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SOLUBLE METALS ASSOCIATED WITH RESIDUAL OIL FLY ASH INCREASE MORBIDITY AND LUNG INJURY AFTER BACTERIAL INFECTION IN RATS

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Inhalation of residual oil fly ash (ROFA) has been shown to impair lung defense mechanisms in laboratory animals and susceptible populations. Bioavailability of soluble transition metals has been shown to play a key role in lung injury caused by ROFA exposure. The goal of this study was to evaluate the effect of soluble metals on lung defense and injury in animals preexposed to ROFA followed by pulmonary challenge with a bacterial pathogen. ROFA was suspended in saline (ROFA-TOTAL), incubated overnight at 37 °C, and separated by centrifugation into soluble (ROFA-SOL) and insoluble (ROFA-INSOL) fractions. A portion of the soluble sample was treated with the metal-binding resin Chelex for 24 h at 37 °C. Sprague-Dawley rats were intratracheally dosed at d 0 with ROFA-TOTAL (1.0 mg/100 g body weight), ROFA-INSOL, ROFA-SOL, saline, saline +Chelex, or ROFA-SOL +Chelex. At d 3, 5×10^5 Listeria monocytogenes were intratracheally instilled into rats from each treatment group. At d 6, 8, and 10, left lungs were removed, homogenized, and cultured to assess bacterial clearance. Histopathological analysis was performed on the right lungs. Pulmonary exposure of ROFA-TOTAL or ROFA-SOL before infection led to a marked increase in lung injury and inflammation at all three time points after inoculation, and an increase in morbidity in comparison to saline control rats. Treatment with ROFA-INSOL, saline +Chelex, or ROFA-SOL +Chelex caused no significant increases in lung damage and morbidity when compared to control. By d 10, the ROFA-SOL and ROFA-TOTAL groups had approximately 200-fold more bacteria in the lung than saline control, indicating the inability of these groups to effectively respond to the infection. None of the other treatment groups had significant impairments in bacterial clearance when compared to saline. In conclusion, exposure to ROFA-TOTAL and ROFA-SOL significantly suppressed the lung response to infection. These results suggest that soluble metals present in ROFA may play a key role in increased susceptibility to pulmonary infection in exposed populations.

Epidemiology studies have demonstrated a positive correlation between increased levels of particulate air pollution and elevated morbidity and mortality

in susceptible human populations (Dockery & Pope, 1994; Pope et al., 1995; Schwartz, 1994). Occupational exposure to ROFA has also been associated with adverse respiratory health effects in humans (Hauser et al., 1995a, 1995b; Levy et al., 1984). Increased infectivity and susceptibility to infection have been correlated with high levels of outdoor air pollutants (reviewed in American Thoracic Society, 1996a, 1996b) as well. Upper and lower respiratory-tract infections that interfere with normal activity have been observed after episodes of elevated air pollution levels. One such particulate pollutant, residual oil fly ash (ROFA), is released into the environment from the combustion of fossil fuels and contributes approximately 2.5×10^5 tons to the ambient air particulate matter burden in the United States annually (Costa & Dreher, 1997). ROFA is a complex mixture of metals, sulfates, acids, fuel contaminants, and other unknown components combined with an insoluble, particulate carbon core (Fisher et al., 1983; reviewed in Ghio et al., 2002).

Animal studies have shown that inhalation of ROFA (Antonini et al., 2002; Hatch et al., 1985; Pritchard et al., 1996) and ambient air particulates (Zelikoff et al., 2002) increases susceptibility to infection. ROFA has been shown to decrease bactericidal activity of alveolar macrophages (Antonini et al., 2002). In vivo studies have also demonstrated that the bioavailability of soluble transition metals in ROFA may be a potential mechanism of lung injury (Dreher et al., 1997; Gavett et al., 1997; Lewis et al., 2003). Soluble metals associated with ROFA have been shown to induce protein leakage and increase the influx of neutrophils and macrophages into the lungs (Kodavanti et al., 1998).

