TOXICOLOGICAL SCIENCES **77,** 263–271 (2004)
DOI: 10.1093/toxsci/kfh035
Advance Access publication December 2, 2003

Suppression of Cell-Mediated Immune Responses to *Listeria* Infection by Repeated Exposure to Diesel Exhaust Particles in Brown Norway Rats

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Received on September 11, 2003; accepted on October 31, 2003

Diesel exhaust particles (DEP) have been shown to alter pulmonary immune responses to bacterial infection. Exposure of rats to 100 mg/m^3 DEP for 4 h was found to aggravate *Listeria monocytogenes* (*Listeria*) infection at 3 days postinfection, but the bacteria were largely cleared at 7 days postinfection due to the development of a strong T cell–mediated immunity. In the present study, we examined the effects of repeated DEP exposure at lower doses on pulmonary responses to bacterial infection. Brown Norway rats were exposed to DEP by inhalation at 20.62 ± 1.31 mg/m^3 for 4 h/day for 5 days, followed by intratracheal inoculation with 100,000 *Listeria* at 2 h after the last DEP exposure. DEP-exposed rats showed a significant increase in lung bacterial load at both 3 and 7 days postinfection. The repeated DEP exposure was shown to suppress both the innate, orchestrated by alveolar macrophages (AM), and T cell–mediated responses to *Listeria*. DEP inhibited AM production of interleukin- (IL-) 1β, tumor necrosis factor- (TNF-) α, and IL-12 but enhanced *Listeria*-induced AM production of IL-10, which has been shown to prolong the survival of intracellular pathogens such as *Listeria*. DEP exposure also suppressed the development of bacteria-specific lymphocytes from lung-draining lymph nodes, as indicated by the decreased numbers of T lymphocytes and their CD4$^+$ and CD8$^+$ subsets. Furthermore, the DEP exposure markedly inhibited the *Listeria*-induced lymphocyte secretion of IL-2 at day 7, IL-10 at days 3 and 7, and interferon- (IFN-) γ at days 3 to 10 postinfection when compared to air-exposed controls. These results show a sustained pattern of downregulation of T cell–mediated immune responses by repeated low-dose DEP exposure, which is different from the results of a single high-dose exposure where the acute effect of DEP aggravated bacteria infection but triggered a strong T cell–mediated immunity.

Key Words: **diesel exhaust particles; inhalation; *Listeria monocytogenes*; alveolar macrophages; lymphocytes; cytokines.**

Exposure to airborne particulate matter (PM) is linked to the development of respiratory diseases. The Committee on the Environmental and Occupational Health Assembly of the American Thoracic Society (1996a,b) have reported a correlation of the daily ambient PM concentrations with increased incidence of respiratory symptoms, hospitalization, and premature mortality among the general population. The fine fractions of PM, with diameters <2.5 μm ($PM_{2.5}$), are predominant in emissions from the combustion of fossil fuels and are more closely associated with mortality and adverse health effects than the coarse fractions (PM_{10}) of PM (Delfino *et al*.,1997; Peters *et al*.,1997). Diesel exhaust particles (DEP), which are generated through extensive industrial use of heavy-duty diesel engines, are the major constituent of the atmospheric $PM_{2.5}$ found in urban and industrialized areas. Thus, DEP may play a key role in the PM-mediated health effects.

Indeed, with diameters <2 μm, these particles can remain airborne for long periods of time and get deposited in great numbers deeply in the lungs. The air concentration of DEP nationwide is relatively low (~2–5 μg/m^3), but in certain urban areas, the air level of DEP can be considerable higher. In the Los Angeles Basin, one estimate has placed the rate of DEP intake by humans at 300 μg/1–3 day(s) (Diaz-Sanchez, 1997). Also, exposure of truckers, railroad and construction workers, and engine mechanics to DEP is an occupational health concern. A report from the Department of Labor showed that the worst-case mean exposures to DEP in underground metal and nonmetal mines are about 2000 μg/m^3, with maximum measurements as high as 3650 μg/m^3 (Department of Labor, Mine Safety, and Health Administration, 1998). To date, DEP exposure has been shown to induce pulmonary inflammation (Nagai *et al*., 1996), increased susceptibility to bacterial infection (Yin *et al*., 2002, 2003), allergic asthma (Al-Humadi *et al*., 2002; Takano *et al*., 1997), pulmonary fibrosis, and lung cancer (Mauderly *et al*., 1994) in experimental animals. Because DEP are a major component of particulate air pollution in most

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industrialized urban areas, their effect on pulmonary infections is of great environmental and occupational concern.

