Alteration of Pulmonary Immunity to *Listeria monocytogenes* by Diesel Exhaust Particles (DEPs). I. Effects of DEPs on Early Pulmonary Responses

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It has been hypothesized that diesel exhaust particles (DEPs) aggravate pulmonary bacterial infection by both innate and cell-mediated immune mechanisms. To test this hypothesis, we investigated the effects of DEP exposure on the functions of alveolar macrophages (AMs) and lymphocytes from lung-draining lymph nodes using a rat *Listeria monocytogenes* infection model. In the present study, we focused on the effects of DEP exposure on AM functions, including phagocytic activity and secretion of proinflammatory cytokines. The *Listeria* infection model was characterized by an increase in neutrophil count, albumin content, and acellular lactate dehydrogenase activity in the bronchoalveolar lavage (BAL) fluid at 3 and 7 days postinfection. Short-term DEP inhalation (50 and 100 mg/m^3, 4 hr) resulted in a dose-dependent suppression of lung clearance of *Listeria*, with the highest bacteria count occurring at day 3. This aggravated bacterial infection was consistent with the inhibitory effect of DEPs on macrophage functions. DEPs suppressed phagocytosis and *Listeria*-induced basal secretion of interleukin-1β (IL-1β) and IL-12 by AMs in a dose-dependent manner. The amount of IL-1β and IL-12 in the BAL fluid was also reduced by DEP exposure. In addition, DEPs decreased *Listeria*-induced lipopolysaccharide-stimulated secretion of tumor necrosis factor-α (TNF-α), IL-1β, and IL-12 from AMs. These results suggest that DEPs retard bacterial clearance by inhibiting AM phagocytosis and weaken the innate immunity by inhibiting AM secretion of IL-1β and TNF-α. DEPs may also suppress cell-mediated immunity by inhibiting AM secretion of IL-12, a key cytokine for the initiation of T helper type 1 cell development in *Listeria* infection. *Key words:* alveolar macrophages, cytokine production, diesel exhaust particles, inhalation exposure, *Listeria monocytogenes*, occupational exposure, phagocytosis. *Environ Health Perspect* 110:1105–1111 (2002). [Online 17 September 2002] *http://ehpnet1.niehs.nih.gov/docs/2002/110p1105-1111yin/abstract.html*

Diesel exhaust particles (DEPs), generated by heavy-duty diesel engines in various industries, can adsorb over 450 different organic compounds, including mutagenic and carcinogenic polycyclic aromatic hydrocarbons (*1*). With diameters < 2 μm, these fine respirable particles can remain airborne for long periods of time and deposit in great numbers deeply in the lungs. For these reasons, exposure of truckers, railroad and construction workers, and engine mechanics to DEPs is an occupational health concern. A report from the U.S. Department of Labor showed that the worst-case mean exposures to DEPs in underground metal and nonmetal mines are about 2,000 μg/m^3, with maximum measurements as high as 3,650 μg/m^3 (*2*). Epidemiologic studies have also shown a consistent association between elevated levels of particulate matter in ambient air and increased incidence of pulmonary infections (*3*) or increased respiratory mortality and morbidity in high-risk groups (*4,5*). Because DEPs are a major component of particulate air pollution in most industrialized urban areas, their effect on pulmonary infections is of great environmental and occupational concern.

The principal function of pulmonary host defense mechanisms is to clear inhaled particles or microorganisms from the lungs and prevent infections. Among the various cell types involved in the innate immune system, alveolar macrophages (AMs) are responsible for the clearance of inhaled particles and/or microorganisms from the distal airways and alveolar spaces. These cells engulf inhaled particles or microorganisms and become activated to release reactive oxygen species (ROS), cytokines, and a variety of mediators that are capable of killing microorganisms (*6,7*). It has been well documented that AM-derived proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), provide innate resistance to bacterial infection, promote the inflammatory process by recruiting neutrophils into the air spaces, and stimulate these phagocytes to release ROS and enzymes (*7,8*). A successful pulmonary host defense, on the other hand, also needs specific cell-mediated immunity (*9*). In this aspect, studies have already shown that AMs, through their secretion of cytokines in response to specific antigen exposure, provide a critical link between these two systems. For example, Hsieh et al. (*10*) showed that the production of IL-12 by macrophages is a key process for the development of the appropriate CD4$^+$ T helper (Th) subset during the immune response to *Listeria monocytogenes* infection. IL-12, in fact, not only initiates but also plays an important role in maintaining the Th1 response (*11*). This cytokine is produced very rapidly after infection, thus serving as an early marker for the study of DEP effect(s) on cell-mediated immunity.

Studies from our laboratory as well as from others have suggested that DEPs may suppress host immunity by suppressing mucociliary clearance and the phagocytic activity of AMs (*12,13*), reducing interferon production in response to viral infection (*14*) and depressing immune responsiveness to bacterial antigenic stimulation (*15,16*). In addition, DEPs were also shown to be capable of potentiating antigen sensitization with increased production of antigen-specific immunoglobulin E (*17–19*). In a recent study, we further demonstrated that exposure to DEPs, but not to carbon black, decreased pulmonary bacterial clearance in rats, suggesting that DEPs may have an adverse influence on both the innate and the T-cell–mediated immune responses (*20*). Although we have previously shown that AMs from DEP-exposed rats were less responsive to *ex vivo* challenge with lipopolysaccharide (LPS) in the production of IL-1 and TNF-α (*16*), how DEPs impair pulmonary host defense mechanisms is not yet well understood. Whether the cytokine production by AMs is indeed decreased during a bacterial infection after DEP exposure remains to be determined.