The objective of this study was to determine what component of ROFA may affect the susceptibility to pulmonary infection. We hypothesized that soluble metals associated with ROFA will suppress lung defense responses and slow clearance of bacteria from the lungs. In this study, rats were preexposed to ROFA by intratracheal instillation. In addition, the ROFA sample was separated and rats were intratracheally treated with the water-soluble or -insoluble fractions. Following exposure to the ROFA samples, rats were intratracheally inoculated with *Listeria monocytogenes*. Animal morbidity, lung injury, and pulmonary clearance of *L. monocytogenes* after ROFA exposure were assessed. To determine the effects of the soluble metals on these parameters, metals were then removed from the soluble sample with a chelating agent, Chelex, and a group of animals was exposed to the chelated soluble sample of ROFA, followed by bacterial inocluation.

METHODS

Animals

Male Sprague-Dawley [Hla:(SD)CVF] rats (Hilltop Laboratories, Scottdale, PA) weighing 250–300 g, approximately 10 wk old, were used for all experiments. They were given the ProLab 3500 diet and tap water ad libitum, housed in a clean air and viral- and antigen-free room with restricted access in an AAALAC-

approved animal facility, and allowed to acclimate for 1 wk before use. The rats were monitored and found to be free of endogenous viral pathogens, parasites, mycoplasms, *Helicobacter*, and CAR bacillus.

Materials

Listeria monocytogenes (strain 10403S, serotype 1) was obtained as a gift from Dr. 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 number 4, Everett, MA. The chelating resin, Chelex 100 (iminodiacetic acid), was purchased from Sigma-Aldrich Co., St. Louis, MO.

Experimental Design

At d 0, animals were preexposed to ROFA samples or saline (vehicle control) by intratracheal instillation. At d 3, the animals were inoculated with 5×10^5 *monocytogenes.* At d 6, 8, and 10, the left lungs of animals were removed, homogenized, and the colony forming units (CFUs) were counted. The right lungs of the same animals were fixed in formalin for histopathological analysis.

ROFA Characterization

Particle size of the ROFA sample was determined by scanning electron microscopy (JSM-5600 SEM, JEOL Ltd., Peabody, MA) and previously characterized (Antonini et al., 2002). ROFA particles were of respirable size with a count mean diameter of 2.2 µm. The metal constituents of the ROFA samples were analyzed using inductively coupled argon plasma, atomic-emission spectroscopy (NIOSH, 1994).

ROFA Treatment

The ROFA sample (ROFA-TOTAL) was suspended in sterile saline (6 mg/ml), sonicated for 1 min with a Sonifier 450 cell disruptor (Branson Ultrasonics, Danbury, CT), and allowed to shake and incubate for 24 h at 37°C. The sample was further divided into soluble and insoluble components by centrifugation at $12,000 \times g$ for 30 min. The supernatant of the sample was recovered and filtered (ROFA-SOL). The pellet was resuspended in saline (ROFA-INSOL). Chelex was added to the ROFA-SOL sample or to saline (20 mg Chelex/0.1 mg ROFA), incubated on a rotor overnight, centrifuged, and the chelated supernatant was recovered (ROFA-SOL +Chelex or saline +Chelex).

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 1.0 mg/100 g body weight of ROFA in 300 µl of saline, according to the method of Brain et al. (1976). In addition, ROFA-SOL, ROFA-INSOL, ROFA-SOL +Chelex, or saline +Chelex (Chelex control) was administered by intratracheal instillation using quantities equivalent to those in the ROFA-TOTAL instillate. Animals in the vehicle control group were intratracheally dosed with 300 µl sterile saline. The ROFA dose chosen was previously

shown to induce inflammation (Antonini et al., 2002), and 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).

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 bacterial concentration was determined spectrophotometrically at an optical density of 600 nm. The sample was diluted with the sterile saline to the concentration of 5×10^5 *monocytogenes* in 500 μl sterile saline. *Listeria monocytogenes* was intratracheally instilled 3 d post-ROFA/saline instillation. The dose of *L. monocytogenes* chosen was found to give a uniform infection and did not result in mortality in untreated naive Sprague-Dawley rats in a previous study (Antonini et al., 2001).

