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Lung

Effect of Silica Inhalation on the Pulmonary Clearance of a Bacterial Pathogen in Fischer 344 Rats

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Abstract. Silica inhalation predisposes workers to bacterial infection and impairments in pulmonary defense function. In this study, we evaluated the effect of pre-exposure to silica on lung defense mechanisms by use of a rat pulmonary *Listeria monocytogenes* infection model. Male Fischer 344 rats were exposed by inhalation to filtered air or silica (15 mg/m^3 × 6 h/day × 5 days/wk). After 21 or 59 days of silica exposure, the rats were inoculated intratracheally with 5 × 10^3 *L. monocytogenes*. At 0 (noninfected controls), 3, and 7 days after infection, the left lungs were removed, homogenized, and the number of viable *L. monocytogenes* was counted after an overnight culture at 37° C. Bronchoalveolar lavage (BAL) was performed on the right lungs. Alveolar macrophages (AM) were collected, and the AM production of chemiluminescence (CL), an index of reactive oxygen species generation, was measured. The number of lavagable neutrophils (PMNs) and acellular BAL lactate dehydrogenase (LDH) activity were determined as indices of inflammation and injury, respectively. Pre-exposure to silica for 59 days caused substantial increases in PMN number and LDH activity compared with the air controls, whereas silica inhalation for both 21 and 59 days significantly enhanced the pulmonary clearance of *L. monocytogenes* compared with air controls. Dramatic elevations were also observed in zymosan- and phorbol myristate acetate (PMA)–stimulated CL production by lung phagocytes recovered from rats pre-exposed to silica for 59 days. These results demonstrate that short-term exposure to inhaled silica particles activates lung phagocytes, as evidenced by increases in reactive oxygen species. This up-regulation in the production of antimicrobial oxidants is

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likely responsible for the enhancement in pulmonary clearance of *L. monocytogenes* observed with short-term silica inhalation.

Key words: Silica—Macrophage—*Listeria monocytogenes*—Pulmonary clearance—Chemiluminescence.

Introduction

It has been well documented that inhalation of silica particles predisposes workers to bacterial infection and impairments in lung defense function [18, 21]. Mycobacterial infection in workers exposed to silica has a great potential to hasten the development of respiratory impairment and shorten the worker's life. Bacterial infection in silicotic workers remains a significant problem in less-advanced countries and among disadvantaged persons in developed nations [7]. However, the mechanisms by which silica predisposes workers to bacterial infection are mostly unknown.

Silica inhalation induces a severe, acute inflammatory response, injury to the respiratory epithelium and interstitial matrix, and the eventual development of fibrosis [4]. This response is initiated when alveolar macrophages (AMs) phagocytize silica particles and become damaged or activated [8]. It has been clearly demonstrated that silica is highly cytotoxic to AMs [1]. Silica's cytotoxic properties have been shown to be related to the presence of highly reactive radicals on the surface of the particles [19]. Because some AMs die after silica exposure, others are continually stimulated to release reactive oxygen species [2, 3, 6].

A number of laboratories have used animal models to investigate the possible mechanisms by which specific pneumotoxic substances increase the susceptibility to pulmonary infection. Inhalation exposure of certain agents before bacterial challenge has resulted in increased severity of the ensuing infection [12, 20]. The gram-positive, facultative intracellular bacterial agent, *Listeria monocytogenes*, has been commonly used in some of these studies to assess pulmonary host defense mechanisms [11, 15, 20]. *L. monocytogenes* has been shown to be an ideal agent for pulmonary defense studies. The initial immune response of the host after *L. monocytogenes* infection is marked by AM activation and rapid recruitment of AMs and neutrophils (PMNs) to the site of infection [17]. The objective of this study was to examine the effect of silica on innate pulmonary defense mechanisms after pulmonary infection. Male Fischer 344 rats were pre-exposed to silica by inhalation and then infected with the bacterial agent, *L. monocytogenes*. The development of lung injury and inflammation, alterations in reactive oxygen species production by lung phagocytes, and the effects on the pulmonary clearance of *L. monocytogenes* after silica exposure were determined.

Materials and Methods

Test Agents

Crystalline Min-u-sil 5 silica (U. S. Silica Co., Berkeley Springs, WV) was of respirable size with a mass median diameter of 1.40 μm (geometric standard deviation, 1.86). *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.

