

Residual Oil Fly Ash Increases the Susceptibility to Infection and Severely Damages the Lungs after Pulmonary Challenge with a Bacterial Pathogen

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Inhalation of residual oil fly ash (ROFA), a component of ambient particulate matter, has been shown to increase pulmonary morbidity and impair lung defense mechanisms in exposed workers. Our objective was to evaluate the effect of ROFA preexposure on lung defense and injury after pulmonary challenge with a bacterial pathogen. Male Sprague-Dawley rats were dosed intratracheally at day 0 with saline (control) or ROFA (0.2 or 1 mg/100 g body weight). Three days later, a low (5×10^3) or high (5×10^5) dose of *Listeria monocytogenes* was instilled intratracheally into the ROFA- and saline-treated rats. Bronchoalveolar lavage was performed on the right lungs at days 6, 8, and 10. The recovered cells were differentiated, and chemiluminescence (CL) and nitric oxide (NO) production, two indices of alveolar macrophage (AM) function, were measured. At the same time points, the left lung and spleen were removed, homogenized, and cultured, and colony-forming units were counted after an overnight incubation. Exposure to ROFA and the high dose of *L. monocytogenes* led to marked lung injury and inflammation as well as to an increase in mortality, compared with rats treated with saline and the high dose of *L. monocytogenes*. Preexposure to ROFA significantly enhanced injury and delayed the pulmonary clearance of *L. monocytogenes* at both bacterial doses when compared to the saline-treated control rats. ROFA had no effect on AM CL but caused a significant suppression of AM NO production, as compared to the saline control rats. We have demonstrated that acute exposure to ROFA slowed the pulmonary clearance of *L. monocytogenes*. The suppression in AM NO production by ROFA pretreatment likely plays an important role. These results suggest that pulmonary exposure to ROFA may alter AM function and lead to increased susceptibility to lung infection in exposed populations.

Key Words: residual oil fly ash; macrophage; *Listeria monocytogenes*; pulmonary clearance; chemiluminescence.

Epidemiological studies have demonstrated that inhalation of increased levels of particulate air pollution is associated with a decline in lung function, increased respiratory symptoms, and increased morbidity and mortality in susceptible populations

(Dockery and Pope, 1994; Pope *et al.*, 1995; Schwartz, 1994). Residual oil fly ash (ROFA) is an airborne hazard produced by the combustion of fossil fuels. It has been estimated that fly ash contributes more than 2.5×10^5 tons to the ambient air particulate matter burden in the United States annually (Costa and Dreher, 1997). ROFA is chemically complex and composed of metals, sulfates, acids, fuel contaminants, and other unknown materials combined with an insoluble, particulate carbon core (Ghio *et al.*, 2002). Occupational exposure to ROFA has been associated with adverse respiratory health effects in humans (Hauser *et al.*, 1995a,b; Levy *et al.*, 1984). Because of the elemental similarities with ambient air particulate matter and its contribution to air pollution burden, ROFA has been extensively used as a surrogate for studies evaluating the pulmonary responses to particulate matter exposure (Ghio *et al.*, 2002).

Mechanisms of pulmonary toxicity to ROFA have been suggested from animal studies. The bioavailability of soluble transition metals has been implicated as one mechanism for the pulmonary injury induced by ROFA particles (Dreher *et al.*, 1997; Gavett *et al.*, 1997; Kodavanti *et al.*, 1998). The production of reactive oxygen species (Dye *et al.*, 1997; Pritchard *et al.*, 1996), release of inflammatory cytokines (Veronesi *et al.*, 1999), and alterations in signal transduction (Samet *et al.*, 1996, 2000) have also been seen to play important roles in ROFA-induced lung injury. ROFA has been shown to enhance pulmonary infections in mice and to be cytotoxic to alveolar macrophages, AMs (Hatch *et al.*, 1985). In addition, mortality after intratracheal instillation of ROFA in mice with subsequent bacterial exposure correlated with metal concentrations (Pritchard *et al.*, 1996). It has been proposed that exposure to ROFA may increase the susceptibility to infection because of transcriptional inhibition of pathogen resistance pathways (Longphre *et al.*, 2000).

The objective of our study was to examine the effect ROFA has on innate pulmonary defense mechanisms after infection. It is hypothesized that the pulmonary exposure to ROFA may increase the susceptibility to lung infection. Little is known about the effect of co-exposure to ROFA and a bacterial

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pathogen on respiratory defense function. Further studies using ROFA, in which infections are involved, are needed to address this problem. The gram-positive, facultative intracellular bacterial pathogen, *Listeria monocytogenes*, has been commonly used to assess pulmonary defense mechanisms (Antonini *et al.*, 2000, 2001a; Cohen *et al.*, 2001; Jakab, 1993; Van Loveren *et al.*, 1988). In the present study, male Sprague-Dawley rats were preexposed to ROFA by intratracheal instillation and then infected with *L. monocytogenes*. The development of lung injury and inflammation, alterations in AM function, and the effects on the pulmonary clearance of *L. monocytogenes* after ROFA exposure were assessed.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Hilltop Laboratories, Scottsdale, PA) weighing 250–300 g were used for all experiments. They were given a conventional laboratory diet and tap water *ad libitum*, housed in a clean air and viral- and antigen-free room with restricted access, and allowed to acclimate in an AAALAC-approved animal facility for one week before use.

Materials. *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. Residual oil fly ash (ROFA) was collected from a precipitator at Boston Edison Co., Mystic Power Plant #4, Everett, MA. Particle size of the ROFA sample was characterized by scanning electron microscopy (JSM-#5600 SEM, JEOL Ltd., Peabody, MA). The ROFA particles were of respirable size with a count mean diameter of 2.2 μm . Analysis of the metal constituents of the ROFA sample was determined using inductively coupled argon plasma, atomic-emission spectroscopy (NIOSH, 1994). The amount of each element per instillate of ROFA is as follows: 244.6 μg of Fe, 121.8 μg of Al, 92.00 μg of V, 76.86 μg of Ni, 61.10 μg of Ca, and 10.69 μg of Zn were present.

Experimental design. At day 0, rats were preexposed by intratracheal instillation of ROFA (0.2 or 1.0 mg/100 g body weight [bw]) or saline (vehicle control). At day 3, the animals were further divided into 3 groups and intratracheally inoculated with saline, 5×10^3 *L. monocytogenes* (low dose), or 5×10^5 *L. monocytogenes* (high dose) as previously described (Antonini *et al.*, 2000). At days 6, 8, and 10, bronchoalveolar lavage was performed on the right lungs. The cells recovered were differentiated, and chemiluminescence (CL), nitric oxide (NO) production, and phagocytosis were measured as indices of macrophage function. From the same animals, the left lung and spleen were removed, homogenized, and the number of bacteria colony-forming units (CFUs) was determined.