Morbidity/Histolopathology

Animal weights were monitored over the course of the treatment period as an indicator of morbidity. Histology was performed on the right lungs of the rats from each treatment group. Rats were euthanized with an overdose of sodium pentobarbital, the left bronchus was clamped off, and the right lungs were preserved with 10% neutral buffered formalin by airway fixation at 30 cm water pressure. The right lobes of the lungs were removed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Stained tissue sections were imaged with a SprintScan 35 Plus slide scanner (Polaroid, Waltham, MA).

Pulmonary Clearance of *L. monocytogenes*

At d 6, 8, and 10, the left lungs were removed from all the rats in each treatment group. The excised tissues were suspended in 10 ml sterile water, homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA), and cultured quantitatively on brain–heart infusion agar plates (Becton Dickinson and Co., Cockeysville, MD). The viable CFUs were counted after an overnight incubation at 37°C.

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 different treatment groups for the different parameters at each time point was assessed using analysis of variance (ANOVA). The significance of difference between individual groups was analyzed using the Tukey–Kramer post hoc test with the criterion of significance set at $p < .05$.

RESULTS

ROFA Characterization

Elemental analysis was performed on the ROFA-TOTAL, ROFA-INSOL, ROFA-SOL, and ROFA-SOL +Chelex samples to determine their compositions (Table 1). Significant amounts of Fe, Al, V, Ni, and Ca, as well as a small amount of Zn, were present in the ROFA-TOTAL sample. ROFA-TOTAL was separated into soluble and insoluble fractions. The insoluble fraction was found to consist primarily of Fe, V, and Al, whereas the soluble fraction contained significant amounts of Ni, Al, Ca, Fe, and Zn. Chelation of ROFA-SOL with Chelex resulted in removal of the majority of soluble metals with the exception of a small amount of Fe and V.

Pulmonary Infection: ROFA Fractions

Survival of animals treated with *L. monocytogenes* 3 d after pretreatment with ROFA-TOTAL, ROFA-INSOL, ROFA-SOL, or saline is shown in Figure 1. There was no significant decrease in survival of infected rats administered saline or ROFA-INSOL. An unexpected significant decrease in survival was observed in rats administered ROFA-TOTAL or ROFA-SOL, with approximately 60% and 20% of animals surviving in each group, respectively. The greatest decline in survival occurred between d 7 and 8 post-ROFA exposure.

ROFA-TOTAL and ROFA-SOL slowed the clearance of bacteria from the lungs (Figure 2). Animals treated with ROFA-INSOL and saline were able to clear the majority of bacteria from the lungs by d 10, whereas animals preexposed to ROFA-TOTAL or ROFA-SOL had a significantly greater bacterial lung burden at all three time points postinfection compared to all other groups. Bacterial lung burden for ROFA-TOTAL and ROFA-SOL was approximately 200-fold greater than for saline and ROFA-INSOL on d 10. Numbers of bacterial CFUs in the lungs of the ROFA-TOTAL and ROFA-SOL reported in Figure 2 represent the fraction of animals that survived to those time points. Due to this unexpected decrease in survival, the bacterial lung burdens reported at d 8 and 10 likely underestimate the extent of the infection in those groups.

TABLE 1. Element Mass of ROFA (µg/amount of instillate: 2 mg)

	Total	Insoluble	Soluble	Soluble +Chelex
Fe	244	186	37.2	6.76
Al	121	64.1	46.6	Not detected
V	92.0	83.1	1.17	0.574
Ni	76.9	11.0	55.7	Not detected
Ca	61.1	7.16	45.1	Not detected
Zn	10.7	1.13	8.69	Not detected

