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Diesel Exhaust Particles Suppress Macrophage Function and Slow the Pulmonary Clearance of *Listeria monocytogenes* in Rats

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In this study, we tested the hypothesis that exposure to diesel exhaust particles (DEP) may increase susceptibility of the host to pulmonary infection. Male Sprague-Dawley rats received a single dose of DEP (5 mg/kg), carbon black (CB, 5 mg/kg), or saline intratracheally. Three days later, the rats were inoculated intratracheally with ~5,000 *Listeria monocytogenes* and sacrificed at 3, 5, and 7 days postinfection, and we determined the number of viable *Listeria* in the left lobe of lungs. The remaining lungs underwent bronchoalveolar lavage (BAL) and the retrieved BAL cells were identified and counted. Luminol-dependent chemiluminescence, a measure of reactive oxygen species (ROS) formation, generated by BAL cells was monitored and the levels of nitric oxide and tumor necrosis factor (TNF)-α produced by macrophages in culture were determined. At 7 days postinfection, we excised the lung-draining lymph nodes and phenotyped the lymphocyte subpopulations. Exposure of rats to DEP, but not to CB, decreased the clearance of *Listeria* from the lungs. *Listeria*-induced generation of luminol-dependent chemiluminescence by pulmonary phagocytes decreased by exposure to DEP but not CB. Similarly, *Listeria*-induced production of NO by alveolar macrophages was negated at 3, 5, and 7 days after inoculation in DEP-exposed rats. In contrast, CB exposure had no effect on *Listeria*-induced NO production at 3 days after infection and had a substantially smaller effect than DEP at later days. Exposure to DEP or CB resulted in enlarged lung-draining lymph nodes and increased the number and percentage of CD4+ and CD8+ T cells. These results showed that exposure to DEP decreased the ability of macrophages to produce antimicrobial oxidants in response to *Listeria*, which may play a role in the increased susceptibility of rats to pulmonary infection. This DEP-induced suppression is caused partially by chemicals adsorbed onto the carbon core of DEP, because impaired macrophage function and decreased *Listeria* clearance were not observed following exposure to CB. *Key words*: alveolar macrophages, diesel exhaust particles, *Listeria monocytogenes*, lung clearance, lung-draining lymph nodes, reactive oxidative species, tumor necrosis factor-α, T cells. *Environ Health Perspect* 109:515–521 (2001). [Online 11 May 2001]
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Numerous epidemiologic studies have consistently shown an association between the elevated particulate matter in ambient air and the increased respiratory mortality and morbidity in certain groups of people (*1,2*), evidenced as reduced lung function (*3*) and increased hospitalization and outpatient visits caused by problems such as asthma or pneumonia (*4,5*). Diesel exhaust particles (DEP), the particles with diameters < 2 µm, are a major component of particulate air pollution in the most industrialized urban areas. These fine respirable particles can remain airborne for long periods of time and deposit in great numbers deeply in the lungs, where they may cause pulmonary damage. Diesel particulate levels in mines have been reported as high as 2 mg/m³ (*6*). Because miners are commonly exposed to much higher levels of DEP than those found in ambient air, effects on pulmonary susceptibility of infection is a concern in these workers.

Numerous reports have demonstrated that DEP may sensitize the host immune system, e.g. the adjuvant effect of DEP on an increased antigen-induced production of immunoglobulin E. Such sensitization may enhance the incidence and severity of asthma and of collagen-induced arthritis, an autoimmune disease (*7–9*). On the other hand, reports also suggest that DEP may suppress the host immunity. Early studies have shown that exposure of animals to DEP may retard mucociliary clearance (*10*), depress interferon production in response to viral infection (*11*), and enhance influenza multiplication within the lungs (*12*). Several studies have shown that the phagocytic activity of macrophages is suppressed after exposure to DEP (*13–15*). In addition, we have also demonstrated that *in vitro* or *in vivo* exposure of alveolar macrophages (AM) to DEP depresses their responsiveness to lipopolisaccharide (LPS), evidenced as a decrease in LPS-stimulated secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-1 after exposure to DEP (*16,17*).

Among the most important cells in the innate immune system, AM are the first line of lung defense, eliminating most foreign particulate matter and microorganisms from the distal airways and keeping the alveoli sterile (*18*). Many studies also demonstrate that macrophages act as an important modulator in initiating and developing sequential acquired immune reactions (*19*). Microorganisms or particles can be phagocytosed by AM and cleared from the lungs by the mucociliary escalator system or translocated into the internal milieu, such as the local lymphoid system. DEP are found in the local lung-draining lymph nodes after pulmonary exposure (*20,21*). Because lymphocytes are intimately involved in defense against bacterial, fungal, viral, and toxic assaults, these translocated particles may affect the lymphatic arm of the host immune system.

In this study, we investigated the possible role of macrophages in DEP-induced immune suppression using a rat lung *Listeria monocytogenes* infection model. *Listeria* is a gram-positive, facultative intracellular pathogen. Resolution of *Listeria* infection requires both the nonspecific innate immune system, including macrophages, neutrophils, and natural killer cells, and the specific T-cell–mediated immunity (*22*). Actually, macrophages, through their secreted cytokines, are a critical link between these two systems (*23*). Experimental listeriosis has been a widely accepted method for studying T-cell–mediated macrophage activation (*24*). Several studies have used the rat *Listeria* infection model to assess pulmonary host defense mechanisms (*25–27*). Therefore, we use this animal model to test the hypothesis that exposure to DEP suppresses macrophage function, alters T-cell–mediated immunity, and increases the susceptibility of host-to-lung infection. To evaluate the role of various organic chemicals associated with DEP in this toxic effect, we also exposed rats to carbon black (CB)—particles having a carbonaceous core similar to that of DEP but containing fewer adsorbed chemicals than

DEP, and in lower volumes. Comparing the responsiveness of rats exposed to these different particles may reveal some insights of the mechanisms related to DEP-induced toxicity.