ROFA treatment. ROFA particles were suspended in sterile saline and sonicated for 1 min with a Sonifier 450 Cell Disruptor (Branson Ultrasonics, Danbury, CT). Rats 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 0.2 or 1.0 mg/100 g bw of ROFA in 300 μl of saline, according to the method of Brain *et al.* (1976). The ROFA concentrations were chosen after a pilot study using this particular ROFA sample to include a dose (1.0 mg/100 g) that induces lung inflammation as well as a dose (0.2 mg/100 g) that does not (Antonini *et al.*, 2002). In addition, the intratracheal ROFA concentrations used in this study fell within the range of concentrations consistently used in other animal studies evaluating the pulmonary responses to ROFA (Pritchard *et al.*, 1996; Dreher *et al.*, 1997; Gavett *et al.*, 1997; Kodavanti *et al.*, 1998). Animals in the vehicle control group were intratracheally dosed with 300 μl of sterile saline.

Intratracheal bacteria inoculation. *L. 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 days post-ROFA/saline instillation, the rats were inoculated intratracheally with either a low (5×10^3) or high (5×10^5) dose of *L. monocytogenes* in 500 μl of sterile saline, according to the instillation method described in the previous section. These two doses were found, in a previous pilot study, to give a uniform infection and not to kill untreated naive Sprague-Dawley rats (Antonini *et al.*, 2001a). The intratracheal instillation of 5×10^3 *L. monocytogenes* was selected because it did not elicit an inflammatory response in the lungs, whereas the higher 5×10^5 *L. monocytogenes* was observed to induce a significant influx of neutrophils into lungs soon after instillation.

Morbidity/histopathology. Animal weights and morbidity were monitored over the course of the treatment period. Histopathological analysis was performed on the lungs of rats from each group. Rats were euthanized with sodium pentobarbital, and the lungs were preserved with 10% buffered formalin by airway fixation at total lung capacity. The lobes of the lungs were removed, sectioned, embedded in paraffin, and stained with hematoxylin and eosin. Histopathological analysis was performed by Dr. Val Vallyathan (NIOSH, Morgantown, WV) who was unaware of the experimental design and blinded to the treatment groups of the study.

Bronchoalveolar lavage. At days 6, 8, or 10, 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, as previously described (Antonini *et al.*, 2000).

Pulmonary clearance of *L. monocytogenes*. At days 6, 8, and 10, the left lungs and spleens 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 number of viable CFUs were counted after an overnight incubation at 37°C.

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), and 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 4 ml of a lavage sample, albumin content, a measure to quantify increased permeability of the bronchoalveolar-capillary barrier and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were measured. Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromocresol 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. LDH enzyme reagents were purchased from Roche Diagnostics Systems (Indianapolis, IN).

Luminol-dependent chemiluminescence. Luminol-dependent chemiluminescence (CL), a measure of light generation representing reactive oxidant species (ROS) production, was performed with an automated Berthold Autolumat LB 953 luminometer (Wallace, Inc., Gaithersburg, MD) as described previously (Antonini *et al.*, 1994). Non-opsonized, insoluble zymosan (2 mg/ml; Sigma Chemical Co., St. Louis, MO) was used as a stimulant and was added to the assay immediately prior to measurement of CL. Rat neutrophils have not been shown to respond to unopsonized zymosan in our system, therefore, the zymosan-stimulated CL produced is from AMs. Measurement of CL was recorded for 15 min at 37°C, and the integral of counts per min (cpm) versus time was calculated. CL was calculated as the cpm of stimulated cells minus the cpm of the corresponding resting cells. CL generated from non-

stimulated AMs was not significantly different among the different treatment groups.

Alveolar macrophage nitric oxide production. BAL cells were suspended at a concentration of 1×10^6 cells/ml in essential minimum eagle medium (EMEM, BioWhittaker, Walkersville, MD) supplemented with 2 mM glutamine, 100 g/ml streptomycin, 100 units/ml penicillin and 10% heat-activated fetal calf serum, and seeded onto each well of a 24-well tissue culture plate. BAL cells were allowed to adhere to the plates for 2 h at 37°C at 5% CO₂. After the incubation, non-adherent cells were removed by washing 3 times with EMEM medium. The adherent cells, which were found to be >90% AMs, were then incubated in fresh EMEM for 18 h at 37°C at 5% CO₂. The AM-conditioned media were collected, centrifuged, and stored at -70°C until analysis. The production of nitric oxide was determined as an accumulation of nitrite by a modified microplate assay using the Griess reagent (Green, 1982). Briefly, the samples were incubated with an equal volume of the Griess reagent at room temperature for 10 min. The absorbance at 550 nm was determined with a microplate spectrophotometer reader (SpectraMax 250, Molecular Devices Co., Sunnyvale, CA). Sodium nitrite (Sigma Chemical Co., St. Louis, MO) was used as a standard. The results were expressed as nmol nitrite/ 10^6 AMs.

Alveolar macrophage phagocytosis/bactericidal killing. AM phagocytosis and bacterial killing was determined by a method modified from Ohya *et al.* (1998). AMs were recovered by BAL from rats 3 days after the intratracheal instillation of ROFA (1 mg/100 g bw) or saline. The recovered AMs from each animal were allowed to attach in 24-well plates for 3 h at 37°C in RPMI-1640 media (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum at a concentration of 5×10^5 cells/well. After the incubation period, the wells were washed 4 times with media to remove non-adherent cells. The adherent cells (found to be >90% AMs) were treated with 1×10^7 *L. monocytogenes* at 37°C for 1.5 h. After this second incubation, one set of AMs from each animal was washed 6 times with media and lysed in distilled H₂O while sonicating with a Sonifier 450 Cell Disruptor. This first lysate was diluted, cultured overnight at 37°C, and viable CFUs were counted to determine AM phagocytosis. For the remaining AMs from each animal, the cells were incubated for an additional 4 and 18 h at 37°C. The AMs were continually washed every 2 h during the incubation period with media containing 10 µg/ml of chloramphenicol (Sigma Chemical Co., St. Louis, MO) to kill any extracellular bacteria that had not been taken up by the AMs after the first incubation. After the incubations, the AMs were lysed, diluted, cultured overnight at 37°C, and the number of viable CFUs counted. The number of bacteria killed by the AMs was determined by subtracting the number of CFUs of the lysates at 4 and 18 h from the number of CFUs from the first lysate at 1.5 h.