Increasing evidence shows that DEP may exert a strong effect on the pulmonary immune system. In allergic asthma, DEP exposure was shown to skew the immune response toward immunoglobulin E production and augment allergen-induced airway inflammation and eosinophil infiltration (Al-Humadi *et al*., 2002; Nel *et al*.,1998, 2001). While DEP enhance the T helper (Th) 2 responses to allergic sensitization, the same particles have been shown to suppress the host defense mechanism against bacterial infection, which may involve a downregulation of the $CD4^+$ Th1 and $CD8^+$ immunity (Yin *et al*., 2002). The DEP-altered immune responses can be readily seen through changes in cytokine production by alveolar macrophages (AM) and lymphocytes. Under allergic sensitization, such as by ovalbumin, the adjuvant effect of DEP on interleukin- (IL-) 4 and IL-5 production by lymphocytes has been demonstrated (Steerenberg *et al*., 2003; Takano *et al*., 1997). On the other hand, DEP, through the organic components, have been shown to inhibit bacteria- or lypopolysaccharide- (LPS-) mediated secretion of tumor necrosis factor-α (TNF-α), IL-1β, and IL-12 (Yang *et al*., 1997, 1999; Yin *et al*., 2002, 2003) by AM. IL-12 is rapidly produced by AM in response to bacterial infection and is known to play a key role in initiating and maintaining a Th1 response to clear the bacteria (Trinchieri, 1995, 1998). The suppression of production of IL-12 and other cytokines by AM exposed to DEP weakens the host defense and may lead to diminished development of bacteria-specific lymphocytes that secret interferon-γ (IFN-γ).

Although exposure of rats to DEP resulted in particle distribution in the alveolar region as well as in the lung-draining lymph nodes (LDLN) through particle translocation in the local lymphoid system (Chan *et al*., 1981; Yu and Yoon, 1991), the *in vivo* effect of DEP exposure on the T lymphocyte–mediated immune responses against bacterial infection has not been clearly demonstrated. Studies from our laboratory have shown that in Brown Norway rats exposed to 100 mg/m^3 DEP for 4 h and then to *Listeria monocytogenes* (*Listeria*), DEP strongly aggravated *Listeria* infection at 3 days postinfection, resulting in a 10-fold increase in bacterial count when compared to the air-exposed, *Listeria*-infected rats. But, at 7 days postinfection, the DEP-exposed rats showed a strong T cell–mediated immunity and were able to clear the bacteria as efficiently as the air-exposed rats (Yin *et al*., 2002, 2003). This is despite the fact that DEP exhibit a direct inhibitory effect on lymphocyte production of key cytokines including IL-2 and IFN-γ in cell culture.

We hypothesized that the alteration of T cell–mediated immunity depends not only on the pharmacological effect of DEP but also on a dynamic relationship between pulmonary responses and exposure conditions. It is possible that with intact host defense mechanism, a normal lung, can effectively respond to an acute toxic insult even when the exposure dose is high. Under this condition, the large increase in the number of bacteria may result in a strong development of bacteria-specific T cell responses to eliminate the bacteria, thus overcoming the inhibitory effect of DEP. Under repeated or chronic exposures at a lower dose, however, where DEP have a moderate effect on AM function, the inhibitory effect of DEP on the development of T lymphocytes may become a determining factor in regulating the pulmonary responses. For this reason, we have examined the effect of DEP on the pulmonary immune responses to *Listeria* infection using a repeated exposure protocol at 20 mg/m^3 for 4 h/day for 5 days. Our objective was to show the inhibitory effects of DEP on AM and T lymphocyte–mediated immune responses, their correlation with the bacterial clearance and inflammatory lung injury, and the altered infection pattern via this exposure protocol in comparison to that of a previous study in which rats were exposed to DEP through a single exposure of 100 mg/m^3 for 4 h (Yin *et al.*, 2002, 2003).

MATERIALS AND METHODS

Materials. A standardized DEP sample (standard reference material 2975) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). *Listeria monocytogenes* used was a strain 10403S, serotype 1, routinely cultured in our laboratory (Yin *et al*., 2002, 2003).

Animals. Male Brown Norway rats [BN/CrlBR] weighing 200–250 g were purchased from Charles River Laboratories (Wilmington, MA). 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 for 1 week before use in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. During the week before inhalation exposure, the animals were conditioned to the exposure unit. Animals were placed in the tubes of the exposure unit for increasing time periods from 1–4 h/day for 4 successive days.