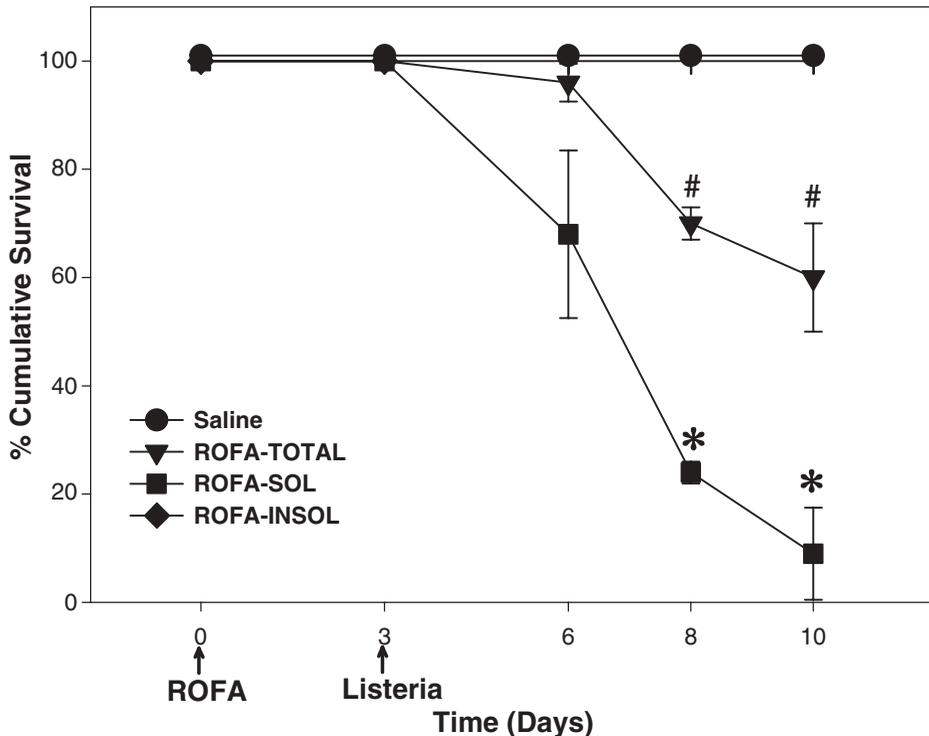*Note.* Trace elements: Ba, Cd, Co, Cr, Cu, Mn, Pb.

FIGURE 1. Survival rate of rats that were pre-exposed to ROFA-TOTAL, ROFA-SOL, or ROFA-INSOL, or saline by intratracheal instillation 3 d prior to intratracheal inoculation with 5×10^5 *L. monocytogenes*. Values are means ±SE; asterisk indicates significantly different from all other groups ($p < 0.05$); #, significantly different from ROFA-SOL, ROFA-INSOL, and saline groups ($p < .05$).

Pulmonary Infection: Metal Chelation

Survival of animals inoculated with *L. monocytogenes* 3 d after treatment with ROFA-SOL, ROFA-SOL +Chelex, saline +Chelex, or saline is shown in Figure 3. There was no significant decrease in survival of rats administered saline, saline +Chelex, or ROFA-SOL +Chelex. A significant decrease in survival was observed in rats administered ROFA-SOL, with approximately 50% of the rats surviving. Again, the greatest decline in survival occurred between d 7 and 8 post-ROFA-SOL exposure.