Materials and Methods

Particle sample preparations. We purchased a standardized DEP sample with a mass median aerodynamic diameter of approximately 0.5 μm from the National Institute of Standards and Technology (Standard Reference Material 1650, Gaithersburg, MD). We obtained carbon black (Elftex-12 furnace black), with particles ranging from 0.1 to 0.6 μm in diameter, from Cabot (Boston, MA). Particles were autoclaved, suspended in pyrogen-free sterile saline (Baxter Healthcare Corporation, Deerfield, IL), and sonicated for 5 min using an ultrasonic processor with a micro tip (Heat System-Ultrasonics, Plainview, NY) before intratracheal instillation.

Animals and exposures. Male Sprague-Dawley rats (250–300 g) purchased from Hilltop Lab (Scottsdale, PA) were kept in cages upon arrival and housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals had free access to food and water. They were exposed to particles by intratracheal instillation as follows: Rats were lightly anesthetized by intraperitoneal (ip) injection with 1% methohexital sodium (Brevital; Eli Lilly Co., Indianapolis, IN), and DEP or CB suspensions (5 mg/kg body weight) were injected into the trachea through a curved ball-tipped cannula (18-gauge). Control animals were instilled with sterile saline (the vehicle) only.

Listeria monocytogenes, strain 10403S, was kindly provided by R. Schafer (West Virginia University, Morgantown, WV) and grown in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C overnight. The *Listeria* concentration was determined spectrophotometrically and diluted with sterile saline to a desired concentration for dosing. Three days after particle instillation, half of the rats were intratracheally inoculated with approximately 5,000 *Listeria*, according to the procedure described above for particle instillation. The other half of the rats received sterile saline only. There were six different treatment groups in this study, including saline:saline, DEP:saline, CB:saline, saline:*Listeria*, DEP:*Listeria*, and CB:*Listeria*.

Bronchoalveolar lavage. At 3, 5, and 7 days after *Listeria* inoculation, the rats were deeply anesthetized with an overdose of sodium pentobarbital (ip, Butler Co., Columbus, OH) and exsanguinated by cutting of the abdominal aorta. The trachea was cannulated, the left lobe of the lungs was clamped, and bronchoalveolar lavage (BAL) was performed on the right lungs. The lungs were lavaged with 4 mL Ca^{2+}/Mg^{2+} free phosphate-buffered solution (PBS, 145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH = 7.4). Infusion and aspiration of Ca^{2+}/Mg^{2+} free PBS was continued until 50 mL bronchoalveolar lavage (BAL) fluid were collected from each rat. The samples were centrifuged for 10 min at 500 *g* and the cell-free lavage fluid was discarded. The cell pellets were suspended in 1 mL of PBS. The number of AM and neutrophils in the BAL cell suspension was determined using an electronic cell counter equipped with a cell sizing attachment (Coulter Electronics, Hialeah, FL). The remaining BAL cells were used for primary cell culture to determine functional activity of the cells.

Clearance of Listeria from the lungs. After BAL, the left lobe of the lungs was excised and homogenized in sterile water using a tissue grinder. Serial dilutions of the tissue homogenate were plated in triplicate on BHI agar plates and incubated at 37°C overnight. Colony-forming units (CFUs), an index of viable bacteria, were counted on each plate. We averaged the counts and corrected them for dilution to yield the CFUs per left lobe of lungs.

Luminol-dependent chemiluminescence. We performed luminol-dependent chemiluminescence (CL) of BAL cells, a measure of reactive oxygen species (ROS) formation, with a Berthold LB953 Luminometer (Berthold, Wildbad, Germany) as described previously (*28*). CL generated by BAL cells, adjusted to 1×10^6 AM/mL, was measured before and after stimulation with nonopsonized zymosan (2 mg/mL final concentration; Sigma Chemical Company, St. Louis, MO), a particle stimulant that stimulates macrophages. We monitored CL for 15 min at 37°C and calculated total CL by integrating the CL versus time response using a Berthold AutoLumat PC-control computer program. The results were presented as total counts/15 min/10^6 AM. Zymosan-stimulated CL was calculated as the total counts in the presence of stimulant minus the corresponding basal counts.

Measurements of nitric oxide and TNF-α secreted by AM. BAL cells (1×10^6 AM/mL) were suspended 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-inactivated calf serum, and seeded onto each well of a 24-well tissue culture plate. BAL cells were allowed to adhere to plastic plates for 2 hr in a humidified incubator (37°C and 5% CO_2); nonadherent cells were then removed by rinsing the monolayer with EMEM media 3 times. These adherent cells, more than 90% macrophages, were then incubated (37°C and 5% CO_2) in fresh EMEM for 18 hr. The AM-conditioned media were collected and centrifuged (8,000 rpm for 4 min). The supernatants were aliquoted and stored at –70°C until analysis of nitric oxide and TNF-α. The production of nitric oxide was determined using a Griess assay (*29*), as the accumulation of nitrite measured by a modified microplate assay. Briefly, samples were incubated with an equal volume of Griess reagent at room temperature for 10 min. We determined the absorbance at 550 nm with a microplate spectrophotometer reader (SPECTRAMaxTM 250; Molecular Devices Co., Sunnyvale, CA). Sodium nitrite (Sigma) was used as a standard. The results were expressed as micromoles of nitrite per 10^6 AM. We quantified the content of TNF-α in AM-conditioned supernatants by an enzyme linked immunosorbent assay (ELISA) using a commercial kit (BioSource International, Inc. Camarillo, CA.). The production of TNF-α was expressed as ng/10^6 AM.