Inhalation exposure of rats to DEP. The inhalation exposure system and DEP exposure procedure used in this study have been previously described and characterized (Yin *et al*., 2002, 2003). Briefly, rats were exposed to either filtered air or DEP (20.62 $\pm$ 1.31 mg/m^3) for 4 h/day for 5 consecutive days using a nose-only directed flow exposure unit (CH Technologies, Inc., Westwood, NJ). DEP concentrations in the exposure unit were monitored by both gravimetric sampling of dust collected on a polycarbonate membrane filter (37 mm, 0.45 μm, Poretics Corporation, Livermore, CA) at a sampling rate of 1 l/min and a Grimm Model 1.108 portable dust monitor (GRIMM Technologies, Inc., Douglasville, GA), allowing simultaneous measurements of the particle concentration in the exposure unit in real time. The estimated mean lung deposit of DEP for the inhalation exposure, according to the calculation of Leong *et al*. (1998), was 389 $\pm$ 25 μg/rat.

Listeria culture and intratracheal instillation. *Listeria* was cultured overnight in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) at 37°C in a shaking incubator. Diluted solution of the *Listeria* culture was further cultured for 3 h to achieve log growth. Following incubation, the bacterial concentration was determined spectrophotometrically at 600 nm. For the preparation of heat-killed *Listeria monocytogenes* (HKLM), the bacteria were incubated at 80° C for 1 h, washed, and resuspended in sterile PBS. An aliquot of the HKLM was plated overnight on BHI plates to ensure that there were no viable bacteria. For animal infection, the culture was diluted with sterile saline to the desired concentration; 2 h after the last DEP exposure, rats were lightly anesthetized with methohexital sodium (25 mg/kg body weight, ip; Eli Lilly Co., Indianapolis, IN) and inoculated intratracheally with 100,000 colony-forming units (CFU) of *Listeria* in 500 μl of sterile saline or 500 μl of the vehicle alone, as described previously (Antonini *et al*., 2000). To ensure

that the number of *Listeria* given to the rats was suitable, the bacterial sample used for animal infection was diluted and plated on BHI plates and the colonies were counted after being cultured overnight at 37°C. According to the combination of inhalation exposure and bacterial instillation, there were four different treatment groups in this study, namely, air + saline, DEP + saline, air + *Listeria*, and DEP + *Listeria*.

Bronchoalveolar lavage (BAL) and biochemical assays. At 3, 7, and 10 days after bacterial inoculation, rats were deeply anesthetized with an overdose of sodium pentobarbital (50 mg/kg, ip; Butler, Columbus, OH) and euthanized by exsanguinations through the abdominal aorta. The lungs were lavaged with Ca^{2+}/Mg^{2+}-free, phosphate-buffered saline (PBS, pH 7.4) at a volume of 6 ml for the first lavage and 8 ml for the subsequent lavages until a total of 80 ml of BAL fluid was collected. The BAL fluid samples were centrifuged at 500 × *g* for 10 min at 4°C, and the cell-free supernatant from the first lavage was analyzed for various biochemical parameters. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 ml PBS. The numbers of AM and neutrophils in the BAL cell suspension were determined according to their unique cell diameters using an electronic cell counter equipped with a cell-sizing unit (Coulter Electronics, Hialeah, FL).

Albumin content, a measure to quantify 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 a COBAS MIRA auto-analyzer (Roche Diagnostic Systems, Montclair, NJ). Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromcresol green using an albumin BCG diagnostic kit (Sigma Chemical Co., St. Louis, MO). LDH activity was determined by measuring the oxidation of lactate to pyruvate coupled with the formation of a reduced form of nicotinamide adenine dinucleotide at 340 nm using the Roche Diagnostic reagents and procedures (Roche Diagnostic Systems).

Isolation of lymphocytes. All LDLN from each rat were collected and a single cell suspension was prepared as described previously (Yin *et al*., 2003). The cells were washed twice with PBS and lymphocytes were isolated by Histopaque (density, 1.083; Sigma Chemical Co.) gradient centrifugation. Briefly, the samples were centrifuged for 30 min at 2500 rpm and lymphocytes were collected, washed twice, and resuspended in 1 ml PBS. The number of lymphocytes was counted by a standard hemocytometer and the cell viability was assessed by the trypan blue dye exclusion technique. The cell samples thus prepared showed both the lymphocyte content and viability of greater than 98%.

Differential counts of T cell subsets. The numbers of $CD4^+$ and $CD8^+$ T cell subsets in lymphocytes recovered at 7 and 10 days postexposure were determined by flow cytometry, as described previously (Yin *et al*., 2003). Lymphocytes were stained with the addition of FITC-labeled $CD4^+$ or $CD8^+$ monoclonal antibody (mAb, BD Pharmingen, San Diego, CA) for 30 min on ice in the dark. The flow cytometric data were collected with a Becton-Dickinson FACScan using FACScan Research software (version B; Becton-Dickinson Immunocytometry System, San Jose, CA), and analyzed using the PC-LYSYS (v 1.0) software (Becton-Dickinson). The absolute numbers of cells in each lymphocyte subpopulation were calculated by multiplying the total number of cells by the percentage of the total within each phenotype, as determined by flow cytometry.