ROFA-SOL slowed the clearance of bacteria from the lungs (Figure 4). Animals treated with saline, saline +Chelex, or ROFA-SOL +Chelex were able to clear the majority of bacteria from the lungs by d 10 and did not differ significantly from each other at any time point postinfection. Animals preexposed to ROFA-SOL had a significantly greater bacterial lung burden at d 6, 8, and 10 compared to the saline control. Numbers of bacterial CFUs in the lungs of animals pretreated with ROFA-SOL reported represent the fraction of animals that survived to that time point.
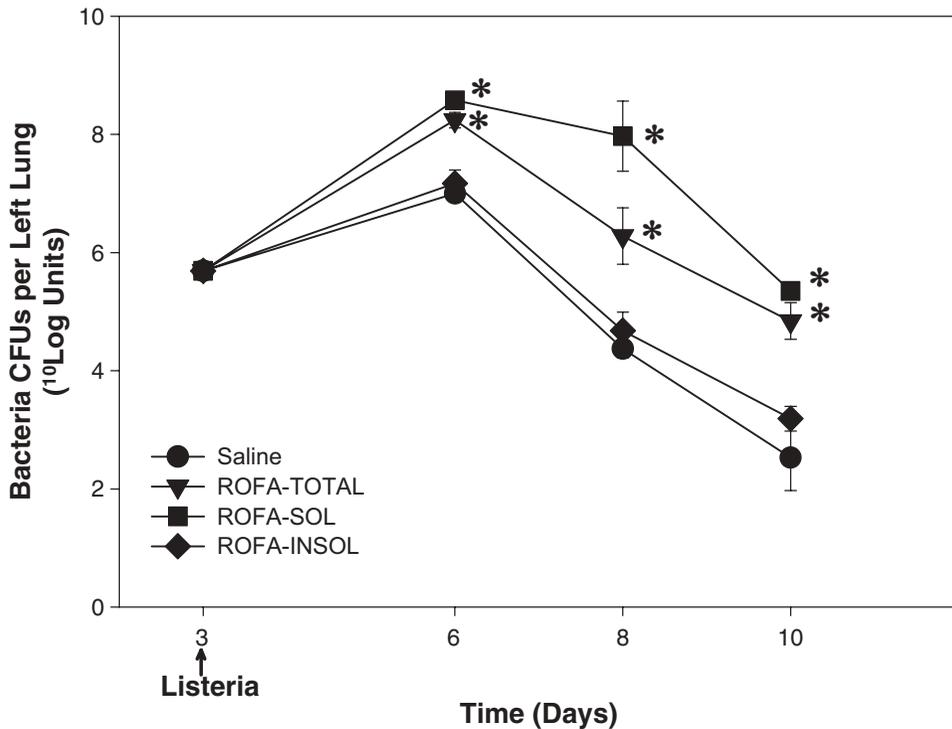
FIGURE 2. Number of bacterial CFUs in the left lung of rats that were preexposed to ROFA-TOTAL, ROFA-SOL, ROFA-INSOL, or saline by intratracheal instillation 3 d prior to intratracheal inoculation with 5×10^5 *L. monocytogenes*. Values are means ±SE; asterisk indicates significantly different from ROFA-INSOL and saline groups ($p < .05$).

Lung Histopathology

Hematoxylin and eosin-stained tissue sections from the lower right lung of animals treated with ROFA-SOL, ROFA-SOL +Chelex, or saline, followed by inoculation with 5×10^5 *monocytogenes*, were scanned at d 6, 8, and 10 (Figure 5). Infection with *L. monocytogenes* led to edema, inflammation, and the formation of granulomatous lesions (areas of dark purple) characterized by the presence of amorphous tissue debris in the lungs of saline and ROFA-SOL + Chelex groups. The lesions that were observed in the lungs of rats that were pretreated with ROFA-SOL were more extensive and significantly more pronounced than those observed in the other groups. Lung injury and inflammation in the ROFA-SOL group was still progressing at d 10, whereas the lung response of the other groups had subsided by this time point.