AMs from all treatments at each time point were allowed to adhere to glass cover slips and imaged using a Sarastro 2000 laser scanning confocal microscope (Molecular Dynamics, Inc., Sunnyvale, CA). Bacteria were immunolabeled using rabbit anti-*L. monocytogenes* primary IgG antibody (Biodesign International, Saco, ME) and FITC-labeled goat anti-rabbit secondary IgG antibody (Sigma Chemical Co., St. Louis, MO).

Statistical analysis. Results are expressed as means $\pm$ 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 $p < 0.05$.

RESULTS

Pulmonary clearance of *L. monocytogenes*. ROFA high- and low-dose groups slowed the clearance of *L. monocytogenes* from the lungs at both bacteria doses (Fig. 1). A significant

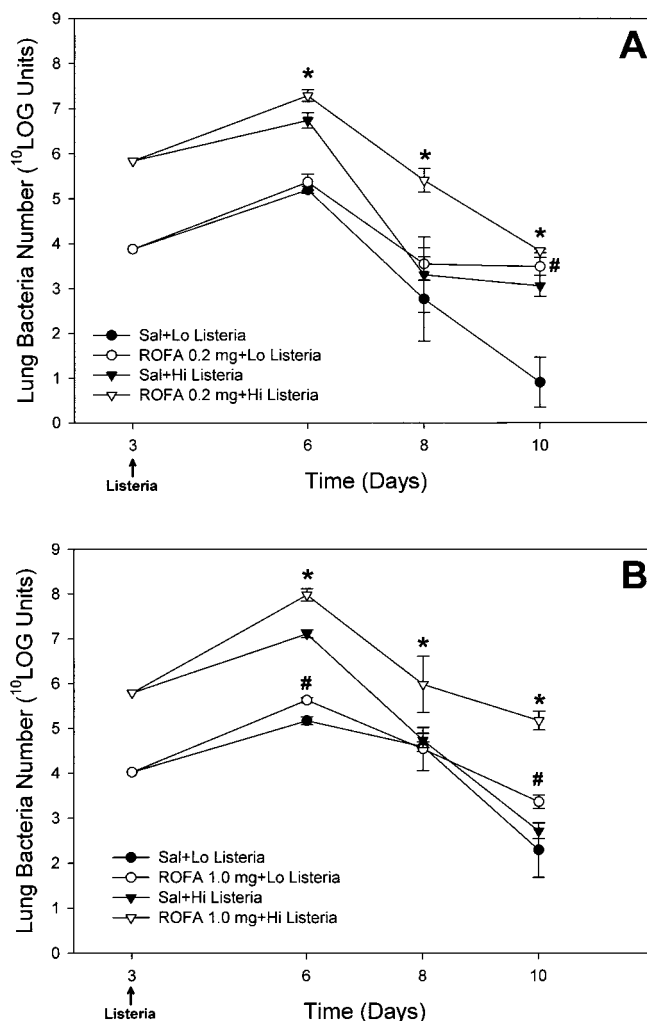


FIG. 1. Number of bacteria CFUs in the left lung of rats preexposed to (A) 0.2 mg or (B) 1.0 mg/100 g bw of ROFA by intratracheal instillation 3 days prior to intratracheal inoculation with *L. monocytogenes* (Lo, 5×10^3 bacteria; Hi, 5×10^5 bacteria). Values are means in Log₁₀ base units $\pm$ SE ($n = 5-10$); *, ROFA + Hi Listeria group is significantly greater than Sal + Hi Listeria group at each time point; #, ROFA + Lo Listeria group is significantly greater than Sal + Lo Listeria group at each time point ($p < 0.05$).

icant elevation in pulmonary bacterial CFUs was observed at day 10 for the group preexposed to the low-dose ROFA before treatment with the low dose of bacteria, whereas significant increases in bacterial CFUs were measured at all time points after treatment with the high dose of *L. monocytogenes* (Fig. 1A).

For the lower dose of *L. monocytogenes*, there were significantly more lung bacterial CFUs counted for the high dose ROFA group at days 6 and 10 as compared to the saline + low dose *L. monocytogenes* group (Fig. 1B). For the higher dose of *L. monocytogenes*, there were significantly more lung bacterial CFUs measured for the high-dose-ROFA group at all three time points as compared to the saline + high dose *L. monocytogenes* group. At day 10, highly significant increases in bacteria number of 288- and 12-fold were observed for the

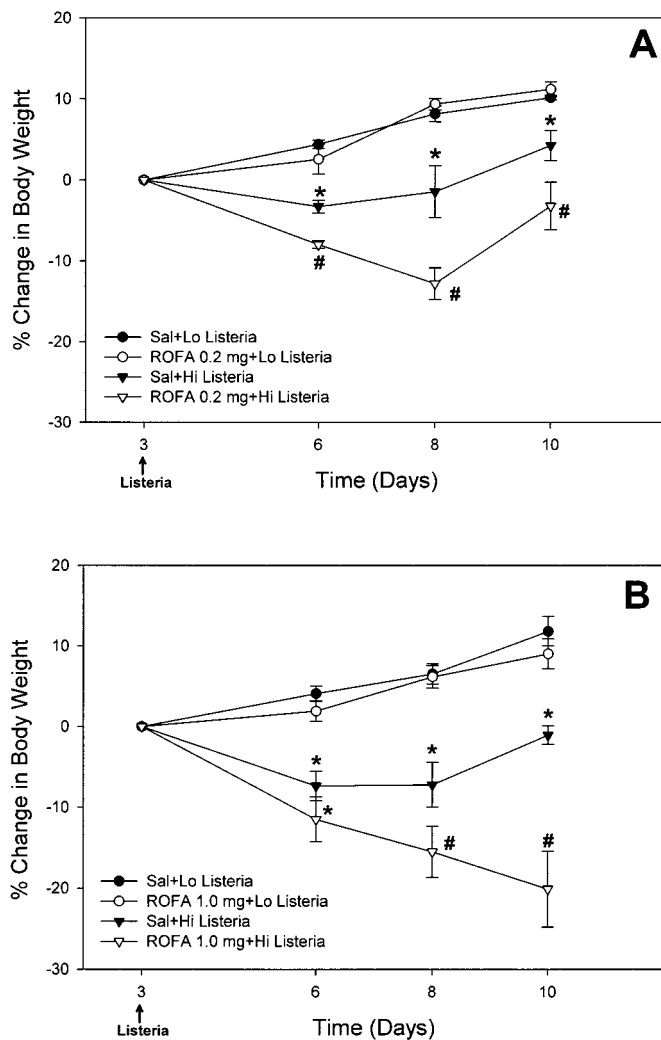


FIG. 2. Percent change in bw of rats preexposed to (A) 0.2 mg and (B) 1.0 mg/100 g bw of ROFA by intratracheal instillation 3 days prior to intratracheal inoculation with *L. monocytogenes* (Lo, 5×10^3 bacteria; Hi, 5×10^5 bacteria). Values are means $\pm$ standard error (SE; $n = 9-16$); *, significantly different from saline + Lo Listeria and ROFA + Lo Listeria groups at each time point; #, significantly different from all other groups at each time point ($p < 0.05$).