Differential counts of T cells, CD4+ and CD8+ cells. At 7 days after *Listeria* inoculation, we excised two lung-draining lymph nodes from each rat and prepared single-cell suspensions. We determined total lymphocytes by using an electronic cell counter (Coulter Electronics). To enumerate the T cells, CD4+ and CD8+ cells, we labeled each of the respective cell types with an appropriate monoclonal antibody, which is conjugated with a fluorescent probe for visualization. We collected the cells by centrifugation and suspended them in PBS, pH 7.4, containing 1% bovine serum albumin and 0.1% sodium azide to a cell density of 1.5×10^6/mL. We used antirat CD3 monoclonal antibody conjugated with fluorescein isothiocyanate to enumerate T cells. For the T-cell subsets, we incubated the cells with antirat CD4+ monoclonal antibody conjugated with fluorescein isothiocyanate and antimouse CD8+ monoclonal antibody conjugated with phycoerythin. We also incubated the cells with their respective isotype control to correct for autofluorescence. After incubation with the conjugated monoclonal antibodies, the cells were washed once with staining buffer and incubated for 5 min with propidium iodide as a viability stain. The cells were again washed and then enumerated with a FacsVantage Flow Cytometer (Becton Dickinson, San Jose, CA). Fluorescence was gated on propidium iodide to eliminate dead cells. The values were expressed as the percentage of gated live cells and the absolute number of cells staining positive for each cell surface marker.

Statistics. We analyzed the data using JMPs, a statistical package obtained from SAS Institute, Inc. (Cary, NC). Most data were expressed as the mean ± standard error of mean (SE) of experimental values, except for CFUs in the lung lobe, for which the individual values were expressed. We used one-way analysis of variance (ANOVA) to analyze difference among the treatments at the same postinfection day; for multiple comparisons among means we used Tukey-Kramer's Honestly Significant Different (HSD) Test. We analyzed the interactive effects of particulate preexposure and *Listeria* challenge by two-way ANOVA. If the response of the combined treatment is the sum of a separate function for exposure to particles alone and exposure to *Listeria* alone (additive effect), then it indicates no interaction. If the response of the combined treatment is not the sum of a separate function of each treatment, either lower or higher (nonadditive effect), then an interaction, either inhibition or synergist, occurred. We tested all data for homogeneity of variance before ANOVA using a Bartlett's test; wherever the variance was heterogeneous, such as the results of CFUs and TNF-α, data were log-transformed. The significance was set at $p < 0.05$.
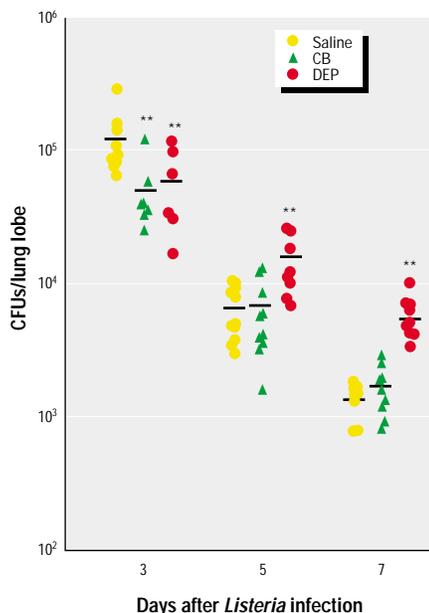

Figure 1. Effect of particulate exposure on the lung clearance of *Listeria* in rats. Rats received a single dose of DEP (5 mg/kg), CB (5 mg/kg), or saline intratracheally. Three days later, the rats were intratracheally inoculated with ~5,000 bacteria. At 3, 5, and 7 days after *Listeria* infection, the rats were sacrificed and the number of viable *Listeria* in the left lobe of lungs was determined. Results are expressed as CFUs, an index of viable bacteria, in the lungs for each individual rat. The bars represent the mean for each group. Each group had at least eight animals.
**Significant difference from the saline:*Listeria* controls, $p < 0.01$.

Results

Effect of particulate exposure on the Listeria ***clearance in lungs***. The effects of DEP and CB exposure on the clearance of *Listeria* from lungs are presented in Figure 1. The pattern of pulmonary *Listeria* clearance was different among the DEP-, CB-, and saline-pretreated rats. Compared to the saline controls, fewer bacteria were found in the lungs of DEP-treated rats at 3 days postinfection, whereas more bacteria were detected in the lungs of DEP-treated rats at 5 days. By 7 days, approximately 1,000 CFUs were counted from the left lobe of lungs of the saline-pretreated rats, while approximately 6,000 were recovered from the lungs of rats exposed to DEP. The difference was significant. As with DEP, rats treated with CB had fewer bacteria in the lungs compared to the saline controls at 3 days; such difference, however, was not observed at 5 and 7 days after infection. These data indicate that exposure of rats to DEP, but not to CB, slowed intrapulmonary *Listeria* killing.