Silica Aerosol Exposure of Rats

Pathogen-free male Fischer 344 rats (200–225 g) were exposed at the Division of Applied Research and Technology at NIOSH in Cincinnati, OH. The rats were housed in individual cages in two 5-m^3 Hinners-type inhalation chambers, in which one chamber was used for filtered-air exposures (control) and the other for exposures to 15 mg/m^3 of silica. Exposures were conducted for 6 h/day, 5 days/wk for a total treatment period of 21 and 59 exposure days. Food and water were available ad libitum except during exposures, when only water was available. The rats were on a 12-h light-dark schedule and were exposed during the dark cycle to coincide with their most active period.

Each chamber had 10 to 12 air changes per hour and an inside pressure of 25 Pa less than the ambient laboratory pressure. The air was rough filtered, heated, or chilled to approximately 16° then reheated as necessary to maintain a temperature between 22.2° and 25.6°C within the chambers. The air next passed through a high-efficiency particle filter, a charcoal bed, and a medium-efficiency filter before humidification with a steam humidifier. Temperature (22°–26°C), humidity (40–70%), and ammonia (≤5 ppm) were monitored continuously with a computer data acquisition system and showed little deviation from the target levels during the study. Because aerosol concentration in the chamber was monitored continuously, both optically with a RAS-2 particle sensor and gravimetrically with polyvinyl chloride, membrane filters (37 mm, 5-μm pore size) were changed at 1-hour intervals throughout the study.

Intratracheal Bacteria Inoculation

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

After 21 or 59 days of silica or air exposure, the rats were lightly anesthetized with an intraperitoneal injection of 0.6 mL of a 1% solution of sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN) and inoculated intratracheally with 5×10^3 *L. monocytogenes* in 500 μL of sterile saline, according to the method of Brain et al. [5]. This dose of bacteria was selected because it did not elicit an inflammatory response or significantly affect body weight or mortality in control animals as determined in a previous pilot study.

Bronchoalveolar Lavage

At 0 (noninfected controls), 3, 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 bronchoalveolar lavage (BAL) was performed on the right lungs of rats from each group. The 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 sample was centrifuged at 500 × *g* for 10 min, the supernatant filtered with 0.22-μm sterile filters, and the resultant cell-free supernatant 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 discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 mL of PBS buffer and evaluated as described in the following.

Evaluation of Lung Injury and Inflammation

The total number of alveolar macrophages (AMs) and neutrophils (PMNs) recovered by BAL was determined by means of a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL).

Lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, was determined by measuring the reduction of pyruvate coupled with the oxidation of NADH with a wavelength of 340 nm in the acellular BAL fluid according to the method of Wroblewski and LaDue [23]. LDH enzyme reagents were purchased from Roche Diagnostic Systems (Indianapolis, IN).

Pulmonary Clearance of L. monocytogenes

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

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 according to the method of Antonini et al. [2]. Luminol was used as an amplifier to enhance detection of the light, and 2 mg/mL of unopsonized zymosan (Sigma Chemical Company, St. Louis, MO) or 3 μM phorbol myristate acetate (PMA; Sigma Chemical Company, St. Louis, MO) was added to the assay immediately before the measurement of CL to activate the cells. Because rat PMNs do not respond to unopsonized zymosan, the zymosan-stimulated CL produced is from AMs, whereas activation with the soluble stimulant PMA activates both PMNs and AMs to generate a reactive oxidant species [2]. Measurement of CL was done with 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. The production of CL was calculated as the cpm of stimulated cells minus the cpm of the corresponding resting cells.

Statistical Analysis

Results are expressed as means ± standard error of measurements (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 was assessed by use of an analysis of variance (ANOVA). The significance of difference between individual groups was determined with the Tukey-Kramer post-hoc test. For all analyses, the criterion of significance was set at $p < 0.05$.

Results

As a measure of inflammation, the number of PMNs recovered from the lungs of the rats from each group was determined (Fig. 1). Inhalation of 15 mg/m^3 of silica by noninfected rats for 59 days led to a dramatic increase in lung PMNs compared with the other groups. The number of PMNs recovered from noninfected animals exposed to silica for 21 days was not significantly different from the air control groups. The PMN number remained elevated for the group exposed to silica for 59 days at 3 and 7 days after intratracheal inoculation with *L. monocytogenes*. At 3 days after bacteria instillation, the group pre-exposed to silica for 21 days had a significant elevation in PMNs compared with the 21-day air controls; and at 7 days, a greater number of PMNs was recovered from the 21-day silica group compared with both air control groups.