To test the hypothesis that DEPs aggravate pulmonary bacterial infection by both innate and cell-mediated immune mechanisms, we have characterized the effect of short-term DEP exposure on macrophage functions critical in the immune response to *Listeria* infection. Specifically, these functions include AM phagocytosis, macrophage secretion of IL-1β and TNF-α for innate immune responses, and AM production of IL-12, a proinflammatory cytokine that bridges the innate resistance and antigen-specific adaptive immunity. The acute effect of DEP exposure

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was studied by exposing rats to 50 or 100 mg/m^3 DEPs for 4 hr. Although these doses appear to be high compared with reported environmental and occupational levels, the estimated lung deposits of DEPs under such short duration were typically 10–20 times less than those in rats under chronic studies (*21–23*). The present study is intended to assess the early responses to acute DEP exposure, which should provide more insight into the mechanisms by which DEPs aggravate pulmonary infection.

Materials and Methods

Materials. A standardized DEP sample (standard reference material 1650a), representative of heavy-duty engine emissions, was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA). This sample had a mass median aerodynamic diameter of approximately 0.5 µm. *Listeria* was of strain 10403s and serotype 1. Male Brown-Norway rats (200–250 g body weight) were purchased from Harlan Laboratories (Indianapolis, IN, USA). They were housed in a clean-air and viral-free room with restricted access, given a conventional laboratory diet and tap water *ad libitum*, and allowed to acclimate in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care for 1 week before use.

DEP inhalation and intratracheal bacterial inoculation. Groups of rats were exposed to either purified air or DEP-containing air (50 and 100 mg/m^3) for 4 hr using a nose-only inhalation system that consisted of a nebulizer, a diffusion dryer, and an inhalation chamber with 12 ports for animal holding tubes. DEP suspension in water was sonicated for 10 min, aerosolized using a nebulizer, and carried by generated air (carrier air) through a diffusion dryer. The carrier air was mixed with clean air (dilution air) from a different air source before entering the inhalation chamber. Effluent from the inhalation chamber was passed through a HEPA filter to remove particles. The total air flowing through the inhalation chamber was regulated by the flow rates of the carrier air and dilution air. DEP concentrations in the chamber were monitored by gravimetric sampling of dust collected on a filter at a sampling rate of 1 L/min. The estimated lung deposits of DEPs for the 4-hr inhalation exposure, according to the method of Leong et al. (*24*), were 194 and 384 µg/rat for 50 and 100 mg/m^3 dose groups, respectively.

Listeria was cultured overnight in brain–heart infusion broth (Difco Laboratories, Detroit, MI, USA) at 37°C in a shaking incubator. After incubation, the bacteria concentration was determined spectrophotometrically at an optical density of 600 nm and diluted with sterile saline to the desired concentrations. Two hours after DEP exposure, rats were lightly anesthetized with methohexital sodium (35 mg/kg body weight, intraperitoneally; Eli Lilly Co., Indianapolis, IN, USA) and inoculated intratracheally with approximately 100,000 *Listeria* in 500 µL of sterile saline or with 500 µL of the vehicle alone, according to the method of Antonini et al. (*25*).

Bronchoalveolar lavage (BAL) and biochemical assay of BAL fluid. At 3 and 7 days after bacteria instillation, rats were deeply anesthetized with an overdose of sodium pentobarbital (50 mg/kg, intraperitoneally; Butler, Columbus, OH, USA) and then exsanguinated by severing the abdominal aorta. The trachea was cannulated, and the lungs were lavaged with 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) at a volume of 6 mL for the first lavage and 8 mL for the subsequent lavages. The supernatant of the first lavage (~4 mL/rat) was kept separately from the others and saved at –70°C for various assays. From the subsequent lavages, a total of 80 mL of bronchoalveolar lavage (BAL) fluid was collected from each rat and centrifuged at 500 × *g* for 10 min at 4°C. All cell pellets from an individual rat were combined, washed, and resuspended in 1 mL PBS buffer. The numbers of AMs and neutrophils in the BAL cell suspension were determined according to their unique cell diameters (*26*) using an electronic cell counter equipped with a cell-sizing unit (Coulter Electronics, Hialeah, FL, USA). The remaining BAL cells were used for primary cell culture to determine functional activity of the cells, as described below.

Albumin content, a measure to quantitate increased permeability of the bronchoalveolar–capillary barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were determined in the acellular BAL fluid from the first lavage. Measurements were performed with an automated Cobas Fara II analyzer (Roche Diagnostic Systems, Indianapolis, IN, USA). The albumin content was determined colorimetrically based on albumin binding to bromcresol green using an albumin BCG diagnostic kit (Sigma Chemical Co., St. Louis, MO, USA). The LDH activity, expressed as units per liter of BAL fluid, was determined by measuring the formation of reduced form of nicotinamide adenine dinucleotide using the Roche Diagnostic reagents and procedures (Roche Diagnostic Systems).

***Pulmonary clearance of* Listeria.** After BAL, the lungs were removed from all *Listeria*-infected rats and homogenized in 10 mL sterile water using a Polytron 2100 homogenizer (Brinkmann Instruments, Westbury, NY, USA). The tissue homogenates or their dilutions were quantitatively plated in triplicate on brain–heart infusion agar plates and incubated at 37°C overnight. For each plate, the colony-forming units (CFU), an index of viable bacteria, were counted using a scanner. The counts were averaged and corrected for dilution to yield the CFU per milliliter by a computer-based program (CIA-BEN V2.2; Spiral Biotech, Inc., Norwood, MA, USA), through which the CFU per lung from each treatment group was determined.