Effect of particulate exposure and/or Listeria ***infection on retrievable phagocytes by BAL.*** Phagocytes, including AM and neutrophils, are the major effector cells against pulmonary *Listeria* during the early infectious stage. To determine whether the exposures had any effect on the retrievable phagocytes from the alveolar space, rats underwent bronchoalveolar lavage at 3, 5, and 7 days after *Listeria* infection. The rats infected with *Listeria* alone at this dosage had no significant change in the yield of lavageable AM or neutrophils compared to the saline controls (Table 1). At 3 days postinfection, exposure to DEP alone resulted in an approximately 2-fold or 8-fold increase, respectively, in the number of lavageable AM or neutrophils. This increase, however, did not occur with the combined treatment of DEP and *Listeria*; the total number of lavageable phagocytes was slightly higher than that of the rats treated with *Listeria* alone, but much lower than that of

the rats treated with DEP alone. Similarly, exposure to CB alone also increased the yield of lavageable AM and neutrophils; the combined treatment of CB and *Listeria* also diminished the total number of AM plus neutrophils at 3 days postinfection compared to rats treated with CB alone. However, the extent of the decrease was much lower than that with DEP. The yield of phagocytes from rats treated with CB and *Listeria* was significantly higher than that in rats treated with *Listeria* alone. The effect of particle exposures on the yield of these lavageable phagocytes was transient. No significant differences were noted among the various treatment groups by 5 and 7 days after infection.

Effect of particulate exposures on Listeria-***induced ROS production by phagocytes***. Reactive oxygen species (ROS) generated by phagocytes are important in killing various bacteria, including *Listeria*. CL is a measure of light production that represents the generation of ROS by cells. As shown in Figure 2, differences in CL production were found among the rats treated with DEP, CB, *Listeria*, or saline at 3 days postinfection. Without any *ex vivo* stimulation, the basal production of CL was not affected by intratracheal exposure to particles, but was slightly increased by *Listeria* inoculation compared to the saline controls. After preexposure to DEP, *Listeria*-induced basal CL production was significantly decreased, whereas it was not significantly affected by preexposure to CB. This DEP-related suppressive effect also occurred in CL production of macrophages in response to zymosan. Preexposure to either DEP or CB increased zymosan-stimulated CL. Exposure to *Listeria* alone also significantly increased CL production after zymosan stimulations. However, *Listeria*-induced CL was depressed following DEP exposure but not after CB exposure. These effects were transient; no treatment-related differences were observed at 5 and 7 days after infection (data not shown).

Table 1. Effects of particulate exposure and/or *Listeria* infection on the yield of lavageable AM or neutrophils in rats.[a]

	3 Days postinfection		5 Days postinfection		7 Days postinfection	
	AM	Neutrophils	AM	Neutrophils	AM	Neutrophils
Without *Listeria*						
Saline	4.21 ± 0.55	0.60 ± 0.06	5.98 ± 0.93	0.87 ± 0.16	4.93 ± 0.63	0.75 ± 0.05
DEP	10.87 ± 0.57*	5.04 ± 0.70*	6.46 ± 0.96	1.82 ± 0.32	3.21 ± 0.56	0.76 ± 0.05
CB	10.90 ± 1.92*	5.30 ± 1.14*	5.98 ± 1.60	1.03 ± 0.10	4.52 ± 1.01	0.86 ± 0.07
With *Listeria*						
Saline	3.91 ± 0.75	1.12 ± 0.32	4.77 ± 0.74	0.85 ± 0.09	3.77 ± 0.53	0.63 ± 0.06
DEP	4.44 ± 0.65‡	2.08 ± 0.51*‡	5.55 ± 1.28	1.26 ± 0.16	3.12 ± 0.67	0.70 ± 0.10
CB	8.87 ± 1.02*	2.99 ± 0.86*‡	6.05 ± 1.07	1.04 ± 0.08	5.43 ± 0.84	0.65 ± 0.07

[a]Data are presented as mean ± SE of cell numbers (× 10^6). Each group had at least 6 animals. Rats received a single dose of DEP (5 mg/kg), CB (5 mg/kg), or saline intratracheally. Three days later, the rats were intratracheally inoculated with ~5,000 *Listeria*. At 3, 5, and 7 days after bacteria infection, the rats were sacrificed, bronchoalveolar lavage was performed, and differential counts of BAL cells were determined. *Significant difference from the saline:saline controls, $p < 0.05$. ‡Significantly less than the expected additive effects of particles alone (DEP or CB) and *Listeria* alone (analyzed by two-way ANOVA), $p < 0.05$.

This DEP-elicited suppression of phagocyte activity after *Listeria* infection is also demonstrated by monitoring nitric oxide (NO), another ROS, produced by AM. Compared to saline controls, the production of NO by AM was consistently increased in *Listeria*-inoculated rats through all examining time points, whereas NO production increased slightly in DEP-treated rats only at 3 days postinfection (Figure 3). Preexposure to DEP inhibited *Listeria*-induced NO production at 3, 5, and 7 days postinfection—*Listeria*-induced NO production was abolished thoroughly in the rats. The response of rats to CB was different from their response to DEP. CB consistently increased NO production throughout this 7-day experimental period. At 3 days postinfection, the amount of NO produced by the rats treated with both CB and *Listeria* was the expected additive sum of CB alone and *Listeria* alone, indicating that no interaction occurred. The combined treatment of CB and *Listeria* caused an inhibitory reaction at 5 and 7 days postinfection, because the level of NO fell significantly below the expected additive sum of CB alone and *Listeria* alone. However, unlike the rats treated with both DEP and *Listeria*, the rats treated with CB and *Listeria* produced no less NO compared to the rats treated with *Listeria* alone.