DISCUSSION

ROFA is a particulate pollutant emitted into the environment as a product of the combustion of fossil fuel. Its composition consists of a complex mixture
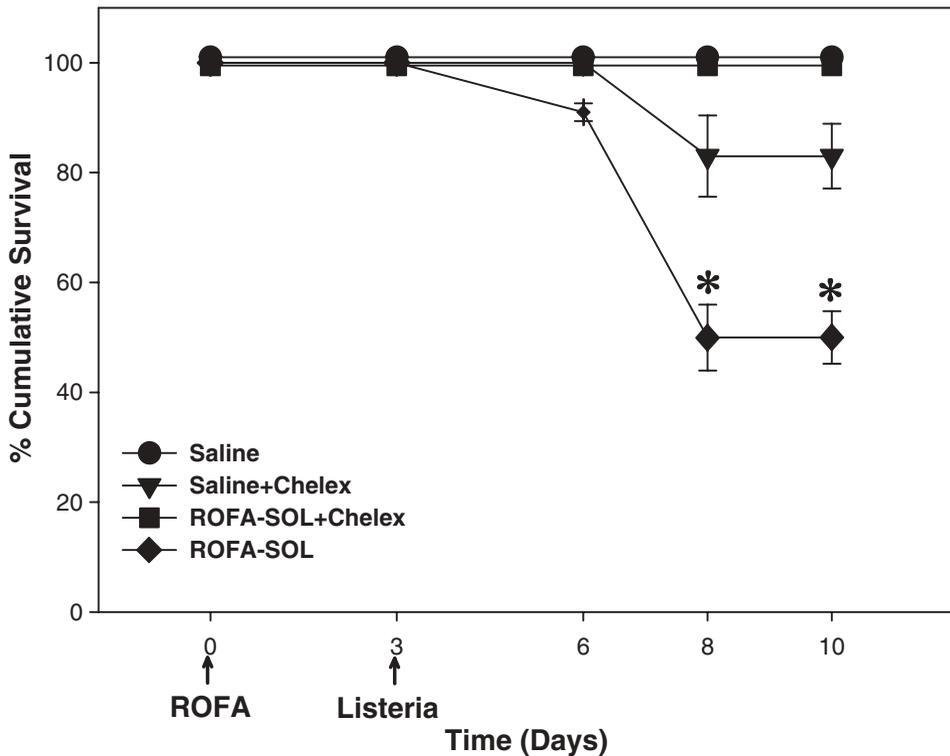
FIGURE 3. Survival rate of rats that were preexposed to ROFA-SOL, ROFA-SOL +Chelex, saline +Chelex, or saline by intratracheal instillation 3 d prior to intratracheal inoculation with 5×10^5 *L. monocytogenes*. Values are means ±SE; asterisk indicates significantly different from all other groups ($p < .05$).

of metals and other fuel contaminants complexed to an insoluble carbon core (Fisher et al., 1983). ROFA is known to induce a variety of respiratory responses in vivo and in vitro, including, but not limited to, alterations in transcription factor activation (Samet et al., 2002), increased airway hyperreactivity (Goldsmith et al., 1999; Hamada et al., 1999), increased mucin secretion (Jiang et al., 2000; Longphre et al., 2000), increased oxidative stress (Becker et al., 2002; Ghio et al., 2002; Huang et al., 2003; Pritchard et al., 1996), altered cytokine expression (Carter et al., 1997; Tao & Kobzik, 2002), and altered gene expression (Nadadur et al., 2000; Nadadur & Kodavanti, 2002). The presence of soluble transition metals of ROFA has been implicated in the development of adverse respiratory responses, and has been shown to play a role in lung inflammation and injury (Dreher et al., 1997; Dye et al., 1999; Gavett et al., 1997; Ghio et al., 1999; Kodavanti et al., 1998; Lewis et al., 2003).

The metal composition of ROFA is dependent on the fuel source, the combustion conditions in the power plant (including temperature, pressure changes, gas velocities, and duct work), and the efficiency of emission control devices (Burckle, 1977; El-Mogazi et al., 1988). Responses to ROFA may vary
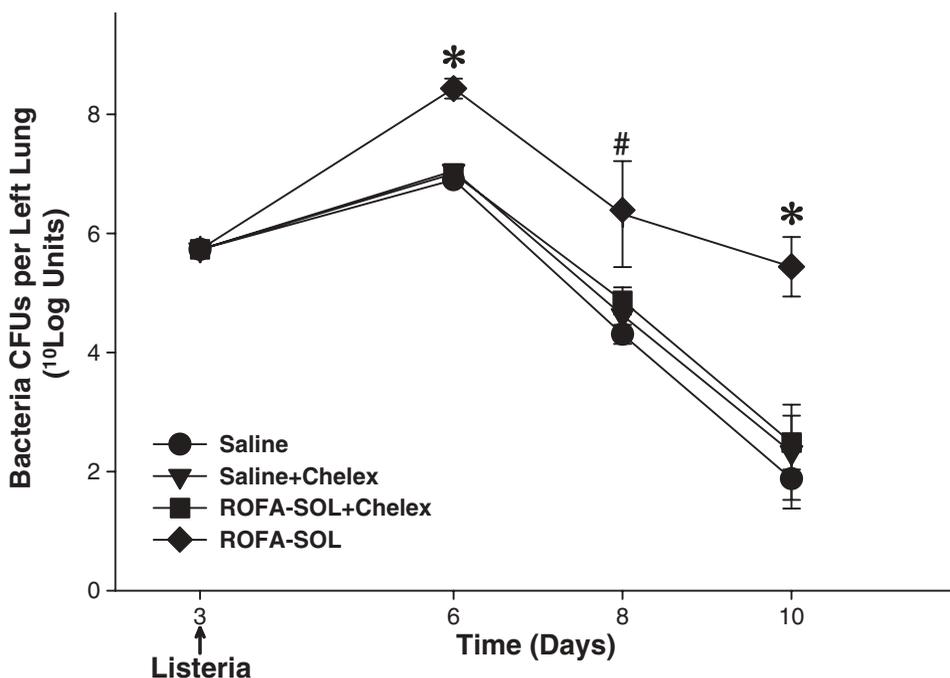
FIGURE 4. Number of CFUs in the left lung of rats that were preexposed to ROFA-SOL, ROFA-SOL +Chelex, saline +Chelex, or saline by intratracheal instillation 3 d prior to intratracheal inoculation with 5×10^5 *L. monocytogenes*. Values are means ±SE; asterisk indicates significantly different from all groups ($p < .05$); #, significantly different from saline +Chelex and saline groups ($p < .05$).