Measurement of phagocytosis. The BAL cells, 5 × 10^5 cells/well, were incubated at 37°C for 1 hr in a 24-well plate in PBS with 1% fetal bovine serum (FBS) to allow cell attachment to glass coverslips. After the incubation period, nonadherent cells were removed by washing with PBS. The adhered AMs were treated with carboxylate-modified, yellow-green FluoSpheres (2.0 µm; Molecular Probes, Eugene, OR, USA) and rocked at 37°C for 1 hr at a concentration of 30 beads/cell. After the second incubation period, AMs were washed twice with PBS to remove any free beads, fixed with 2% paraformaldehyde overnight, and stained with 0.1 µg/mL of fluorochrome nile red (Molecular Probes) for 5 min. The glass coverslips were mounted on microscope slides, and images were recorded from a Sarastro 2000 laser scanning confocal microscope fitted with an argon-ion laser (Molecular Dynamics, Inc., Sunnyvale, CA, USA) using an excitation light of 514 nm. This technique allowed for discrimination of AMs and neutrophils that adhered to the coverslips. The phagocytic activity of AMs was expressed using a weighted phagocytic index (WPI) determined as follows. Two hundred AMs per rat for each of the treatment groups were evaluated and scored as having 0, 1–4, 5–9, 10–14, or > 15 beads/cell. These scoring groups were assigned to have a numerical factor of 0, 1, 2, 3, and 4, respectively. The number of AMs in each scoring group was first multiplied by its numerical factor and then added together for all scoring groups. This summation was then divided by 200 (total number of cells) to give the WPI.

To ascertain whether the change in phagocytic activity was due to a direct interaction of AMs with DEPs, an *in vitro* assay was also performed. Briefly, 5 × 10^5 AMs isolated from normal male Brown-Norway rats were treated with 25–200 µg/mL DEPs for 2 hr or 100 µg/mL DEPs for 1–24 hr in a rocked culture tube in a humidified incubator (37°C and 5% CO_2). We used dimethylsulfoxide as a solvent control. Viability was assessed by trypan blue exclusion assay, and measurement of phagocytosis was carried out as described above.

Determination of cytokines. BAL cells recovered from rats were suspended in the RPMI-1640 medium (Gibco BRL Life Technologies, Gaithersburg, MD, USA) containing 2 mM glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin, 5 × 10^{-5} M

2-β-mercaptoethanol, 5 mM HEPES, and 10% heat-inactivated FBS. Aliquots of 1 mL cell suspensions, adjusted to 4×10^6 AMs, were added to each well of 24-well tissue culture plates (Costar, Cambridge, MA, USA) and incubated in a humidified incubator (37°C and 5% CO_2) for 1 hr to allow cell attachment to plastic plate. The nonadherent BAL cells were then removed by rinsing the monolayers three times with RPMI medium. These AM-enriched cells were then treated with or without LPS (1 μg/mL; Sigma Chemical Co.) for 24 hr. The AM-conditioned media were collected and centrifuged (1,200 × *g* for 4 min) and the supernatants were aliquoted and stored at –70°C until assayed. To ensure that the number of adherent cells was the same in various culture samples, studies were carried out to determine the cellular protein levels after incubation. The adherent cells were treated with 0.5% Triton X-100 at 37°C for 30 min, and the media were collected and centrifuged. The protein contents in supernates were determined using Sigma Diagnostic reagents and procedures (Sigma Chemical Co.) on a Cobas Fara II analyzer (Roche Diagnostic Systems). The results did not show a significant difference among the samples from various treatment groups (data not shown).

The concentrations of IL-1β, IL-12, and TNF-α in the culture media were quantified by the enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (BioSource International, Inc., Camarillo, CA). The level of detection for each cytokine measured using the ELISA kit was 31.2–2,000 pg/mL for IL-1β, 7.8–500 pg/mL for IL-12, and 15.6–1,000 pg/mL for TNF-α. Absorbance was read at 450 nm with a microplate spectrophotometer reader (SpectraMax 250; Molecular Devices Co., Sunnyvale, CA, USA). The concentrations of these cytokines in the first BAL fluid were also quantified to provide an assessment of *in vivo* cytokine production by AMs.

Statistical analysis. Results are expressed as mean ± SE of multiple measurements. Statistical analyses were carried out with the JMP IN statistical program (SAS, Inc., Cary, NC, USA). 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's honestly significant different (HSD) test. For all analyses, the criterion of significance was set at $p < 0.05$.

Results

***Pulmonary inflammatory responses to DEPs and* Listeria.** Intratracheal inoculation of 100,000 *Listeria* caused a significant increase in the number of lavageable neutrophils at 3 and 7 days postinfection compared with the noninfected controls. Significantly increased yield of lavageable AMs, however, was observed only at 7 days postinfection in these *Listeria*-infected animals (Table 1). Exposure to DEPs alone also resulted in significant increases in the number of AMs as well as in neutrophil infiltration. These alterations persisted through 7 days, and the magnitudes of change increased with increasing DEP exposure doses. At the higher DEP dose (100 mg/m^3), the number of AMs and neutrophils in *Listeria*-infected rats were 2- and 4–7-fold that of the air and noninfected controls, respectively. The yield of BAL neutrophils from rats exposed to 100 mg/m^3 DEPs and *Listeria* was significantly higher than those from rats treated with either DEPs or *Listeria* alone.