ROFA high and low *L. monocytogenes* groups as compared to their respective saline control groups.

Body weight/survival. The percent change in rat bw after the intratracheal inoculation of *L. monocytogenes* was assessed (Fig. 2). A significant loss in bw was observed for both the high- and low-dose ROFA groups instilled with the high dose of *L. monocytogenes* (Figs. 2A and 2B). The percent change in bw for the high dose ROFA + high dose *L. monocytogenes* group continued to decline (Fig. 2B), whereas the percent change in bw for rats of all other groups had begun to rise by 10 days (Figs. 2A and 2B). Without infection, the two doses of ROFA caused no change in bw.

All the rats from each of the treatment groups, except for the

high-dose ROFA + high-dose *L. monocytogenes* group, survived for the duration of the experiment period (data not shown). One rat from the high-dose ROFA + high-dose *L. monocytogenes* group expired within 24 h after intratracheal inoculation of the bacteria. By day 7, 30% of the rats from this group had died.

Histopathology. Histopathological analyses were performed on the lungs of each treatment group at day 6 (Fig. 3). Lungs appeared normal for the saline + low dose *L. monocytogenes* group (Fig. 3A). A mild pneumonitis, characterized by a peribronchiolar accumulation of neutrophils, and some particle accumulation were observed in the terminal bronchiolar regions of the lungs in the high dose ROFA + low dose *L. monocytogenes* group (Fig. 3C). At a higher magnification, particle-containing macrophages were observed throughout the alveolar region for the high dose ROFA + low dose *L. monocytogenes* group (Fig. 3E, arrowheads). For the saline or high dose ROFA groups treated with the high dose of *L. monocytogenes*, edema, inflammation with significant infiltration of neutrophils, and many granulomatous lesions with amorphous tissue debris were observed (Figs. 3B and 3D). The lesions that were observed in the high-dose ROFA + high-dose *L. monocytogenes* were much more extensive and significantly more pronounced than those observed in the saline + high-dose *L. monocytogenes*. Several areas of particle accumulation were observed in the lungs of the high-dose ROFA + high-dose *L. monocytogenes* group (Figs. 3D and 3F, arrows).

Bronchoalveolar lavage fluid injury and inflammation parameters. Two indicators of lung damage (albumin content and LDH activity) were assessed within the recovered BAL fluid (Table 1). At day 3, ROFA treatment (1.0 mg/100 g bw) caused significant elevations in albumin and LDH as compared to its corresponding saline control. In the animals that received a second instillation of saline on day 3, high-dose ROFA pretreatment caused a significant increase in albumin at day 6 and in LDH at all three time points, as compared to the saline + saline group. Intratracheal inoculation of the low dose of *L. monocytogenes* to rats pretreated with saline had no effect on either of the BAL indices at any of the time points post-bacterial treatment to the saline + saline control. Pulmonary instillation of the low dose of *L. monocytogenes* to ROFA-pretreated rats caused significant elevations in both parameters at various time points as compared to the saline + saline group. By day 10, only the LDH values were elevated in the ROFA-pretreated animals.

Intratracheal inoculation of the high dose of *L. monocytogenes* to rats pretreated with saline caused significant increases in both parameters as compared to the saline + saline control at days 6 and 8. A significant elevation in LDH was observed at day 10. Both indices of lung damage were elevated in the rats treated with high-dose ROFA and the high dose of *L. monocytogenes* as compared to the saline + saline group at all three time points. At day 10, LDH was significantly elevated in