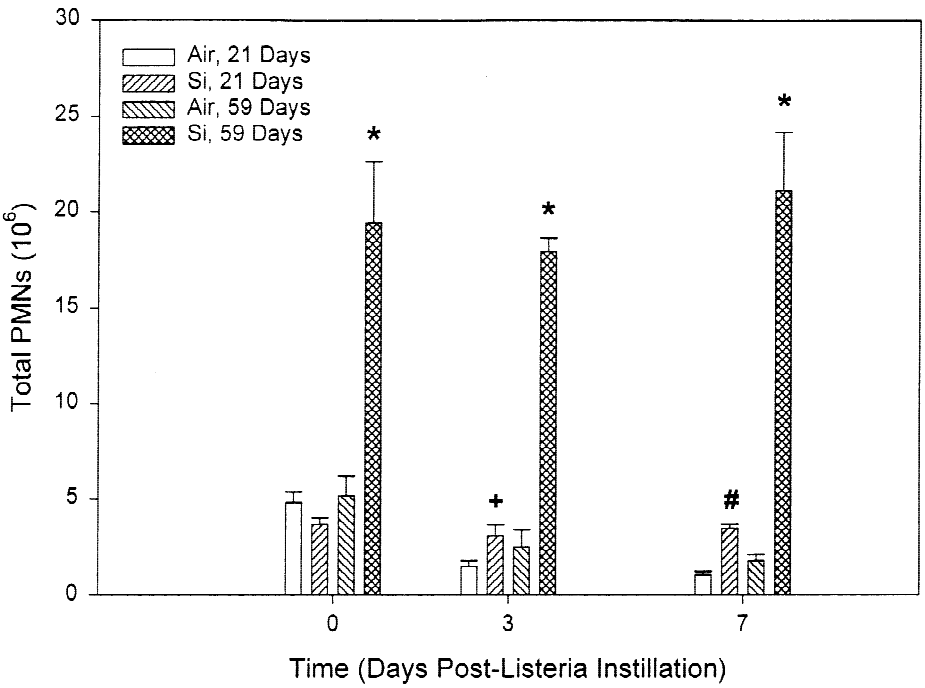

Fig. 1. Total number of neutrophils (PMNs) recovered from rats pre-exposed to silica (15 mg/m^3) or filtered air at 0 (noninfected controls), 3, and 7 days after intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE ($n = 5$ rats/treatment group); *, significantly greater than other groups within each time point; +, significantly greater than air, 21-day group at 3 days; #, significantly greater than air, 21 and air, 59-day groups at 7 days ($p < 0.05$).

Neither silica nor the bacteria treatment significantly altered the total number of AMs recovered from the lungs at any of the time points (data not shown).

As an index of lung injury, the LDH activity of BAL fluid recovered from each group was measured (Fig. 2). LDH was substantially elevated in both noninfected and infected rats that had been pre-exposed to silica for 59 days when compared with the other groups. A significant increase in LDH was observed in the 21-day silica group at 3 and 7 days after bacteria instillation compared with the air control groups, which was indicative of a synergistic cytotoxic effect between silica and the bacteria.

The ability of animals pre-exposed to silica or air to clear a bacterial pathogen from the lungs was determined (Fig. 3). At both 3 and 7 days after the intratracheal inoculation of *L. monocytogenes*, pre-exposure to silica for either 21 or 59 days significantly enhanced the pulmonary clearance of the bacteria as measured on a log scale when compared with the air control groups. At 3 days after *L. monocytogenes* instillation, the bacteria were also cleared more effectively from the lungs of the 59-day silica group compared with the 21-day silica group.

Zymosan- and PMA-stimulated CL of lung phagocytes collected from the different treatment groups were measured as an indices of reactive oxygen species production (Fig. 4*A*,*B*). The CL produced by phagocytic cells recovered from the 59-day silica

