill *L. monocytogenes* in vitro. There are several explanations that can be given to address this discrepancy between our study and theirs in assessing silica's effect on macrophage function. In their study, extremely high in vitro doses of silica were used, which caused a significant decrease in cell viability. A significant suppression in phagocytosis and killing of *L. monocytogenes* was only observed at the cytotoxic silica doses used in their study. Also, with the design of their in vitro study, the contribution of other immune cells, that is, lymphocytes and neutrophils, and inflammatory cytokines, that is, tumor necrosis factor and interleukin-1, which aid the macrophage in defense against bacterial insult, was absent.

Other components of the innate immune response were affected in our current study. Preexposure to silica also markedly increased the number of neutrophils recruited to the lungs, thereby increasing the phagocyte-to-bacteria ratio and substantially elevating the auxiliary phagocytic capacity of the lungs. The involvement of neutrophils in killing extracellular, pyrogenic bacteria has been well documented. However, there is limited information about the role of neutrophils in resistance to intracellular pathogenic bacteria, such as *L. monocytogenes.* Using neutrophil-depleting monoclonal antibodies, Rogers and Unanue (1993) have demonstrated the direct involvement of neutrophils in defense against *L. monocytogenes* infection. Total NK cell number recovered from the lung-associated lymph nodes as well as the percentage of lymphocytes that were NK cells were also elevated after exposure to silica in our study. Early production of gamma interferon by NK cells from lung-draining lymph nodes has been shown to be crucial for activating the bactericidal mechanisms of macrophages (Dunn & North, 1991). Depletion of NK cells by treatment with anti-NK cell antibodies resulted in severe exacerbation of *L. monocytogenes* infection.

Because of the role T lymphocytes play in *L. monocytogenes* infection, the use of *L. monocytogenes* in immune studies continues to serve as a model to investigate cell-mediated immunity mechanisms (Shen et al., 1998). Silica has also been shown to affect acquired T-cell-mediated immune responses (Hubbard, 1989; Li et al., 1992). Silica exposure then would likely alter the pulmonary fate of *L. monocytogenes.* In this current study, preexposure of silica led to a marked increase in the number of B lymphocytes, CD4+ T-helper cells, and CD8+ T-cytotoxic cells recovered from the lung-associated lymph nodes. The percentage of lymphocytes that were CD8+ T-cytotoxic cells was also significantly elevated after silica pretreatment. Since T-lymphocyte numbers were elevated by silica, it was

not surprising that *L. monocytogenes* was cleared more quickly from the lungs as compared to controls. Hubbard (1989) demonstrated that T lymphocytes modulate the silica-induced immune response by maintaining macrophage response and influencing the termination of neutrophil inflammation—two actions that would affect bacteria clearance. It has also been observed that lymphokines, such as macrophage—derived growth factor, gamma interferon, and tumor necrosis factor released from activated lymphocytes, could possibly stimulate the secretion of mitogenic factors from AMs (Li et al., 1992). This process is facilitated by cell-to-cell contact between lymphocytes and AMs, implying that the lymphokines are expressed on the surface of activated lymphocytes or secreted at sites of cell-to-cell contact.

In summary, we have demonstrated that acute and subchronic preexposure to silica accelerates the pulmonary clearance of *L. monocytogenes* in rats. This is likely due to an activation of local pulmonary immune responses. Acute silica pretreatment enhanced AM function and increased pulmonary neutrophil and lung-associated lymph-node NK-cell and T-lymphocyte numbers. In comparison with other particle studies in our laboratory, this finding is unique for silica. We have shown that diesel exhaust particles (Yang et al., 1999) and residual oil fly ash (Antonini et al., 2000) slow the pulmonary clearance of *L. monocytogenes* in rats.

A question still remains as to whether the pulmonary clearance of *L. monocytogenes* and immune defense against silica is affected differently at more chronic exposures to silica. It has been shown that at lower more acute exposures, when alveolar clearance mechanisms are intact, workers are usually free of disease (Green et al., 1989). However, once a certain exposure threshold has been breached, silica injures the lymphatic vessels and impedes lung clearance mechanisms, thus explaining the characteristic distribution of parenchymal silicotic nodules along the lymphatic routes in the pleura, interlobular septa, and bronchopulmonary rays (Murray et al., 1991). Chronic silica inhalation studies, as well as studies using different particulates, such as residual oil fly ash and diesel exhaust particles, are currently ongoing in our laboratory to address this question.

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