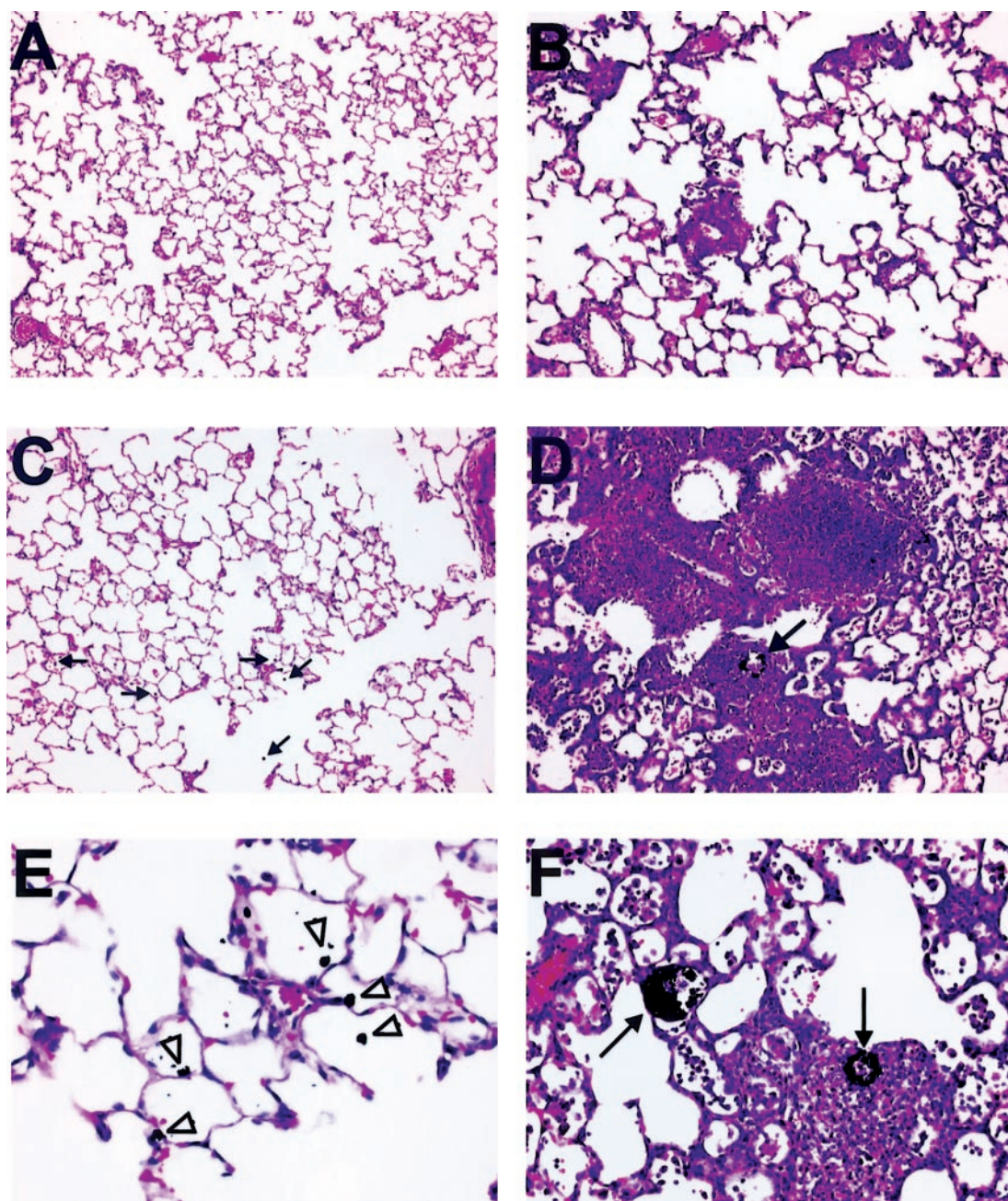


FIG. 3. Micrographs of rat lungs stained with hematoxylin and eosin at day 6 after exposure to (A) saline + Lo *L. monocytogenes*, original magnification is $\times 10$; (B) saline + Hi *L. monocytogenes*, $\times 10$; (C) ROFA 1.0 mg/100 g bw + Lo *L. monocytogenes*, $\times 10$; (D) ROFA 1.0 mg/100 g bw + Hi *L. monocytogenes*, $\times 10$; (E) ROFA 1.0 mg/100 g bw + Lo *L. monocytogenes*, $\times 40$; and (F) ROFA 1.0 mg/100 g bw + Hi *L. monocytogenes*, $\times 20$. Areas of particle accumulation were observed in the lungs of the ROFA groups (E, arrowheads; C, D, and F, arrows).

the high-dose ROFA + high dose *L. monocytogenes* group when compared with the saline + high dose *L. monocytogenes* group.

In the assessment of the cellular response, the number of AMs and neutrophils was determined after BAL (Fig. 4). No significant differences were observed among any of the groups in the number of AMs recovered at days 3, 6, and 8 (Fig. 4A). By day 10, significantly more AMs were recovered from the

ROFA and saline groups treated with the high dose of *L. monocytogenes* as compared to the groups that were not treated with the bacteria. A significant elevation in neutrophil number was observed for the high-dose ROFA + saline group as compared to its respective saline control at each time point (Fig. 4B). The two groups that were inoculated with the high dose of bacteria had significant increases in neutrophil number as compared to the non-bacteria groups. At day 10, the high

TABLE 1
Bronchoalveolar Lavage Fluid Injury Parameters

Time points	Treatments	Albumin (mg/ml)	LDH (U/L)
Day 3	Sal only	0.18 ± 0.02	74.6 ± 11.2
	ROFA only	0.38 ± 0.04*	266 ± 62.5*
Day 6	Sal + Sal	0.14 ± 0.01	69.2 ± 3.10
	ROFA + Sal	0.35 ± 0.11*	181 ± 14.1*
	Sal + Lo Listeria	0.15 ± 0.01	58.8 ± 3.97
	ROFA + Lo Listeria	0.45 ± 0.08 ^{a,*}	249 ± 40.4 ^{a,*}
	Sal + Hi Listeria	0.40 ± 0.04*	224 ± 27.4*
	ROFA + Hi Listeria	0.54 ± 0.07*	437 ± 66.8 ^{b,*}
Day 8	Sal + Sal	0.12 ± 0.01	69.6 ± 4.79
	ROFA + Sal	0.16 ± 0.01	143 ± 14.8*
	Sal + Lo Listeria	0.18 ± 0.03	78.6 ± 4.85
	ROFA + Lo Listeria	0.17 ± 0.02	119 ± 11.2*
	Sal + Hi Listeria	0.45 ± 0.07*	289 ± 21.7*
	ROFA + Hi Listeria	0.68 ± 0.05 ^{b,*}	472 ± 39.0 ^{b,*}
Day 10	Sal + Sal	0.12 ± 0.02	54.0 ± 8.17
	ROFA + Sal	0.12 ± 0.02	97.3 ± 2.95*
	Sal + Lo Listeria	0.11 ± 0.01	55.0 ± 3.18
	ROFA + Lo Listeria	0.16 ± 0.02	91.1 ± 7.91 ^{a,*}
	Sal + Hi Listeria	0.15 ± 0.03	118 ± 7.04*
	ROFA + Hi Listeria	0.24 ± 0.04*	233 ± 41.2 ^{b,*}

Note. Rats preexposed to 1.0 mg/100 g bw of ROFA by intratracheal instillation 3 days prior to intratracheal inoculation with *L. monocytogenes* (Lo, 5×10^3 bacteria; Hi, 5×10^5 bacteria). Values are means ± SE ($n = 5-10$).

^aSal + Lo Listeria and ROFA + Lo Listeria groups are significantly different within each time point.

^bSal + Hi Listeria and ROFA + Hi Listeria groups are significantly different within each time point ($p < 0.05$).

*Significantly different from Sal only or Sal + Sal control groups within each time point.

dose ROFA + high dose *L. monocytogenes* group had a significant elevation in neutrophil number as compared to the saline + high bacteria group, which returned to control levels.

Alveolar macrophage function. The generation of ROS was assessed by measuring the CL of recovered BAL cells (Fig. 5). In the analysis of non-opsonized, zymosan-stimulated CL, a measure of ROS production by AMs, there was no significant difference between the saline + saline and high-dose ROFA + saline groups at any of the time points after instillation of the bacteria (Fig. 5A). The intratracheal inoculation of the high dose of *L. monocytogenes* significantly increased zymosan-stimulated CL at days 6 and 8. Preexposure to high dose ROFA had no effect on the zymosan-stimulated CL after inoculation with *L. monocytogenes*.