Effect of particulate exposures and/or* Listeria *infection on TNF-α production by AM. TNF-α is an important cytokine involved in listeriocidal activity. The effects of particles and *Listeria* on the production of TNF-α by AM were assessed 3 days after *Listeria* inoculation. The content of TNF-α in cultured cell supernatants was ~0.67 ng/10⁶ AM in rats treated with saline only (Figure 4). Exposure to DEP alone or *Listeria* alone at this dosage and this time did not show a significant change in the secretion of TNF-α. However, CB stimulated macrophage production of TNF-α. There was more than a 10-fold increase in TNF-α production in the CB-treated rats compared to rats treated with either DEP or saline. This particulate-related effect was not affected by the combined treatment; the production of TNF-α was not significantly different between the group treated with particles alone and those treated with both particles and *Listeria*.

Effect of particulate exposures and/or* Listeria *infection on the lymphocyte subpopulations in the lung-draining lymph nodes. Exposure to DEP appeared to decrease the ability of rats to clear *Listeria* from the lungs. Since the clearance of *Listeria* partially depends on T-cell mediated immunity, the question is whether exposure of rats to DEP and/or *Listeria* could alter the subpopulations of lymphocytes. As shown in Table 2, at 7 days after bacteria inoculation, the total

number of lymphocytes recovered from the lung-draining lymph nodes, which were markedly enlarged, increased significantly in DEP-, CB-, or *Listeria*-treated rats. The rats exposed to either DEP and *Listeria* or CB and *Listeria* had an expected additive number of total lymphocytes. Flow cytometric analysis showed that the number of T cells increased approximately 3-fold in rats treated with DEP alone, CB alone, or *Listeria* alone compared

to the saline controls. However, the percentage of T cells increased in the DEP- or CB-treated rats, whereas it did not change in the *Listeria*-treated rats. These particulate-induced changes in both percentage and number apparently diminished after coexposure to particles (either DEP or CB) and *Listeria*. The percentage and absolute number of T cells in the rats treated with particles and *Listeria* fell significantly below the
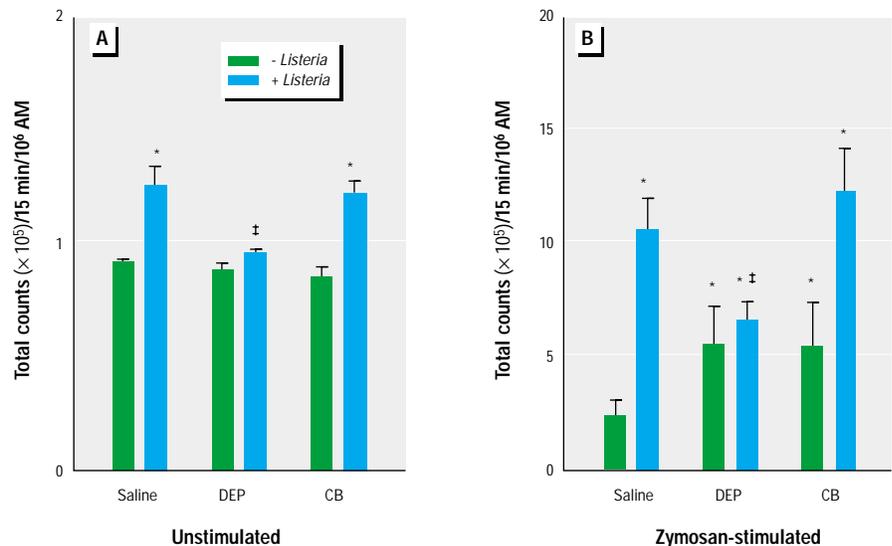

Figure 2. Effect of DEP or CB exposure on *Listeria*-induced luminol-dependent CL produced by BAL cells 3 days after infection. Rats received a single dose of DEP (5 mg/kg), CB (5 mg/kg) or saline intratracheally. Three days later, the rats were intratracheally inoculated with ~5,000 *Listeria*. At 3 days after bacteria infection, the rats were sacrificed, BAL cells were collected, and CL was performed on the BAL cells. Data are expressed as the mean ± SE ($n > 6$).