As indices of lung injury, albumin content and LDH activity were measured in the acellular BAL fluid (Table 2). Exposure to DEPs alone did not increase BAL fluid albumin levels or LDH activity except at the higher dose and the 7-day postexposure time point. The results also show that *Listeria* infection alone caused increases in both albumin content and LDH activity compared with the noninfected control. In the high-dose DEP plus *Listeria* group, higher albumin levels and LDH activity were noted above *Listeria* alone at 3 days postexposure and for LDH at 7 days postexposure. There was, however, a descending trend in these elevations at day 7, suggesting that the lungs were recovering from the inflammatory injury.

***Pulmonary clearance of* Listeria.** The effects of DEP exposure on lung clearance of *Listeria* are shown in Table 3. Rats exposed to clean air showed an increased bacterial count (from 1×10^5 to 4.3×10^5) in the lungs at day 3 but substantial bacterial clearance at day 7. In rats exposed to DEPs at 50 and 100 mg/m^3, the bacterial counts at day 3 were more than 2- and 10-fold that of the air control, respectively. Both increases were statistically significant. At day 7, the bacteria count for rats exposed to the higher dose of DEPs was much lower than that at day 3 but remained significantly elevated compared with air control.

Table 1. Effects of DEP exposure and/or *Listeria* infection on the yield of lavageable AMs and neutrophils in rats.

DEP dose (mg/m^3)	3 Days postexposure		7 Days postexposure	
	AM (× 10^6)	Neutrophils (× 10^6)	AM (× 10^6)	Neutrophils (× 10^6)
Without *Listeria*				
Air	4.48 ± 0.76	1.55 ± 0.12	5.63 ± 0.64	1.78 ± 0.17
50	5.17 ± 0.91	3.16 ± 0.46[a]	8.70 ± 1.60[a]	4.10 ± 0.41[a]
100	8.97 ± 1.04[a]	10.64 ± 2.15[a]	10.42 ± 1.89[a]	7.72 ± 1.79[a]
With *Listeria*				
Air	4.88 ± 0.49	9.46 ± 0.88[b]	16.53 ± 1.76[b]	8.02 ± 0.74[b]
50	5.79 ± 1.10	8.88 ± 1.39[b]	15.21 ± 3.31[b]	7.55 ± 1.42[b]
100	9.05 ± 1.62[a]	14.44 ± 1.99[a,b]	16.68 ± 1.31[b]	16.10 ± 1.50[a,b]

See "Materials and Methods" for details. Values are expressed as mean ± SE ($n = 5$) of cell numbers (× 10^6); data were analyzed by one-way ANOVA followed by Tukey-Kramer's HSD test for multiple mean comparisons for each treatment group at the same dose and exposure time.
[a]Significantly different from air controls, $p < 0.05$. [b]Significantly different from noninfected controls, $p < 0.05$.

Table 2. Effects of DEP exposure and/or *Listeria* infection on albumin content and LDH activity in BAL fluid from rats.

DEP dose (mg/m^3)	3 Days postexposure		7 Days postexposure	
	Albumin (mg/mL BAL fluid)	LDH (U/L BAL fluid)	Albumin (mg/mL BAL fluid)	LDH (U/L BAL fluid)
Without *Listeria*				
Air	0.22 ± 0.03	90.50 ± 4.37	0.19 ± 0.02	68.20 ± 11.80
50	0.27 ± 0.04	116.80 ± 22.52	0.17 ± 0.01	60.00 ± 6.93
100	0.32 ± 0.07	116.00 ± 19.91	0.25 ± 0.03	104.80 ± 7.14[a]
With *Listeria*				
Air	0.39 ± 0.02[b]	118.20 ± 6.83[b]	0.31 ± 0.04[b]	92.00 ± 17.18[b]
50	0.35 ± 0.05[b]	130.60 ± 19.57[b]	0.30 ± 0.04[b]	113.60 ± 17.47[b]
100	0.59 ± 0.05[a,b]	170.60 ± 18.12[a,b]	0.44 ± 0.01[b]	151.40 ± 9.04[a,b]

See "Materials and Methods" for details. Values are expressed as mean ± SE ($n = 5$); data were analyzed by one-way ANOVA followed by Tukey-Kramer's HSD test for multiple mean comparisons for each treatment group at the same dose and exposure time.
[a]Significantly different from air controls, $p < 0.05$. [b]Significantly different from noninfected controls, $p < 0.05$.

Table 3. Effects of DEP exposure on pulmonary clearance of *Listeria*.

DEP dose (mg/m^3)	CFU/lung (× 10^5)		
	Initial infection	3 Days postinfection	7 Days postinfection
Air	1.0	4.3 ± 1.2	0.2 ± 0.0
50	1.0	9.5 ± 3.1[a]	0.2 ± 0.1
100	1.0	42.2 ± 7.6[a]	0.4 ± 0.1[a]

See "Materials and Methods" for details. Values are expressed as mean ± SE ($n = 5$).
[a]Significantly different from air control, $p < 0.05$.

Macrophage phagocytosis. The *in vivo* effects of DEP exposure and/or *Listeria* infection on the phagocytic activity of AMs are shown in Figure 1. These results indicate that at both 3 and 7 days postexposure, the WPI of AMs was significantly lowered as a result of DEP exposure in a dose-dependent manner. *Listeria* infection did not appear to alter the phagocytic activity of AMs from rats exposed to either clean air or DEPs. Figure 2 shows the dose- and time-dependent effects of DEPs on AM phagocytosis *in vitro*: the WPI of AMs decreases with increasing DEP dose and increasing incubation time. The cells treated with 100 μg/mL DEPs for 24 hr showed slightly decreased viability (79%), whereas the others showed no marked cytotoxicity after treatment with DEPs at the designed doses at each time point (viability ranging from 87% to 98%).