The production of NO, another measure of AM function, was determined (Fig. 5B). AM NO production was significantly reduced in the high-dose ROFA + saline group as compared to the saline + saline group at all three time points. The intratracheal inoculation of the high dose of *L. monocytogenes*

genes significantly enhanced AM NO production at each time point after bacteria treatment. However, high dose ROFA preexposure significantly suppressed the bacteria-induced elevation in AM NO production at all three time points post-*L. monocytogenes* inoculation.

The phagocytic and bacterial killing activities of AMs recovered from rats intratracheally instilled with saline or high dose ROFA were compared (Fig. 6; Table 2). A significant increase of 3.7-fold was observed in the uptake of the *L. monocytogenes* at 1.5 h by the AMs recovered from the ROFA group as compared with the saline group. There were 6.5- and 8-fold increases in the number of bacteria present in the AMs of the ROFA animals as compared to the AMs of the saline

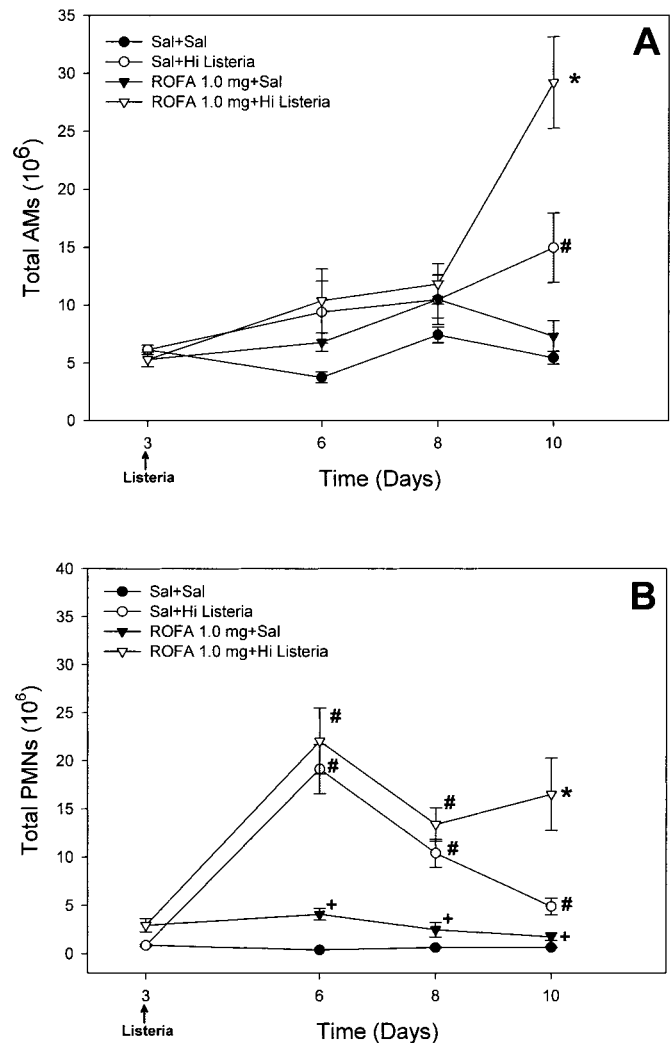


FIG. 4. (A) Alveolar macrophages (AMs) and (B) neutrophils (PMNs) recovered from rats preexposed to ROFA (1.0 mg/100 g bw) by intratracheal instillation 3 days prior to intratracheal inoculation with the high dose 5×10^5 *L. monocytogenes*. Values are means ± SE ($n = 5-10$); *, significantly greater than all groups at each time point; #, significantly greater than Sal + Sal and ROFA + Sal groups at each time point; +, significantly greater than Sal + Sal group at each time point ($p < 0.05$).

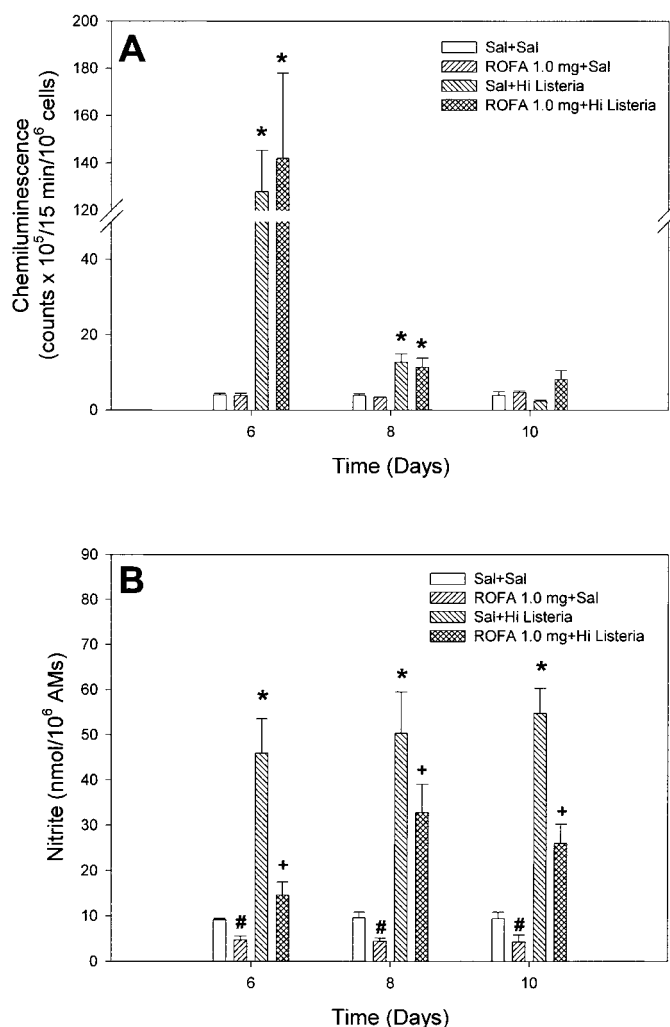


FIG. 5. (A) Zymosan-stimulated CL and (B) NO production by AMs recovered from rats preexposed to ROFA (1.0 mg/100 g bw) by intratracheal instillation 3 days prior to intratracheal inoculation with the high dose 5×10^5 *L. monocytogenes*. Values are means $\pm$ SE ($n = 5-10$); *, significantly greater than Sal + Sal and ROFA + Sal groups at each time point; #, significantly less than Sal + Sal group at each time point; +, significantly less than Sal + Hi Listeria group at each time point ($p < 0.05$).

animals at 4 and 18 h, respectively. These elevations in the numbers of intracellular bacteria for the high dose ROFA group were easily observed microscopically at each time point (Fig. 6). A significant decrease in the percentage of bacteria killed was determined for the AMs collected from the ROFA group at 4 and 18 h as compared to the AMs from the saline group.