*Significant difference from the saline:saline controls, $p < 0.05$. ‡Significantly less than the expected additive effects of particles alone (DEP or CB) and *Listeria* alone (analyzed by two-way ANOVA), $p < 0.05$.
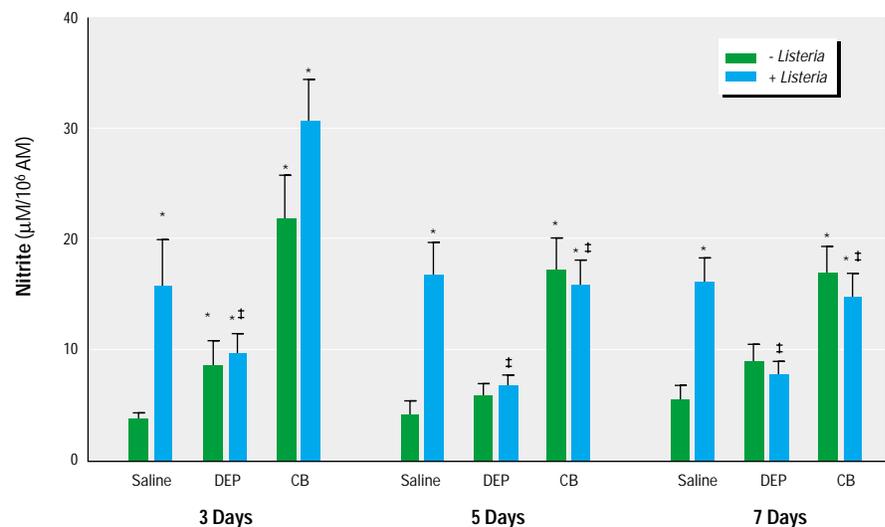

Figure 3. Effect of DEP or CB exposure on *Listeria*-induced NO production by AM at 3, 5, and 7 days after infection. Rats received a single dose of DEP (5 mg/kg), CB (5 mg/kg), or saline intratracheally. At 3 days after exposure, rats were intratracheally inoculated with ~5,000 *Listeria*. At 3, 5, and 7 days after bacteria infection, the rats were sacrificed and AM were isolated. The level of nitrite in the AM-cultured medium was measured. Data are expressed as the mean ± SE of at least six rats.

*Significant difference from the saline:saline controls at the same testing day, $p < 0.05$. ‡Significantly less than the expected additive effects of particles alone (DEP or CB) and *Listeria* alone (analyzed by two-way ANOVA) at the same postinfection day, $p < 0.05$.

expected additive sum in those treated with particles alone and *Listeria* alone, suggesting that an inhibitory interaction may occur.

The results of absolute numbers and percentages of T cell subsets CD4^{+} and CD8^{+} among these different treatment groups are presented in Figure 5. The rats instilled with saline had approximately 30% CD4^{+} cells and 10% CD8^{+} cells, whereas the rats instilled with DEP had approximately 50% CD4^{+} and 20% CD8^{+} (Figure 5A). *Listeria* alone increased the proportion of CD8^{+} cells but not CD4^{+} cells. However, coexposure to DEP and *Listeria* seemed to negate DEP-induced effects; the percentage of CD4^{+} or CD8^{+} cells reverted to the number for rats treated with *Listeria* alone. As shown in Figure 5B, DEP and *Listeria* both increased the absolute numbers of CD4^{+} and CD8^{+} cells. The rats coexposed to DEP and *Listeria* had the expected additive sum of CD4^{+} or CD8^{+} cells. There were more CD4^{+} and CD8^{+} cells available in these rats than in rats treated with either *Listeria* alone or DEP alone. Such changes in the numbers of CD4^{+} and CD8^{+} cells were also observed in the CB-treated rats. Interestingly, the alterations were almost identical to those for DEP.

Discussion

In this study, we demonstrated for the first time that exposure to DEP increased the susceptibility of rats to *Listeria* monocytogenes pulmonary infection. The rats exposed to DEP and then inoculated with *Listeria* intratracheally were less able to clear bacteria from the lungs than were those not exposed to DEP, such as those treated with saline or CB 5 and 7 days after infection. CB has a

carbonaceous core similar to that of DEP, but contains fewer organic chemicals, and in lower volume. The different responsiveness of rats to DEP and CB suggests that the organic components of DEP play a role in this reaction. Many studies have shown that the chemicals associated with DEP are actually responsible for various DEP-induced effects, including mutagenicity (*30*), adjuvant activity for IgE production (*31*), production of reactive oxygen radicals (*32*), induction of macrophage apoptosis (*33*), and secretion of cytokines by macrophages (*16*). Although the results of this study further indicate the importance of these organic compounds, the role of particles as a carrier that delivers these chemicals to the phagocytes and to the deep lung should not be ignored. Jakab and colleagues (*26,34*) have shown that coexposure of animals to CB and acrolein lessens macrophage phagocytosis, suppresses TNF-α secretion, and impairs *Listeria* clearance from the lungs, whereas exposure to either CB alone or acrolein alone has no such effects.

To determine the possible underlying mechanism involved in this compromised defense against respiratory infection, we

focused on the effects of particle exposure on macrophage functions. *Listeria* is a Gram-positive, facultative intracellular bacterium that can be destroyed mainly by one of the most important effector cells, the activated macrophages (*35,36*). Macrophages play an important role not only in the innate immunity as the first line of host defense against *Listeria*, but also through the acquired immune system, acting as a modulator to induce appropriate sequential reactions (*19,37*). Therefore, using a *Listeria*-resistant animal model to study T-cell–mediated macrophage activation has been recommended by the National Toxicology Program (*24*). Various particles present in different occupational and environmental settings affect macrophage host defense mechanisms, such as decreased phagocytosis and oxidant generation and decreased production of inflammatory cytokines (*13,15,38*). Indeed, we have previously shown that preexposure of rats to DEP, both *in vitro* and *in vivo*, decreases LPS-stimulated macrophage secretion of IL-1 and TNF-α (*16,17*).