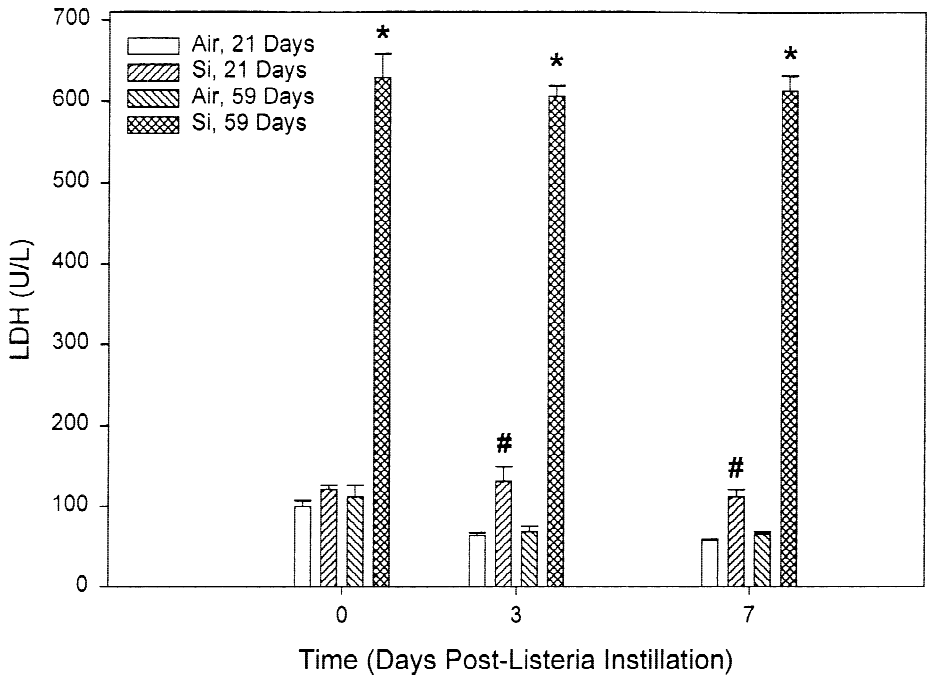

Fig. 2. Lactate dehydrogenase (LDH) activity in the acellular BAL fluid recovered from rats pre-exposed to silica (15 mg/m^3) or filtered air at 0 (noninfected controls), 3, and 7 days after intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE ($n = 5$ rats/treatment group); *, significantly greater than other groups within each time point; #, significantly greater than air, 21 and air, 59-day groups at 3 and 7 days ($p < 0.05$).

group before and after inoculation with *L. monocytogenes* was dramatically elevated compared with the other groups. At 7 days after bacteria infection, the CL was significantly increased in the silica 21-day group compared with the air control groups. No difference in the pattern of response of the four treatment groups was found when the recovered cells were activated with either zymosan or PMA.

Discussion

Studies have shown that pulmonary exposure to pneumotoxic agents causes alterations in the pulmonary host defense. Hatch et al. [10] observed an excess mortality of ~50% in mice instilled intratracheally with residual oil fly ash particles before exposure to aerosolized *Streptococcus*, whereas silica did not significantly alter the infection. They also observed both residual oil fly ash and silica particles to be highly cytotoxic to AMs. Van Loveren et al. [20] have shown that inhalation of ozone decreases the clearance of *L. monocytogenes* from the lungs, concluding that this was most likely caused by a suppression of AM activity, as well as significantly decreasing AM phagocytosis of *L. monocytogenes*. Because inhalation of the highly cytotoxic particle,