DISCUSSION

It has been suggested that inhaled particulate matter may exacerbate preexisting health conditions (Dockery and Pope, 1994) and augment pulmonary infection (Schwartz, 1994).

Animal studies have demonstrated that a significant component of particulate matter, ROFA, is highly toxic to the lungs (Dreher *et al.*, 1997; Kodavanti *et al.*, 1998). ROFA exposure has been shown to affect lung defense responses in laboratory animals (Hatch *et al.*, 1985; Longphre *et al.*, 2000; Pritchard *et al.*, 1996). However, mechanisms for these altered lung responses are not well characterized. The goals of this current investigation were to (1) assess whether preexposure of rats to ROFA before intratracheal inoculation with a bacterial pathogen suppresses non-specific local pulmonary defense function and (2) identify potential mechanisms by which rats preexposed to ROFA may be more susceptible to pulmonary infection.

Intratracheal instillation of ROFA to laboratory animals induces lung injury and inflammation (Dreher *et al.*, 1997; Kodavanti *et al.*, 1998). This pneumotoxic response was shown to be dose-dependent and transient, declining by 96 h after exposure. In our current study, we also observed an acute lung response in rats dosed intratracheally with ROFA that was characterized by neutrophilia and *in vivo* cytotoxicity that subsided over time. In addition, we found that the clearance of the bacteria from the lungs of rats preexposed to ROFA was dramatically slowed after intratracheal inoculation with either a non-inflammatory or a highly inflammatory dose of *L. monocytogenes*. In comparing ROFA treatment without bacteria to ROFA + the low *L. monocytogenes* dose, only slight differences were observed in lung damage with no effect on animal survival. However, a significant loss in bw and a substantial increase in morbidity were observed in the rats preexposed to ROFA and then infected with the high dose of *L. monocytogenes*. Severe edema, a dramatic infiltration of neutrophils, and massive pulmonary infection were observed in the ROFA-exposed rats that had expired. Are the injury and inflammation induced by ROFA then responsible for increasing the susceptibility of the lungs to infection?

In earlier studies, an excess mortality was observed in mice instilled intratracheally with ROFA prior to exposure to aerosolized *Streptococcus* (Hatch *et al.*, 1985; Pritchard *et al.*, 1996). Hatch *et al.* (1985) observed the ROFA particles to be highly cytotoxic to AMs. However, in the assessment of other particulate samples, not all the particles that were highly cytotoxic to AMs caused elevations in bacterial infectivity. Corroborating this work, we have shown previously that the pulmonary clearance of *L. monocytogenes* was actually enhanced in inflamed and fibrotic lungs of animals treated with the highly cytotoxic mineral particle, crystalline silica, thus indicating that significant lung injury may not play a significant role in affecting lung defense after concomitant exposure to particulates and bacteria (Antonini *et al.*, 2000). It was observed instead that acute exposure to high doses of silica enhanced lung defense mechanisms by activating AMs and increasing phagocytosis and the production of reactive oxygen species.

In a related infectivity study, Van Loveren *et al.* (1988) have shown that inhalation of ozone decreases the clearance of *L.*