Our data show that the rats pretreated with DEP exhibited slowed clearance of *Listeria* in the lungs at 5 and 7 days after

Table 2. Effect of particulate exposure and/or *Listeria* infection on the number of total cells and the number and percentage of T cells in the lung-draining lymph nodes.[a]

	Total cells (1×10^6)	T cells (1×10^6)	Percentage
Without *Listeria*			
Saline	21.01 ± 4.09	8.86 ± 2.08	38.18 ± 3.55
DEP	51.55 ± 9.61*	30.47 ± 5.35*	59.57 ± 1.86*
CB	50.01 ± 6.14*	26.10 ± 3.12*	49.63 ± 2.46*
With *Listeria*			
Saline	70.75 ± 9.37*	28.03 ± 3.09*	41.31 ± 3.42
DEP	100.39 ± 14.03*	40.09 ± 5.92*‡	41.48 ± 4.15‡
CB	109.41 ± 6.40*	38.69 ± 2.56*‡	41.71 ± 1.51‡

[a]Rats received a single dose of DEP or CB (5 mg/kg) intratracheally. At 3 days after exposure, rats were intratracheally inoculated with ~5,000 *Listeria*. At 7 days after *Listeria* infection, the lung-draining lymph nodes were excised and the total number of cells was counted. The number and percentage of T cells were determined by flow cytometric analysis. *Significantly different from the saline:saline controls, $p < 0.05$. ‡Significantly less than the expected additive effects of particle alone (DEP or CB) and *Listeria* alone (analyzed by two-way ANOVA), $p < 0.05$.
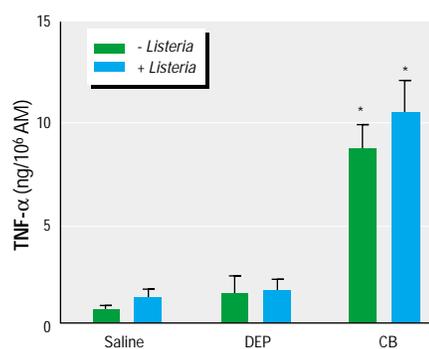

Figure 4. Effect of particulate exposure and/or *Listeria* infection on the secretion of TNF-α from AM at 3 days after infection. Rats received a single dose of DEP (5 mg/kg), CB (5 mg/kg), or saline intratracheally. Three days later, the rats were intratracheally inoculated with ~5,000 *Listeria*. At 3 days after bacteria infection, the rats were sacrificed and AM were isolated. The level of TNF-α in the cultured AM medium was measured by ELISA. Data are expressed as the mean ± SE of at least six rats.
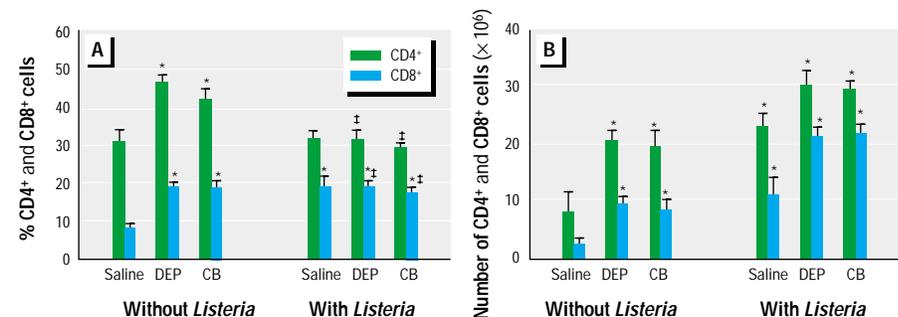*Significant difference from the saline:saline controls, $p < 0.05$.

Figure 5. Effect of particulate exposure and/or *Listeria* infection on the number and percentage of CD4^{+} or CD8^{+} T cells in the lung-draining lymph nodes. Rats received 5 mg/kg of DEP or CB intratracheally. Three days later, the rats were intratracheally inoculated with ~5,000 *Listeria*. At 7 days after *Listeria* infection, cells were isolated from the lung-draining lymph nodes and a single-cell suspension was prepared. The numbers and percentages of CD4^{+} and CD8^{+} cells were determined using flow cytometric analysis. Data are presented as the means ± SE of at least six rats.
*Significant difference from the saline:saline controls, $p < 0.05$. ‡Significantly less than the expected additive effects of particles alone (DEP or CB) and *Listeria* alone (analyzed by two-way ANOVA), $p < 0.05$.

infection, suggesting that macrophage function was suppressed after DEP exposure. This observation was confirmed at least partly by the significant depression of NO production at the same time. ROS play a critical role in the host's defense against *Listeria* (*39,40*). Compared to the saline-treated controls, in the rats treated with DEP *Listeria*-induced production of NO was significantly suppressed, increasing the viable *Listeria* in the lungs; the combination of CB and *Listeria* produced no less NO than *Listeria* alone and therefore did not slow the clearance of *Listeria* from the lungs.