Cytokines in BAL fluid. The concentrations of IL-1β, TNF-α, and IL-12 in BAL fluid obtained from various treatment groups are shown in Figure 3. At 3 days postexposure, *Listeria* infection resulted in a significant increase in IL-1β in both air-exposed and the lower-dose DEP-exposed rats. DEP exposure at the higher dose appeared to enhance IL-1β levels in the BAL fluid. In the combined high-dose DEP/*Listeria* exposure, however, the concentration of IL-1β was significantly lower than those in animals exposed to DEP or *Listeria* alone. The BAL fluid of control rats contained relatively low concentrations of TNF-α. DEP exposure did not affect the production of TNF-α, but the BAL levels of TNF-α from *Listeria*-treated rats were significantly higher than those of the noninfected rats at 3 days postexposure. *Listeria* infection strongly induced the production of IL-12 at 3 days postexposure. DEP exposure, which had little or no effect on the BAL level of IL-12, significantly inhibited *Listeria*-induced IL-12 production. At day 7, the concentrations of various cytokines in the BAL fluid from DEP- and/or *Listeria*-exposed rats were not different from the corresponding cytokine levels obtained from the air-exposed control.

Cytokines in AM-conditioned media. The effects of DEP exposure on the spontaneous release of IL-1β, TNF-α, and IL-12 by AMs from rats with or without *Listeria* infection was determined. The high-dose DEP exposure enhanced the spontaneous release of IL-1β by AMs (Figure 4A). This increase was dose dependent, and the higher dose of DEP exposure (100 mg/m^3) showed significant stimulatory effect at both 3 and 7 days postexposure. AMs from *Listeria*-infected rats also showed increased secretion of IL-1β at 3 days postinfection. This increase was markedly suppressed by DEPs at both exposure doses. The spontaneous release of TNF-α by AMs was not affected by DEP exposure but was significantly enhanced by *Listeria* infection (Figure 4B) at 3 days postexposure. DEPs also had no effect on TNF-α secretion in *Listeria*-infected rats. At day 7, the spontaneous release of TNF-α by AMs from all rats decreased to near basal level. Figure 4C shows that DEP exposure alone had no effect on AM secretion of IL-12. *Listeria*, on the other hand, induced AM secretion of IL-12 at 3 days postexposure. In the combined exposure, however, *Listeria*-induced IL-12 secretion was significantly suppressed by DEPs at both 3 and 7 days postexposure.

Figure 5 shows the secretion of IL-1β, IL-12, and TNF-α by AMs in response to *ex vivo* LPS challenge using cells obtained from various exposure groups. A comparison of Figures 4 and 5 shows that LPS has a general stimulatory effect on AM secretion of these proinflammatory cytokines. *Listeria*-infected AMs, however, showed an increased response to LPS in the secretion of IL-1β compared with the air control (Figure 5A). DEP-exposed AMs showed little to moderate response to LPS stimulation. But in the combined exposure group, the *Listeria*-induced secretion of IL-1β was significantly reduced by DEPs at both exposure doses and at 3 and 7 days postexposure. Figure 5B shows that the secretion of TNF-α by AMs under *Listeria* and/or LPS stimulation was significantly inhibited by DEPs in a dose-dependent manner at both 3 and 7 days postexposure. The effect of DEPs on macrophage secretion of IL-12 is shown in Figure 5C. *Listeria*-infected AMs showed increased response to LPS stimulation in the production of IL-12 compared with AMs obtained from air-exposed rats. DEP exposure, which had no effect on the secretion of IL-12 by noninfected AMs, inhibited the production of the same cytokine by AMs from rats exposed to *Listeria*. This DEP effect was found significant at the higher DEP exposure dose at both 3 and 7 days postexposure.

Discussion

Listeria is a Gram-positive, facultative intracellular bacteria. Unlike most typical extracellular pathogens, *Listeria* induces both innate (nonspecific) and cell-mediated (antigen-specific) immune responses upon infection. This distinguishing feature of *Listeria* infection makes it feasible to serve as an experimental probe to assess how an immunotoxic xenobiotic affects both innate and cell-mediated immunity of the host. Actually, experimental listeriosis has been a widely accepted method for studying cell-mediated immune responses (*27–29*). Several reports have shown that the *Listeria* infection model is also applicable to the respiratory system for assessing pulmonary host defense mechanisms (*20,25,30–32*) and the importance of cytokines in resistance to bacterial infection (*33–35*). The present study demonstrates that *Listeria* induces innate pulmonary immunity and may initiate T-cell–mediated immune responses in Brown-Norway rats, thus allowing one to characterize the mechanism(s)

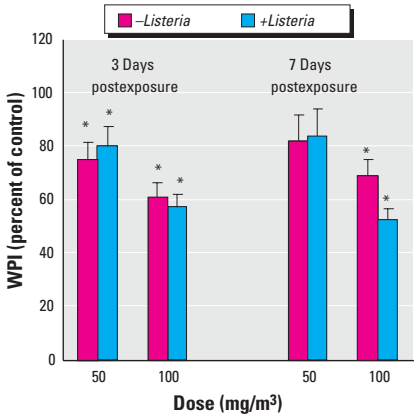

Figure 1. The WPI of AMs recovered from rats exposed to DEPs (50 and 100 mg/m^3) with or without *Listeria* at 3 and 7 days postexposure. Values are expressed as mean ± SE of the percentage of air control.
*Significantly different from the corresponding air controls, $p < 0.05$.