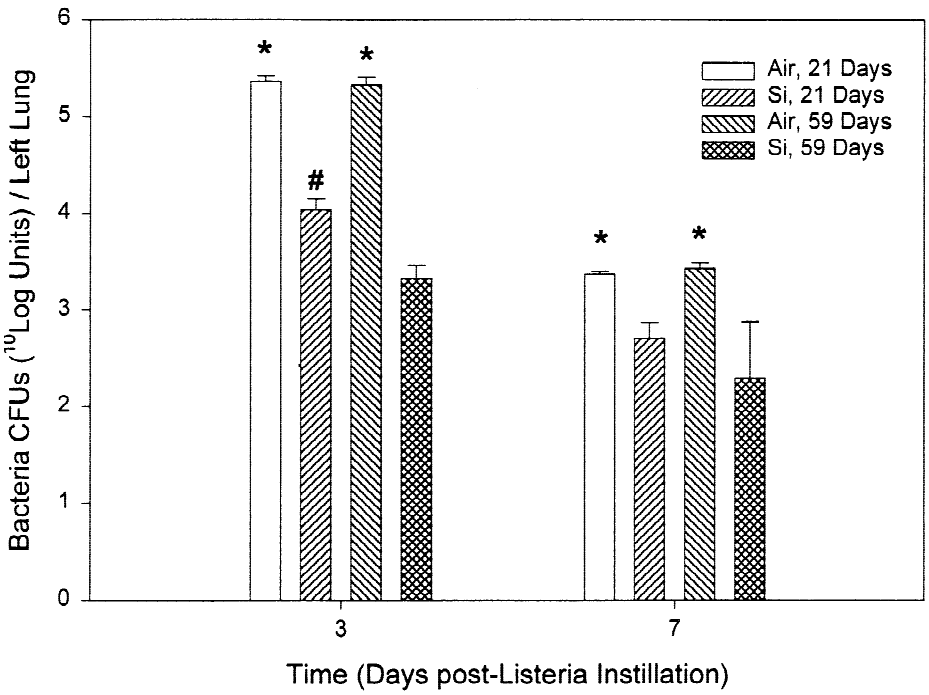

Fig. 3. Number of bacteria CFUs in the left lung of rats pre-exposed to silica (15 mg/m^3) or filtered air at 3 and 7 days after intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means in Log_{10} base units ± SE ($n = 5$ rats/treatment group); *, significantly slower than silica, 21 and silica, 59-day groups within each time point; #, significantly slower than the silica, 59-day group at 3 days ($p < 0.05$).

silica, is associated with increased rates of mycobacterial infection [21], it was the goal of our study to evaluate whether the presence of silica-induced lung injury, inflammation, and alterations in pulmonary defense would have an effect on how the lungs would respond to bacterial challenge with *L. monocytogenes*.

Fischer 344 rats were exposed to silica (15 mg/m^3 × 6 h/day × 5 days/wk) by inhalation for either 21 or 59 days. The silica exposure resulted in inflammation and pulmonary injury that were dramatically more pronounced with the 59 days of treatment compared with the air controls and the 21-day silica exposure. Interestingly, pre-exposure to silica for both 21 and 59 days significantly increased rather than decreased the efficiency at which the bacteria were cleared from the lungs compared with the air controls. This enhancement in bacterial clearance may likely be attributed to an activation of lung AMs and PMNs. CL generation of recovered lung phagocytes was dramatically elevated after silica exposure for 59 days, indicating that the silica-exposed animals were capable of producing more reactive oxygen species to aid in the killing of the bacteria. Ohya et al. [14] have demonstrated that macrophages first activated by immune spleen cells and then infected with *L. monocytogenes* exhibited significantly enhanced bacterial killing. They found that when reactive oxygen intermediate production was blocked by superoxide dismutase, the listericidal ability of the macrophages was significantly diminished.