However, we also noted suppressed macrophage function in the DEP-treated rats at 3 days postinfection, when the lung burden of *Listeria* decreased compared to that in the saline controls. Obviously, other factors contribute to this effect. One of the possibilities is that the particle exposure induces other antimicrobial products. Indeed, increased secretion of IL-1 in response DEP has been reported previously (*17*). This may compensate for the decreased ROS production, resulting in the increased *Listeria* clearance at this time. Another possibility that may account for this decreased bacteria clearance is the elevated ratio of phagocytes to bacteria in DEP-treated rats at day 3 (Table 1). Without *Listeria* infection, DEP-treated rats had a significantly greater amount of lavage-able phagocytes. Both macrophages and neutrophils play a critical role in destroying *Listeria* at the early stage of infection (*41,42*). Therefore, the increased number of phagocytes in the airways initially after particle treatment may make rats initially more resistant to invaders. This increase did not persist at 5 and 7 days, when bacteria clearance was compromised. In the rats treated with both DEP and *Listeria* at 3 days postinfection, the yield of phagocytes was actually lower than the rats treated with particles alone. It is possible that this decreased yield of phagocytes could be related to a decreased lavage efficiency. In a previous study, a decrease in macrophages harvested by lavage was noted in animals exposed to cotton dust; however, such a decrease coincided with an increase in alveolar macrophages, determined by morphologic examination (*43*). These results suggest that initially following cotton dust exposure these activated phagocytes may adhere more tightly to airways and be more difficult to remove by bronchoalveolar lavage. With increasing time of exposure, the discrepancy between lavage and histologic estimates of macrophage number diminishes. A similar observation was also reported by other investigators (*44*). If this is true, there could be more phagocytes in the airways of DEP-exposed rats at 3 days. This increased number of phagocytes (especially

neutrophils) may be responsible for the ability of DEP-pretreated rats to destroy *Listeria*, even though macrophage function was suppressed at that time.

We also noted a different responsiveness of macrophages to DEP and CB in the production of TNF-α. The rats treated with CB but not with DEP exhibited an increased production of TNF-α. TNF-α is one of the important cytokines involved in *Listeria* killing (*45*). TNF-α works in listeriosis primarily through its activation of lymphocytes to produce interferon (IFN)-γ, which in turn activates macrophages, enhancing their listeriocidal activity (*46,47*) through the production of ROS (*39,48*). Thus, the activation of macrophages could be affected by different responsiveness of macrophages in producing TNF-α after DEP and CB exposure, which ultimately would influence the resistance of rats to *Listeria*.

Because sterilizing *Listeria* infection requires the adaptive cellular immune response with the activation of CD4+ and CD8+ T cells (*22*), the decreased clearance of *Listeria* after DEP exposure observed in this study suggests that DEP may depress T-cell–mediated immunity. In this study, we also found that exposure of rats to DEP significantly altered the lymphocyte subpopulations in the lung-draining lymph nodes. Both the absolute number and percentage of CD4+ cells and CD8+ cells in the lymph nodes increased in DEP-exposed rats. Such elevation of T-cell numbers has also been observed in both humans and animals exposed to DEP (*49,50*). Interestingly, the combined treatment of DEP and *Listeria* resulted in a lower percentage of CD4+ cells or CD8+ cells relative to the sum of DEP alone and *Listeria* alone. DEP is likely to induce T-cell–mediated immune disorders by shifting the vulnerable balance in the system and decreasing the resistance of hosts to respiratory infections. However, it is uncertain whether this alteration of lymphocyte subpopulations would increase the susceptibility of rats to *Listeria* infection after DEP exposure, because a similar change in CD4+ and CD8+ cell populations was also noted after CB exposure, which did not decrease the clearance of *Listeria*. Therefore, this particle-related change in the T-cell subpopulation could not account for the different effects of DEP and CB on the clearance of *Listeria*.

Resistance to *Listeria* infection requires symbiosis between macrophages and T cells; such interactions are linked by mediators secreted by these cells, including TNF-α, IL-12, IL-10, and IFN-γ (*45,47,51*). Previously, we have shown that DEP, but not CB, suppressed LPS-induced secretion of IL-1 and TNF-α by macrophages (*17*). In this study, we also demonstrated that DEP and CB

elicited different macrophage responses to *Listeria* in the production of ROS. Because the responses of macrophages to sequential stimuli are different after DEP and CB exposure, this particle-related effect may influence the response of lymphocytes differently. Although the population of lymphocytes was not significantly different between DEP- and CB-treated rats, exposure to DEP or CB may affect T-cell–mediated function differently. Suppressed lymphocyte function by DEP has been shown in a recent study (*52*); the combined treatment of DEP and ragweed decreases gene expression of IFN-γ, which is an active modulator in T-cell–mediated immunity. Van Loveren and colleagues (*25*) also found that exposure of rats to ozone slows the clearance of *Listeria* from the lungs. They demonstrated that ozone suppresses T-cell–mediated immune function; both *Listeria*-elicited delayed-type hypersensitivity response and T-cell proliferation decreased after ozone exposure. The mechanisms by which DEP exposure may affect T-cell–mediated immunity by altering lymphocyte function are currently under investigation in our laboratory.

In summary, we have demonstrated that exposure to DEP, but not to CB, increased the susceptibility of rats to *Listeria* lung infection, indicating that DEP may have an adverse influence on the development of T-cell–mediated immunity. The different responsiveness of macrophages after particulate exposure may account partially for this effect. Exposure to DEP attenuated *Listeria*-induced macrophage activation, determined as CL generation and NO production. In contrast, CB had no effect on *Listeria*-induced CL production and a lesser effect on NO production in response to *Listeria* infection. In addition, CB augmented macrophage TNF-α production, whereas DEP had no such stimulation. These differences in response to DEP versus CB exposure suggest the importance of the organic components of DEP in this reaction. Because DEP suppressed the responsiveness of macrophages to infectious agents, it is possible that people exposed to excessive DEP for long periods in either occupational or environmental settings may be at increased risk of respiratory infections.

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