depending on the composition of soluble metals as well as their interaction with each other. Kodavanti et al. (1997) found that the metal mixture of ROFA, as well as soluble Ni, Fe, and V (the three predominant water-leachable metals), induced lung injury and enhanced cytokine induction (interleukin-1 and -5, and macrophage inflammatory protein-2). They also showed that Ni alone caused the most severe damage, while Fe and V alone produced a less severe pathology. Dreher et al. (1997) demonstrated that the individual soluble metals within a ROFA mixture may interact in an antagonistic fashion; specifically, Ni-induced lung injury was diminished in the presence of soluble Fe or soluble V. Ni has also been shown to be a suppressor of alveolar macrophage (AM) function, whereas V appears to produce a greater oxidative burst in AMs (Graham et al., 1975; Kodavanti et al., 2002). The individual soluble metals of ROFA may have differential effects on lung responses. Therefore, ROFA from various sources that differ in chemical composition may induce different effects. It is of interest to note that the ROFA used in the present study contained significant amounts of soluble Ni, Fe, and Al. Based on observations from previous studies, it is likely that these soluble metals may suppress lung defense responses.
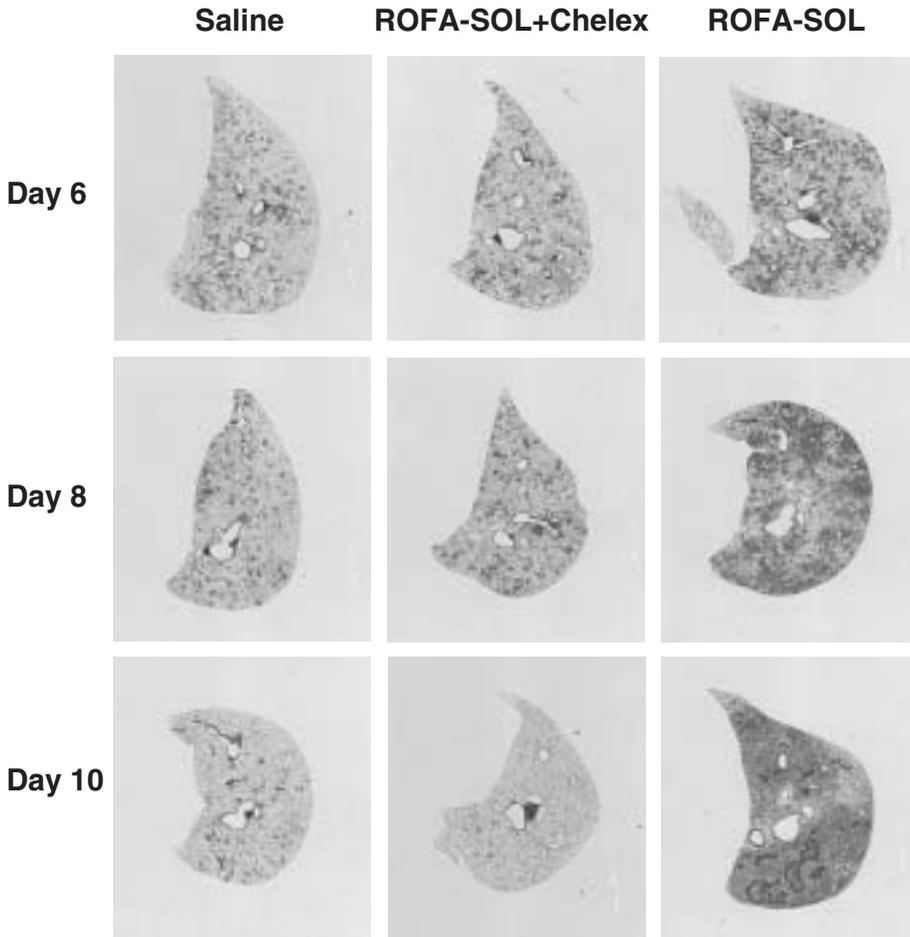
FIGURE 5. Whole slide scans of hematoxylin and eosin-stained right lung tissue sections from animals intratracheally instilled with ROFA-SOL, ROFA-SOL+Chelex, or saline followed by inoculation with 5×10^5 *L. monocytogenes* at d 6, 8, and 10.