Pulmonary clearance of Listeria. The colony forming units (CFU), an index of viable bacteria per lung in all *Listeria*-infected rats, were determined as described previously (Yin *et al*., 2002). The lungs were removed from all *Listeria*-infected rats following BAL and homogenized in sterile water. The tissue homogenates or their dilutions were quantitatively plated in triplicate on brain heart infusion agar plates using an Autoplate 4000 spiral plater (Spriral Biotech, Inc., Norwood, MA). After incubation at 37°C overnight, the CFU in each plate were counted using a scanner. The counts were averaged and corrected for dilution to yield the CFU/ml by a computer-based program (CIA-BEN V2.2; Spiral Biotech, Inc., Norwood, MA), through which the CFU per lung from each treatment group were determined.

Cell culture and cytokine determination. The BAL cells and lymphocytes were suspended in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated fetal bovine serum. Aliquots of 1 ml cell suspensions, adjusted to 2×10^6 AM or lymphocytes, were added to each well of a 24-well tissue culture plate. Before the stimulation, the BAL cells were incubated in a humidified incubator (37°C and 5% CO_2) for 2 h to allow cell attachment to the culture plate. The nonadherent BAL cells were then removed by rinsing the monolayer three times with culture medium. The remaining AM-enriched cells or lymphocytes were then treated with either LPS (1 μg/ml, Sigma Chemical Co.), concanavalin A (ConA, 2 μg/ml, Sigma Chemical Co.), or HKLM (10^7/ml) for 24 or 48 h. The AM- and lymphocyte-conditioned media were collected, centrifuged (1200 × *g* for 4 min), and aliquots of the supernatants were stored at –70°C until assayed.

The amounts of TNF-α, IL-1β, and IL-10 produced by AM and IL-2, IL-6, IL-10, and IFN-γ produced by lymphocytes in cell cultures under various exposure conditions were quantified by the enzyme-linked immunosorbent assay (ELISA) using the OptEIA ELISA sets according to the manufacturer's instructions (BD Pharmingen). Briefly, 96-well ELISA plate (Corning, Corning, NY) was coated with a purified antirat mAb and blocked with an assay diluent (BD PharMingen) before use. Recombinant standards (BD Pharmingen) and samples were added to the plate and incubated for 2 h at room temperature. The plate was then incubated with biotinylated mAb for 1 h and avidin–horseradish peroxidase conjugate for 30 min at room temperature. The plate was developed with tetramethylbenzidine with 50% H_2O_2 in the dark, and color reaction was stopped with 2 N H_2SO_4 and then analyzed at 450 nm with a SpectraMax 250 plate spectrophotometer using Softmax Pro 2.6 software (Molecular Devices Co., Sunnyvale, CA). The levels of IL-12 in the culture media were quantified by ELISA using a commercial ELISA kit (BioSource International, Inc., Camarillo, CA). The range of detection was: 31.3–2000 pg/ml for IL-1β, IL-2, IL-6, and IFN-γ, 15.6–1000 pg/ml for IL-10 and TNF-α, and 7.8–500 pg/ml for IL-12.

Statistical analysis. The experimental results are expressed as means ± standard error (SE) of multiple measurements. Statistical analyses were carried out with the JMP IN statistical program (SAS Institute, Inc., Cary, NC). Values were expressed as means ± SE. 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 Difference (HSD) test. For all analyses, the criterion of significance was set at $p < 0.05$.

RESULTS

The Effects of DEP Exposure on Pulmonary Responses to Listeria *and Bacterial Clearance*

The number of recovered AM and neutrophils in the BAL fluid were counted as an index of inflammation, whereas the LDH activity and albumin content in the acellular BAL fluid were determined to indicate cellular and epithelial lung injury (Fig. 1). *Listeria* inoculation significantly increased AM and neutrophil counts, LDH activity, and albumin content at 3 and 7 days after bacterial challenge. The elevated AM and neutrophil counts from *Listeria*-infected rats were substantially decreased but were still significantly higher than those of the air-exposed, noninfected control rats at 10 days postinfection. At that time point, the LDH activity and albumin content of *Listeria*-infected rats had returned to the control levels. These results show that the Brown Norway rats were capable of recovering from the *Listeria*-induced inflammatory lung injury. The DEP exposure (20.62 ± 1.31 mg/m^3, 4 h/day, 5 days)