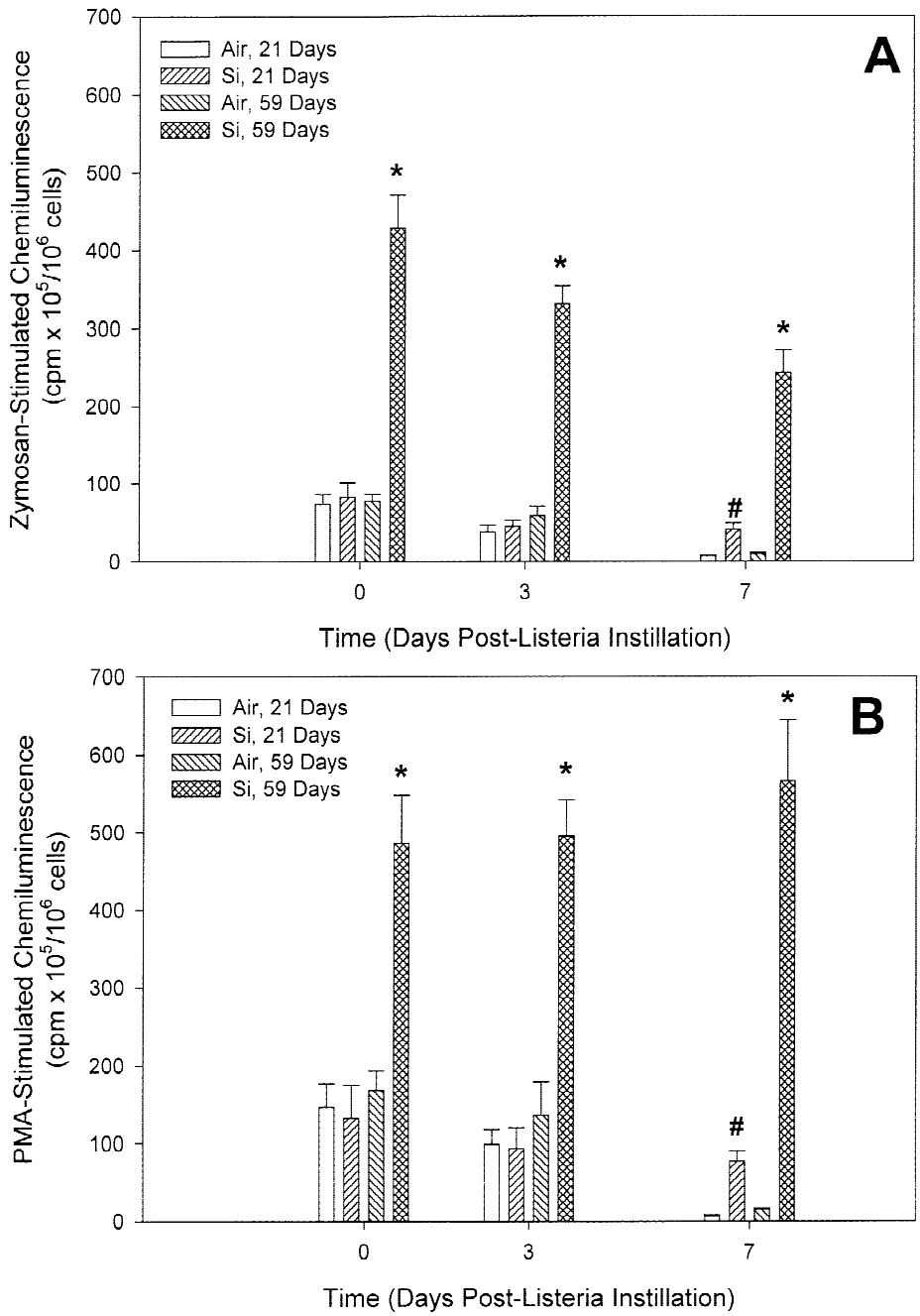

Fig. 4. **(A)** Zymosan- and **(B)** PMA-stimulated chemiluminescence of phagocytes recovered from the lungs of rats pre-exposed to silica (15 mg/m^3) or filtered air at 0 (noninfected controls), 3, and 7 days after intratracheal inoculation with 5×10^3 *L. monocytogenes*. Values are means ± SE ($n = 5$ rats/treatment group); *, significantly greater than other groups within each time point; #, significantly greater than air, 21 and air, 59 day groups at 7 days ($p < 0.05$).

Pre-exposure to silica also increased the number of PMNs recruited into the lungs, thereby increasing the phagocyte/bacteria ratio and enhancing the phagocytic capacity of the lungs. This may be another mechanism by which silica pre-exposure increased pulmonary clearance of *L. monocytogenes*. By the use of monoclonal antibodies to deplete circulating PMNs, Rogers and Unanue [16] have demonstrated the direct involvement of PMNs in defense against *L. monocytogenes* infection. After PMN depletion, both normal mice and immunocompromised SCID mice were unable to eliminate the bacteria and became chronically infected. Despite the PMN depletion, other parameters of nonspecific immune function were unaffected. They observed NK cell and macrophage functions to be normal.

In summary, we have demonstrated that short-term silica inhalation accelerated the pulmonary clearance of *L. monocytogenes* in rats. This is likely due to an activation of innate pulmonary defense responses. Acute silica pre-exposure enhanced AM production of reactive oxygen species and significantly increased pulmonary PMN infiltration. Because of the cytotoxic and fibrogenic nature of silica, the results were somewhat unexpected. Hatch et al. [10] did show that in the assessment of a variety of environmental particulates, not all the particles that were highly cytotoxic caused elevations in bacterial infectivity. Also, with longer, more chronic exposure regimens, the effect on pulmonary clearance of bacteria may be quite different. After low acute silica exposures, workers have been shown to be free of disease in most cases, and alveolar clearance mechanisms are intact [9]. However, it has been reported that once a specific exposure threshold has been reached, silica injures the lymphatic ducts and hinders lung clearance mechanisms [13]. The investigators had observed that parenchymal nodules, characteristic in silicosis, were distributed along the lymphatic routes in the pleura, interlobular septa, and bronchopulmonary rays. In a pilot study, we have demonstrated that 20 to 24 months after rats had inhaled high doses of silica, *L. monocytogenes* clearance and pulmonary host defense mechanisms were significantly impaired [22]. Studies are currently ongoing to evaluate the mechanisms by which chronic silica inhalation may alter pulmonary defense function.

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