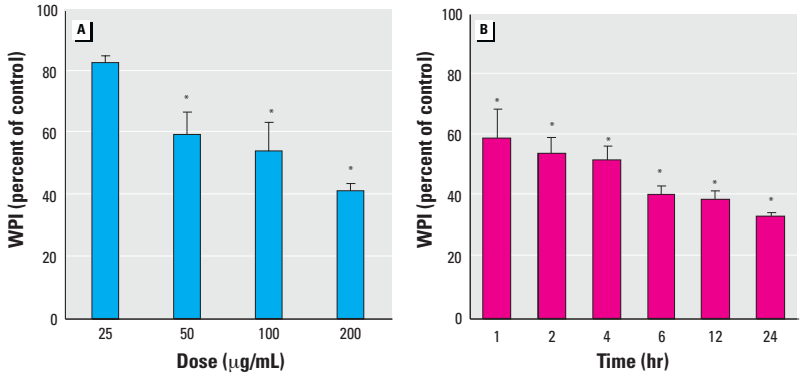

Figure 2. Effect of DEPs on the WPI of AMs *in vitro*. AMs recovered from normal rats were exposed to (*A*) 25–200 μg/mL DEPs for 2 hr or (*B*) 100 μg /mL DEPs for 1–24 hr. Values are expressed as mean ± SE of the percentage of saline control.
*Significantly different from the corresponding saline controls, $p < 0.05$.

by which DEP exposure alters the pulmonary host defense system.

The present study is intended to show the effects of acute DEP exposure on the pulmonary immune/inflammatory responses. Because the exposure duration was short (4 hr), relatively high exposure doses (50 and 100 mg/m^3) were selected so that the DEP effects can be clearly analyzed. These doses may appear to be high compared with the reported environmental and occupational concentrations, but they in fact result in lung deposits that are relevant to both nonoccupational and occupational exposure settings. Based on the reported values of ventilation rate (0.16 L/min) and percentage deposition (~10%) for rats (*24*), the estimated lung deposit of DEPs for a 4-hr exposure to 100 mg/m^3 is 384 µg. This value is among the lowest values reported in DEP studies in rodents. The deposit of 384 µg in the rat lung is equivalent to a deposit of 96,000 µg in the human lung. Although the latter seems to be a large number, it is reachable through chronic exposure to low doses. The air concentration of DEPs nationwide is relatively low (~2–5 µg/m^3), but in certain urban areas, the air level of DEPs can be considerable higher. In the Los Angeles Basin, one estimate has placed the rate of DEP intake by humans at 300 µg every 1–3 days (*36*). Using a percentage deposition of 25% for humans, one can arrive at a daily intake of 75 µg and an accumulative value of 96,000 µg in 3.5 years. This suggests that even at a considerable rate of pulmonary clearance, it is still possible that in urban areas where high concentrations of DEPs are found, there is a significant accumulation of DEPs in the lungs of long-time residents. In occupational settings such as in certain underground mining sites, the air DEP concentration may reach as high as 3.65 mg/m^3 (*2*). Even at 1 mg/m^3, the daily deposit of DEPs would be 2,400 µg (deposition, 25%; ventilation rate, 20 L/min). At this rate, the accumulated lung deposit of DEPs would reach the value of 96,000 µg in 40 working days. These calculations demonstrate that the lung deposits of DEPs from doses used in the present study are within the potential concentration range for both nonoccupational and occupational settings.

Brown-Norway rats were used because of their applicability to investigations involving immunologic reactions such as pulmonary allergic sensitization. These rats exhibited high resistance to *Listeria* infection and survived at initial inoculation doses as high as 600,000 *Listeria*/rat (data not shown). In the present study, rats exposed to DEPs or clean air for 4 hr were inoculated with 100,000 bacteria and maintained for up to 7 days. All rats, including those exposed to the higher dose of DEPs, which resulted in the highest lung burden of bacteria (4.22 × 10^6 *Listeria*/lung) at 3 days postinfection, survived without marked symptoms during the entire experimental period. The short-term DEP exposure via the nose-only inhalation system was shown to cause only a moderate inflammatory injury in the lung. In rats exposed to DEPs only, there was only a slight increase in LDH activity but with no significant change in albumin content in the BAL fluid. This short-term DEP exposure, however, clearly increased the susceptibility of rats to *Listeria* infection because rats exposed to DEPs and then inoculated with *Listeria* were less able to clear bacteria from the lungs than were rats exposed to clean air at 3 and 7 days postexposure. These results indicate that