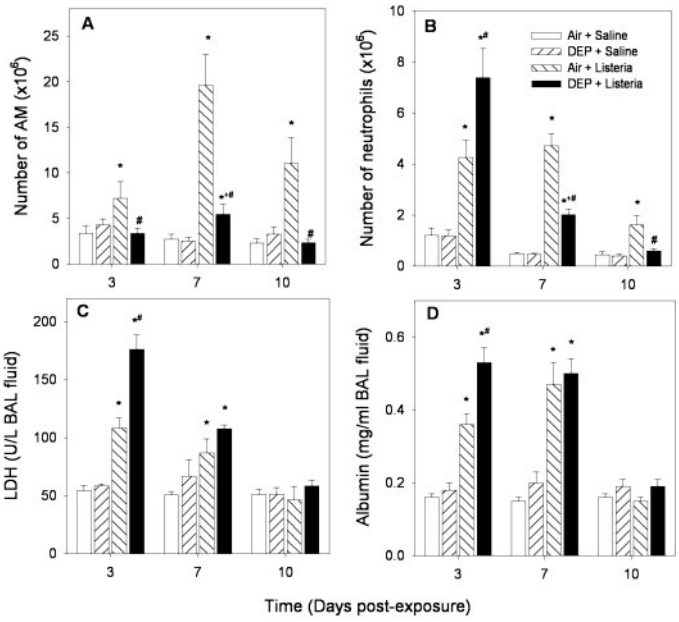

FIG. 1. Yield of (A) AM, (B) neutrophils, (C) LDH activity, and (D) albumin content in bronchoalveolar lavage fluid from rats exposed to DEP and/or *Listeria*. *Significantly different from air + saline group, $p < 0.05$. $^{+}$Significantly different from DEP + saline group, $p < 0.05$. $^{\#}$Significantly different from air + *Listeria* group, $p < 0.05$.

alone did not change AM and neutrophil counts or the LDH activity or albumin content at any time point but significantly augmented *Listeria*-induced neutrophil infiltration, LDH activity, and the albumin content at 3 days postexposure, as seen in the lungs of DEP-exposed and *Listeria*-infected rats. The combined DEP and *Listeria* exposure was also found to decrease the recoverable AM at all time points and neutrophils at 7 and 10 days postinfection, when compared to data from air-exposed and *Listeria*-infected rats.

Table 1 shows the pulmonary handling of *Listeria* infection by rats in various exposure groups at 3, 7, and 10 days after *Listeria* infection. Intratracheal instillation with 100,000 CFU of *Listeria* resulted in increased CFU in the lungs of rats exposed to either filtered air or DEP at 3 days postinfection. Rats exposed to DEP showed significantly greater lung CFU than rats exposed to filtered air. The bacterial count for the DEP-exposed rats at day 7, though much lower than that at 3 days postinfection, remained significantly elevated compared to that of the air-exposed control rats. These results show that DEP, following a 5-day repeated exposure protocol, suppresses pulmonary immunity against *Listeria* up to 7 days post-DEP exposure. At 10 days postinfection, the bacteria count from the lungs of DEP-exposed rats were no different from that of the air-exposed control rats.

TABLE 1
The Effects of Repeated DEP Exposure on Pulmonary Clearance of *Listeria*

	CFU/lungs ($\times 10^5$)			
		Postinfection		
	Initial infection	3 days	7 days	10 days
Air	1.00	221.6 ± 51.1	0.12 ± 0.02	0.09 ± 0.03
DEP	1.00	429.6 ± 66.0*	0.31 ± 0.04*	0.10 ± 0.02

Note. Rats were exposed nose-only to air or DEP at 20.62 ± 1.31 mg/m^3, 4 h/day, for 5 days. At 2 h after the last exposure, the rats were inoculated intratracheally with 1×10^5 *Listeria.* At 3, 7, and 10 days postinfection, the lungs were removed and the colony forming units (CFU) per lungs were determined.

*Significantly different from air control, $p < 0.05$.

The Effects of DEP and Listeria *Exposures on Cytokine Production by AM*

The effects of repeated DEP exposures, *Listeria* infection, and of their combination on AM secretion of cytokines including IL-1β, TNF-α, IL-12, and IL-10 were assessed following *ex vivo* LPS challenge of cells isolated from various exposure groups at 3, 7, and 10 days post-DEP (or *Listeria*) exposure (Fig. 2). LPS was used to enhance cytokine production. *Listeria* infection resulted in an increased response of AM to LPS