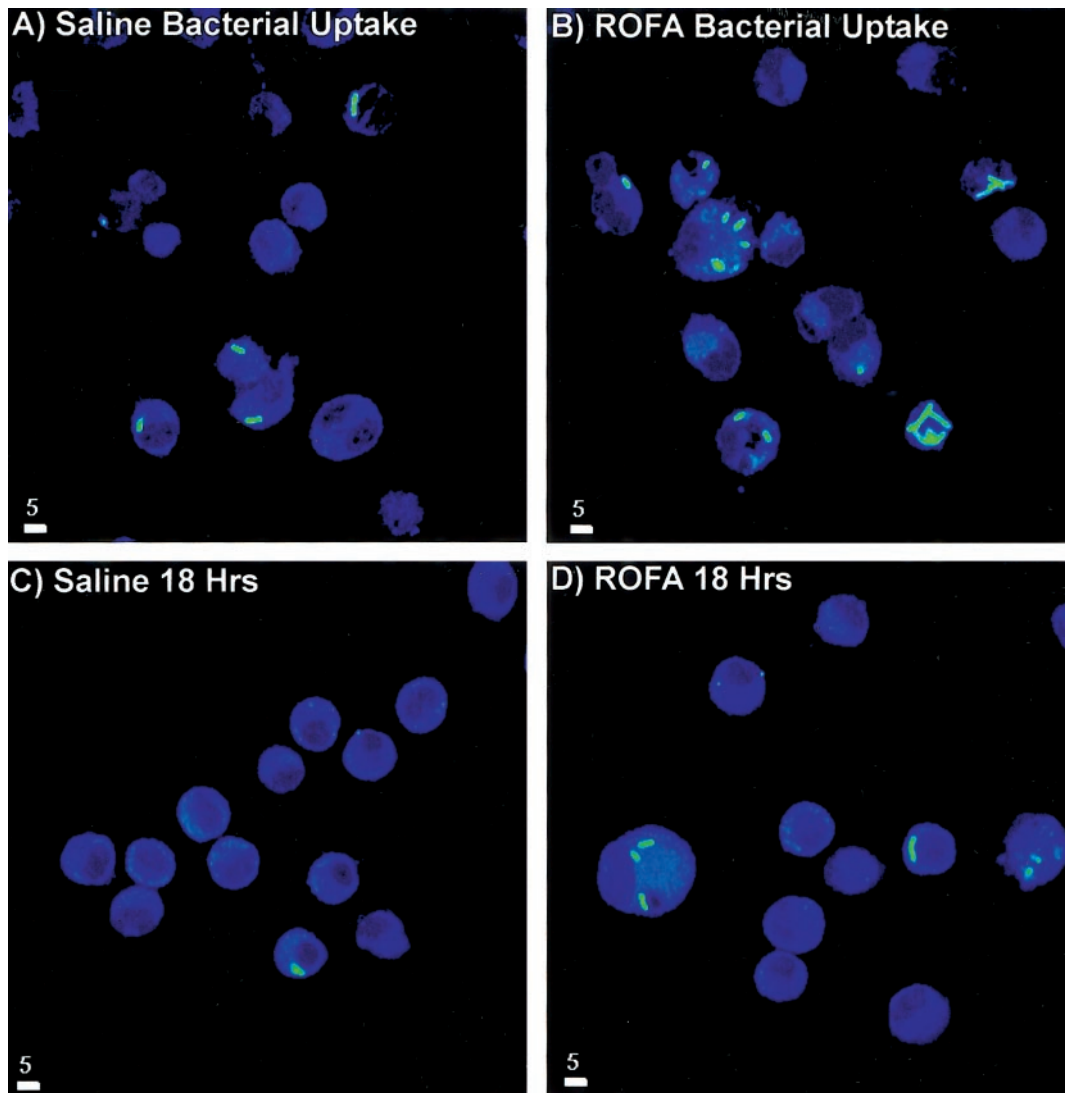


FIG. 6. Confocal micrograph depicting the presence of fluorescent *Listeria monocytogenes* in alveolar macrophages recovered from rats instilled intratracheally with saline or ROFA (1.0 mg/100 g bw) after bacterial uptake at 1.5 h (A and B) and 18 h post-uptake (C and D). Cells are blue; bacteria are green. Bar is 5 μ m; original magnification is $\times 100$.

monocytogenes from the lungs, concluding that this was most likely due to a suppression of AM activity. Ozone exposure was observed to significantly decrease AM phagocytosis of *L. monocytogenes*. Previously, we observed that pretreatment with diesel exhaust particles enhanced the pulmonary infection of *L. monocytogenes* (Yang *et al.*, 2001). This elevation in infection was accompanied by a decrease in the pulmonary clearance of the bacteria likely due to alterations in AM function. Yang *et al.* (1997) have shown the responses of AMs treated with diesel exhaust particles to be suppressed after stimulation with lipopolysaccharide, likely increasing the susceptibility of the lungs to infection after diesel exposure. In addition, susceptible populations may be at increased risk for lung infections. We have shown that advanced age is associated with decrements in lung defense and altered AM function

in rats after *L. monocytogenes* exposure (Antonini *et al.*, 2001b).

Interestingly, ROFA pretreatment had no effect on the generation of reactive oxygen species as measured by CL before and after infection with *L. monocytogenes*. However, CL is a non-specific measure of the light emitted from activated phagocytic cells that accompanies the release of various forms of reactive oxidant species. In studying the modulator effect of ozone on the pulmonary clearance of *L. monocytogenes*, Cohen *et al.* (2001) observed specifically that hydrogen peroxide production by AMs was significantly reduced after ozone exposure. North *et al.* (1997) indicated that reactive nitrogen intermediates play an important role, along with peroxides, in the intracellular killing of *L. monocytogenes*. NO, a highly reactive nitrogen intermediate, which has been shown to play

TABLE 2
Bacteria Uptake and Killing by Macrophages

Time	Group	Bacteria no. (CFUs $\times 10^4$)	Bacteria killed (%)
Bacteria uptake after 1.5 h	Saline	1.94 $\pm$ 0.41	—
	ROFA 1.0 mg	7.21 $\pm$ 0.74*	—
4 h post-bacteria uptake	Saline	0.33 $\pm$ 0.08	81.2 $\pm$ 3.10
	ROFA 1.0 mg	2.15 $\pm$ 0.02*	68.9 $\pm$ 2.34*
18 h post-bacteria uptake	Saline	0.12 $\pm$ 0.03	91.5 $\pm$ 1.93
	ROFA 1.0 mg	0.96 $\pm$ 0.10*	86.4 $\pm$ 1.66*

Note. Values are means $\pm$ SE ($n = 18$).

*Significantly different from saline group ($p < 0.05$).

an important role in AM-mediated defense against infections, was measured specifically in our current study.

The production of NO was significantly suppressed in AMs recovered from non-infected and infected animals that were preexposed to ROFA. NO has been shown to promote the cytotoxic activities of AMs and modulate cell-mediated immunity (Lyons, 1995; Nathan and Xie, 1994). NO combines with the reactive oxygen intermediate, superoxide anion, to form the highly reactive substance, peroxynitrite (Pryor and Squadrito, 1995). Hickman-Davis *et al.* (1999) have demonstrated that peroxynitrite generation by AMs is an important mechanism in the killing of bacterial pathogens in the lungs. In addition, we have shown that AMs from aged rats produced less NO than AMs from young adult rats, this increasing the older rats' susceptibility to infection (Antonini *et al.*, 2001b).

In our current study, a significant suppression in the percentage of bacteria killed by AMs recovered from ROFA-treated animals was observed, even though AM phagocytosis was increased. The suppression in pulmonary clearance of the bacteria that we observed might possibly be explained by the decrease in NO production by AMs recovered from rats exposed to ROFA before the *L. monocytogenes* challenge. Ohya *et al.* (1998) have indicated that reactive oxygen species play a significant role in Listericidal activity only if AMs are activated prior to infection. If the AMs ability to respond to bacteria challenge is suppressed, as we observed with ROFA, reactive nitrogen intermediates such as NO may be involved in the intracellular macrophage killing.

Additional *in vitro* AM studies utilizing antioxidants, inhibitors of reactive oxygen and reactive nitrogen intermediates, and other components of the lung defense system such as surfactant protein A are ongoing in our laboratory to further evaluate the mechanisms by which ROFA may increase the susceptibility to lung infection. Also, numerous animal studies have suggested that the metal components of ROFA are the major determinants of their potential to induce pulmonary injury and inflammation (Gavett *et al.*, 1997; Kodavanti *et al.*, 1998). Dreher *et al.* (1997) have provided evidence for the toxic role of soluble metals in acute pulmonary injury induced by ROFA. Goldsmith *et al.* (1998) demonstrated that the water-

soluble components of ROFA alter AM function by significantly increasing their respiratory burst. Thus, additional *in vivo* studies are needed to assess what components of ROFA may potentially alter defense mechanisms of the lungs. Currently, rat infectivity studies are being conducted in our laboratory, evaluating the role by which the soluble metals of ROFA may alter lung infection susceptibility. Preliminary results indicate that the soluble fraction of ROFA increases mortality and slows the pulmonary clearance of *L. monocytogenes* (Roberts *et al.*, 2001).

In summary, the present study demonstrated that rats preexposed to ROFA have altered lung defenses, making them more susceptible to lung injury, inflammation, and infection after bacterial challenge. In ROFA-treated animals, we observed a dramatic alteration in the pulmonary clearance of a bacterial pathogen along with a reduction in NO production by AMs. Thus, exposure to ROFA in the workplace or from air pollution may suppress the lung's ability to defend against infection.

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