The objective of this study was to determine the component of ROFA that may affect the susceptibility to pulmonary infection. Animals were exposed to ROFA or its soluble or insoluble fractions, followed by pulmonary infection with *L. monocytogenes. Listeria monocytogenes* is a gram-positive, facultative intracellular bacteria that has been commonly used to assess pulmonary defense function (Antonini et al., 2000; Cohen et al., 2001; Jakab et al., 1993; Van Loveren et al., 1988). An effective response to *L. monocytogenes* requires the proper cross-talk between the innate immune system (macrophages, neutrophils, natural killer cells) and the cell-mediated adaptive immune system (CD8^{+}T cells).

The major findings of this study confirmed our hypothesis that the soluble metals associated with ROFA would lead to increased morbidity and infectivity. ROFA and the associated soluble metals slowed the pulmonary clearance of bacteria and decreased survival. Upon removal of the soluble metals from the sample, survival and clearance were restored to control levels. Antonini et al. (2002) showed that ROFA may increase susceptibility to infection by decreasing AM bactericidal activity and the production of nitric oxide (an antimicrobial agent as well as a second messenger) by AMs. The effects of ROFA on AMs may alter innate immune responses and consequently affect adaptive immunity and the ability of the animal to respond to the infection.

This study demonstrated that the soluble fraction of ROFA likely contains elements that are responsible for the increased susceptibility to infection. Zelikoff et al. (2002) exposed *Streptococcus pneumoniae*-infected rats to soluble Fe, Mn, and Ni to examine the effect of metals commonly found in ambient air pollution on immunotoxicity. The investigators found that both Ni and Fe reduced pulmonary clearance of the bacteria in infected rats. Furthermore, soluble Fe was shown to possibly affect both innate and adaptive immunity in uninfected rats, as evidenced by changes in blood lymphocyte profiles and an inhibition of lymphocyte proliferation upon challenge. T cells (cell-mediated immunity) proved to be more sensitive to inhalation of soluble Ni than did B lymphocytes (humoral immunity), whereas soluble Fe had suppressive effects on both populations of lymphocytes. The ROFA used in our study contained significant amounts of soluble Ni and Fe, and it is possible that these metals altered the function of cells involved in both adaptive immune response and innate immune responses to infection. Ongoing investigations in our laboratory involve the evaluation of macrophage- and lymphocyte-derived cytokine and oxidant production important in immune responses.

In summary, soluble metals associated with environmental and occupational particulates likely alter pulmonary defense and increase susceptibility to infection. It has been demonstrated that the soluble metals associated with ROFA increase morbidity and decrease pulmonary clearance of bacteria. Additional studies evaluating the role of individual soluble metals, as well as the mechanisms of their actions, in lung defense are needed. Using the *Listeria* infectivity model, ongoing research in our laboratory is aimed at addressing these questions.

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