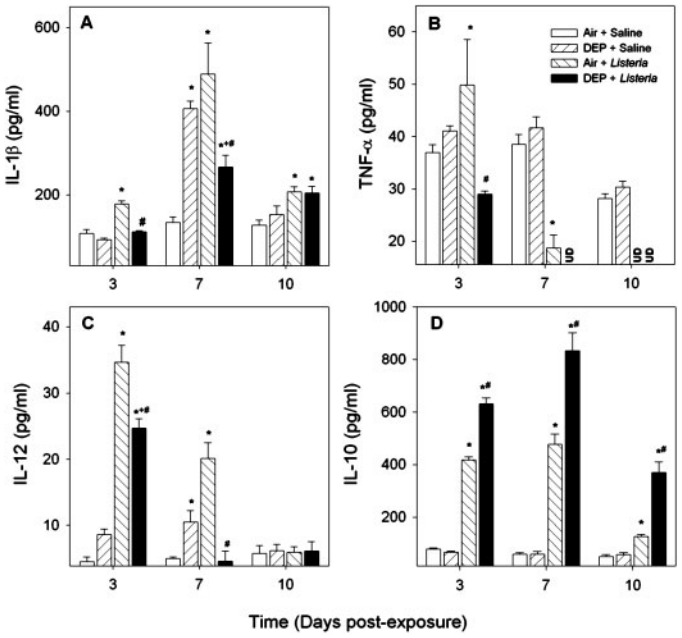

FIG. 2. Production of (A) IL-1β, (B) TNF-α, (C) IL-12, and (D) IL-10 by AM from rats exposed to DEP and/or *Listeria* in responses to *ex vivo* LPS stimulation. AM harvested at 3, 7, and 10 days postinfection were incubated with 1 μg/ml of LPS for 24 h. Concentrations of the cytokines in the culture media were quantified by ELISA. *Significantly different from air + saline group, $p < 0.05$. $^{+}$Significantly different from DEP + saline group, $p < 0.05$. $^{\#}$Significantly different from air + *Listeria* group, $p < 0.05$. UD, under detection limit.

challenge in IL-1β production at all time points with a peak concentration occurring at 7 days postinfection. The DEP exposure alone increased AM production of IL-1β at day 7 but had no effect on AM production of this cytokine at other time points compared to the air-exposed control. However, in comparison to the *Listeria* effect, DEP in the combined exposure significantly lowered the *Listeria*-induced cellular production of IL-1β for up to 7 days (Fig. 2A). AM from *Listeria*-infected rats showed a moderate increase in TNF-α secretion at 3 days postexposure but a decrease at 7 and 10 days postinfection compared to cells from the air-exposed control. DEP, which did not alter AM production of TNF-α as compared to the air-exposed control, were shown in the combined DEP and *Listeria* exposure to lower the production of TNF-α by AM at 3 and 7 days postexposure (Fig. 2B). *Listeria* infection had a profound effect on AM secretion of IL-12 and IL-10. Significant induction of AM secretion of IL-12 in response to LPS challenge occurred at 3 and 7 days postinfection. DEP, which had no or only a moderate effect on this cytokine, strongly inhibited the *Listeria*-induced IL-12 production by AM at 3 and 7 days postexposure (Fig. 2C). At 10 days postexposure, the production of IL-12 by AM was the same for all exposure groups. *Listeria* infection was shown to induce production of IL-10 by AM, with elevated levels measurable up to 10 days postinfection (Fig. 2D). DEP exposure alone did not induce AM production of IL-10, but, in the combined exposure, DEP strongly augmented the *Listeria*-induced IL-10 production by AM at all time points. Figure 2 shows that, under the repeated exposure protocol, DEP downregulated macrophage production of inflammatory cytokines but induced a sustained IL-10 production by AM to dampen the host defense against *Listeria*.

The Effects of DEP and Listeria *Exposures on T Lymphocyte Responses*

The numbers of lymphocytes recoverable from the LDLN were significantly increased by *Listeria* but not by DEP exposure alone (Fig. 3A). However, in the combined DEP and *Listeria* exposure, DEP moderately but not significantly lowered the total number of lymphocytes induced by *Listeria* at 3 and 7 days postexposure. To study the effect of repeated DEP exposures on the development of *Listeria*-specific T cell immunity, which mainly occurs at the late stage (5 days postinfection and later) of *Listeria* infection, the numbers of $CD4^+$ and $CD8^+$ T cell subsets in lymphocytes recovered at 7 and 10 days postexposure were determined. *Listeria* infection was shown to increase the number of total T cells (Fig. 3B) and the development of $CD4^+$ and $CD8^+$ subsets (Figs. 3C and 3D) at both 7 and 10 days postinfection. At 7 days postinfection, the total number of T cells in LDLN from *Listeria*-infected rats was increased to about 4-fold of the air-exposed control, whereas the corresponding increases in $CD4^+$ and $CD8^+$ cells were about 2- and 10-fold, respectively. DEP strongly inhibited the *Listeria*-induced T cell development. Figures 3C and 3D