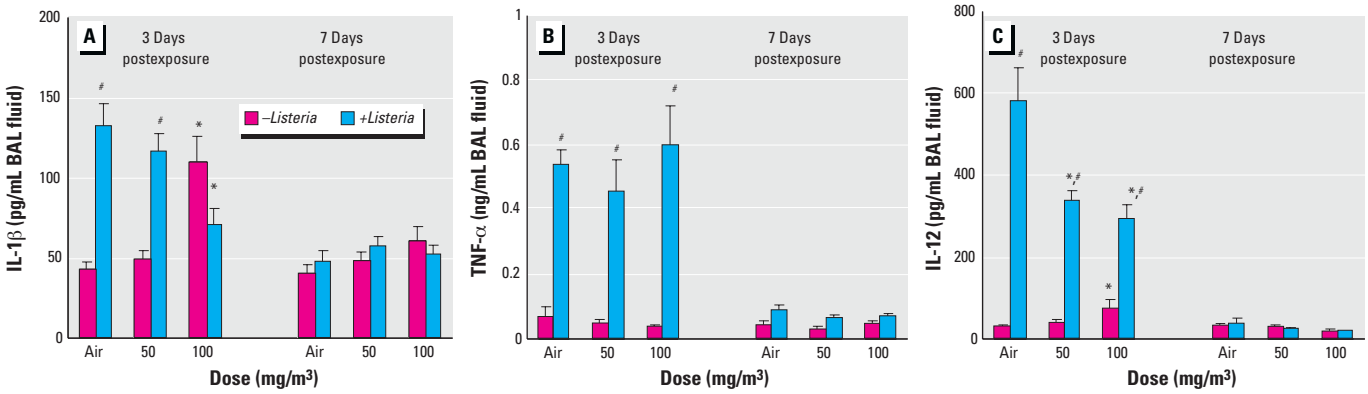

Figure 3. Concentrations of (*A*) IL-1β, (*B*) TNF-α, and (*C*) IL-12 in BAL fluid recovered from rats exposed to air or DEPs (50 and 100 mg/m^3) with or without *Listeria* at 3 and 7 days postexposure. Values are expressed as mean ± SE.
*Significantly different from the corresponding air controls, $p < 0.05$. #Significantly different from the corresponding nonexposed controls, $p < 0.05$.

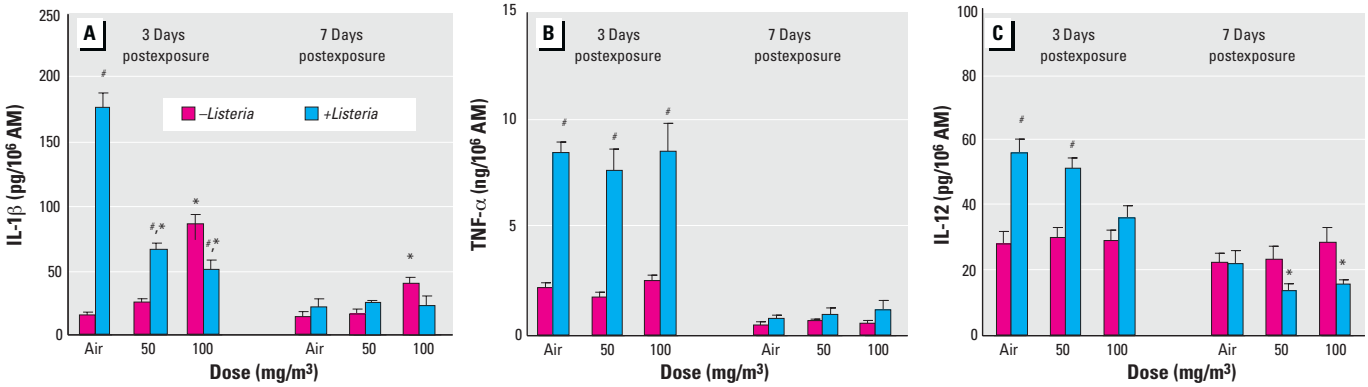

Figure 4. Spontaneous release of (*A*) IL-1β, (*B*) TNF-α, and (*C*) IL-12 by AMs from rats exposed to air or DEPs (50 and 100 mg/m^3) with or without *Listeria* and harvested at 3 and 7 days postexposure. See "Materials and Methods" for details. Concentrations of the cytokines in the culture media were quantified and expressed as mean ± SE.
*Significantly different from the corresponding air controls, $p < 0.05$. #Significantly different from the corresponding uninfected controls, $p < 0.05$.

inhaled DEPs can result in cellular functional changes in rats at concentrations that do not cause substantial inflammatory injury in the alveolar space.

Listeria can live within a variety of host cells, including endothelial and epithelial cells, as well as some macrophages (*37*). Studies have shown that the initial host response to *Listeria* involves rapid recruitment of neutrophils and macrophages to the site of infection and the activation of natural killer (NK) cells (*38*). Activated macrophages have also been shown to play an important role in killing *Listeria* (*27,39*). Indeed, as a key cell type in the innate immune system, AMs serve to provide the primary and first line of defense against bacteria that reach the distal lung. These cells engulf and, through production of oxygen and nitrogen radicals and cytokines, kill the bacteria, thereby sequestering them from the vulnerable respiratory membrane (*6,7,20*). To study the underlying mechanism involved in the DEP-compromised defense against respiratory infection, we have investigated the potential alteration of macrophage functions by DEPs in the presence and absence of *Listeria* infection. The phagocytic activity of AMs is directly linked to bacterial clearance. Van Loveren et al. (*31*) have shown that exposure to ozone slows the pulmonary clearance of *Listeria* in rats and decreases the number of the bacteria ingested and killed by AMs. In the present study, the effect of DEPs on the phagocytic activity of AMs was assessed, and the results demonstrate that AM phagocytosis was significantly suppressed by inhaled DEPs at 3 and 7 days postexposure (Figure 1). This effect may be attributed to direct interaction of DEPs with AMs, as demonstrated by the dose- and time-dependent inhibition of phagocytosis by DEPs in *in vitro* studies (Figure 2). Jakab et al. (*40*) have suggested that such an interaction may involve a suppression of macrophage membrane receptor-mediated phagocytic activity. The impairment of phagocytic activity of DEP-exposed AMs may at least partially account for the decreased pulmonary clearance of *Listeria* in the Brown-Norway rats.