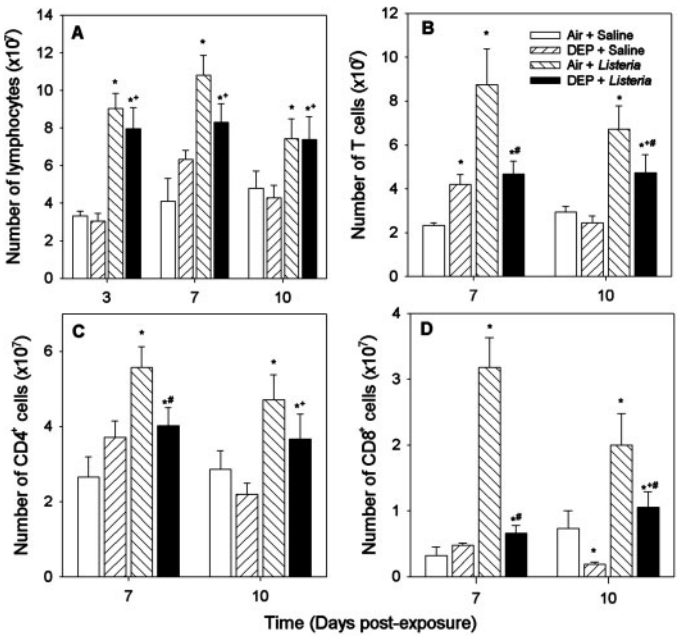

FIG. 3. Lymphocyte differentiation in lung-draining lymph nodes of rats exposed to DEP and/or *Listeria*. *Significantly different from air + saline group, $p < 0.05$. $^+$Significantly different from DEP + saline group, $p < 0.05$. $^\#$Significantly different from air + *Listeria* group, $p < 0.05$.

show that DEP moderately reduced the *Listeria*-induced $CD4^+$ cells at day 7 and strongly inhibited the development of $CD8^+$ cells at 7 and 10 days postinfection. These results also show that, in the combined DEP and *Listeria* exposure, the percentages of total T cells, the $CD8^+$ subset, and the $CD8^+/CD4^+$ ratio in the lymphocyte population were significantly decreased in comparison with those from air-exposed and *Listeria*-infected rats. Figure 3 shows that *Listeria* elicits a T cell response characterized by a strong development of the $CD8^+$ T cell subset, and that the repeated DEP exposure markedly inhibits this immune response.

The effects of repeated DEP exposures, *Listeria* infection, and of their combination on lymphocyte production of IL-2 and IFN-γ in response to *ex vivo* ConA stimulation are shown in Figure 4. ConA was used to enhance lymphocyte production of cytokines. The *Listeria* infection was associated with an elevated lymphocyte production of IL-2 at 3 and 7 days postinfection (Fig. 4A). DEP exposure, which did not affect lymphocyte development or IL-2 production in noninfected rats, was found to inhibit *Listeria*-induced IL-2 production at 7 days postinfection. Lymphocytes from *Listeria*-infected rats showed a marked ability to secret IFN-γ at all time points (Fig. 4B). This ability was significantly inhibited, especially at 7 and 10 days postinfection, by DEP exposure, which alone induced IFN-γ production at day 3 but did not alter lymphocyte population or their secretion of IFN-γ at later time points.

To study the effect of DEP exposure on antigen-specific response against *Listeria* infection, the production of IL-6 and IL-10 by lymphocytes from rats exposed to air, DEP, *Listeria*,

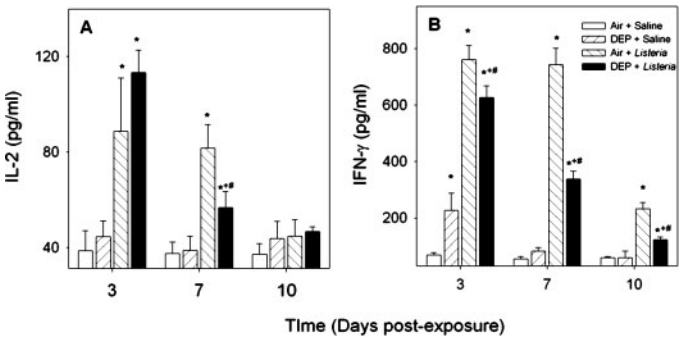

FIG. 4. Production of (A) IL-2 and (B) IFN-γ by lymphocytes from lung-draining lymph nodes of rats exposed to DEP and/or *Listeria* in responses to *ex vivo* ConA stimulation. Lymphocytes isolated at 3, 7, and 10 days postinfection were incubated with 2 μg/ml of ConA for 24 h. Concentrations of the cytokines in the culture media were quantified by ELISA. *Significantly different from air + saline group, $p < 0.05$. $^{+}$Significantly different from DEP + saline group, $p < 0.05$. $^{\#}$Significantly different from air + *Listeria* group, $p < 0.05$.

or their combination was determined following *ex vivo* challenge of cells with HKLM (Fig. 5). In comparison to cells from the air-exposed control, lymphocytes from *Listeria*-infected rats showed increased production of IL-6 with a peak level occurring at 3 days postinfection (Fig. 5A). Lymphocytes from the combined DEP and *Listeria* exposure showed a further increased capacity in IL-6 production at 3 and 7 days postinfection. The DEP-mediated increase was moderate but significant, which may be due to the fact that DEP exposure alone moderately enhanced lymphocyte production of this cytokine. Lymphocytes from *Listeria*-infected rats also exhibited an increased capacity to secrete IL-10 in response to HKLM (Fig. 5B). In comparison, lymphocytes from the combined exposure were less responsive to HKLM in the production of IL-10. DEP exposure alone had no effect on lymphocyte production of IL-10.