Although the innate immune response is efficient at limiting the initial spread of infection, effective clearance of *Listeria* depends on acquired T-cell–mediated immunity (*28*). Several studies have demonstrated the importance of cytokines in resistance to *Listeria* infection (*41,42*). Through cytokine secretion, macrophages play a key role in the acquired immune system to induce appropriate sequential reactions against bacterial infection (*43,44*). We have previously reported that AMs exposed to DEPs, both *in vitro* and *in vivo*, showed decreased response to *ex vivo* LPS stimulation in the production of IL-1 and TNF-α (*15,16*). In the present study, short-term inhalation exposure to DEPs leads to lowered concentrations of IL-1β and IL-12 in the BAL fluid, suggesting that the *in vivo* secretion of these cytokines by lung cells from *Listeria*-infected rats was inhibited by DEP exposure (Figure 3). This is consistent with the data that the spontaneous release of these cytokines by AMs from *Listeria*-infected and DEP-exposed rats was significantly lowered compared with cytokine release by AMs obtained from *Listeria*-infected and air-exposed rats (Figure 4). Furthermore, although *Listeria*-infected AMs showed increased secretion of IL-1β, TNF-α, and IL-12 in response to *ex vivo* stimulation by LPS, AMs from rats also exposed to DEPs were diminished in their ability to respond to *ex vivo* LPS challenge. The DEP effect is particularly clear at the higher DEP exposure dose. These results show that AMs respond to *Listeria* infection with increased secretion of IL-1β, TNF-α, and IL-12. In rats preexposed to DEPs, however, the *in vivo* production of IL-1β and IL-12 was suppressed, and AMs exhibited diminished capacity to respond to an *ex vivo* stimulation with LPS in the secretion of IL-1β, IL-12, and TNF-α.

It has been known that IL-1 and TNF-α secreted by AMs are necessary for the generation of a protective immune response against *Listeria* (*33,45*). Studies have also shown that mice deficient in the 55-kDa TNF receptor are extremely sensitive to *Listeria* and succumb easily to infection (*27,28*), suggesting that TNF-α is necessary for the elimination of *Listeria*. Both IL-1 and TNF-α activate NK cells to release interferon-γ (IFN-γ), which activates macrophages to kill the bacteria. IL-12, on the other hand, has been shown to play a key role for the initiation of T-cell–mediated immunity. IL-12 is a heterodimeric cytokine first described for its ability to stimulate IFN-γ production by NK cells and enhance CD8 cytotoxicity (*46,47*). Among its many functions, IL-12 is involved in initiation of cell-mediated immune responses, development and survival of Th1 cells, and down-regulation of Th2 immune responses. One of the important aspects of IL-12 is its ability to be produced very rapidly after infection, which provides the cytokine with the potential to influence Th1 cell development (*11*). The suppressive effects of DEPs on the production of IL-12 indicate that DEP exposure might elicit an adverse influence on the development of T-cell–mediated immunity. This may disrupt the balance of Th1 and Th2 immune responses, resulting in increased susceptibility to, and severity of, pulmonary bacterial infection and, perhaps, allergic sensitization as well. Our studies on the effects of DEP exposure on T-cell–mediated immunity in response to *Listeria* infection will be reported elsewhere.

In summary, this study demonstrates that exposure to DEPs significantly decreased the rate of bacterial clearance from the lungs compared with the air controls. Exposure to DEPs attenuated *Listeria*-induced activation of AMs and resulted in a diminished capacity in phagocytosis and production of IL-1β, IL-12, and TNF-α.

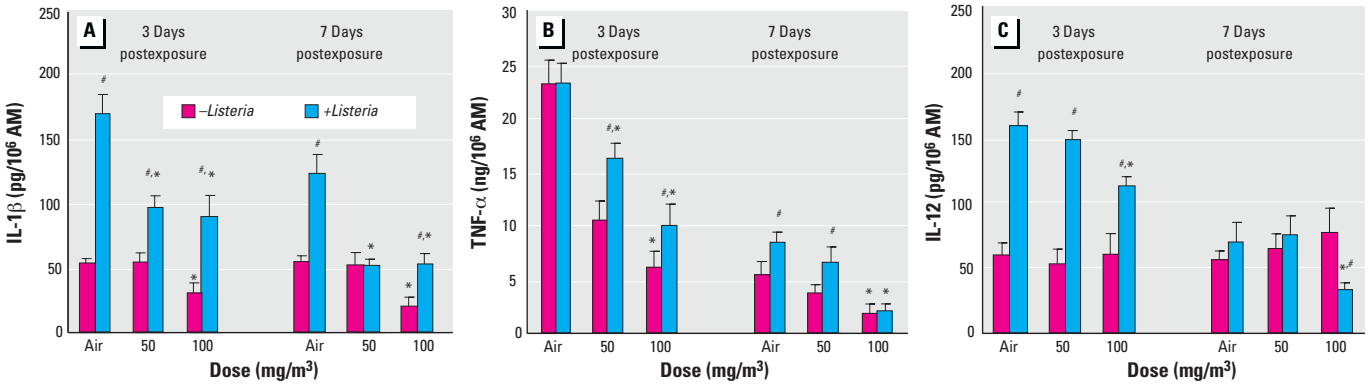

Figure 5. Production of (*A*) IL-1β, (*B*) TNF-α, and (*C*) IL-12 by AMs from rats exposed to air or DEPs (50 and 100 mg/m^3) with or without *Listeria* in responses to *ex vivo* LPS stimulation. AMs harvested at 3 and 7 days postinfection were incubated in RPMI-1640 medium with 1 μg/mL of LPS for 24 hr. Concentrations of the cytokines in the culture media were quantified and expressed as the mean ± SEM.
*Significantly different from the corresponding air controls, $p < 0.05$. #Significantly different from the corresponding noninfected controls, $p < 0.05$.

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