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 (Luster *et al*., 1988, Shen *et al*., 1998). Several reports have shown that the *Listeria* infection model is also applicable to the respiratory system for assessing pulmonary host defense mechanisms (Jakab, 1993; Reasor *et al*., 1996; Yang *et al*., 2001; Yin *et al*., 2002, 2003). Studies from our laboratory have shown that DEP, through inhalation exposure at 50 or 100 mg/m^3 for 4 h, suppressed the early pulmonary defense against *Listeria* in Brown Norway rats, resulting in substantially elevated bacterial growth at 3 days postexposure. The aggravated infection elicited a strong T cell–mediated immune response that allowed the rats to clear the bacteria at 7 days post-DEP exposure. The T cell–mediated immunity was characterized by a significant increase in $CD4^+$ and $CD8^+$ cells, the $CD8^+/CD4^+$ ratio, and lymphocyte production of IL-2, IL-6, and IFN-γ at 7 days postexposure (Yin *et al*., 2002, 2003).

The present study was carried out to elucidate how DEP may affect the development of T cell–mediated immunity under an exposure condition that would not lead to a strong acute pulmonary inflammatory response. Brown Norway rats were exposed to DEP at 20.62 ± 1.31 mg/m^3 for 4 h/day for 5 days. This exposure protocol gave a total DEP inhalation equivalent to that of a single dose exposure at 100 mg/m^3 for 4 h but induced no inflammatory lung injury in noninfected rats, as indicated by the recovered AM, neutrophils, LDH activity, and the albumin content in the BAL fluid. The dose may appear to be high in comparison to the reported environmental and occupational concentrations, but it results in a lung deposit that is relevant to both nonoccupational and occupational exposure settings, as discussed previously (Yin *et al*., 2002). Based on the reported values of minute ventilation (0.16 l/min) and percentage of deposition (~10%) for rats (Leong *et al*., 1998), the estimated lung deposit of DEP for the current exposure is 389 μg, which is equivalent to a deposit of about 97,000 μg in the human lungs according to the ratio of lung surface area of humans and rats (about 1:250). Although the latter seems to be a large amount, it is reachable through chronic exposure to low doses. For example, using a percentage of deposition of 25% for humans, a resident of the Los Angeles Basin area can arrive at a daily intake of 75 μg and an accumulative value of 97,000 μg in 3.5 years, according to one estimate that has placed the rate of DEP intake by humans there at 300 μg/1–3 day(s)

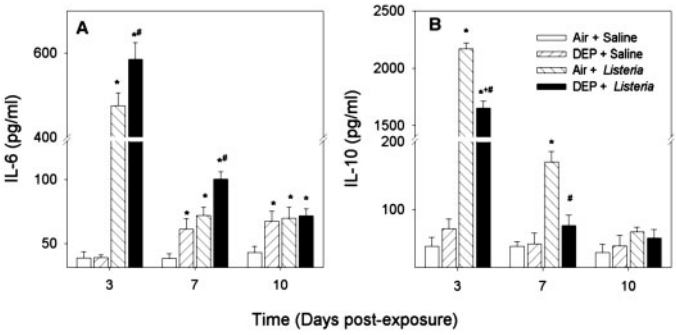

FIG. 5. Production of (A) IL-6 and (B) IL-10 by lymphocytes from lung-draining lymph nodes of rats exposed to DEP and/or *Listeria* in responses to *ex vivo* HKLM stimulation. Lymphocytes isolated at 3, 7, and 10 days postinfection were incubated with HKLM (10^7/ml) for 48 h. Concentrations of the cytokines in the culture media were quantified by ELISA. *Significantly different from air + saline group, $p < 0.05$. $^{+}$Significantly different from DEP + saline group, $p < 0.05$. $^{\#}$Significantly different from air + *Listeria* group, $p < 0.05$.

(Diaz-Sanchez, 1997). This suggests that even at a considerable rate of pulmonary clearance, it is still possible that, in urban areas where a high concentration of DEP is found, there is a significant accumulation of DEP in the lungs of long-time residents. In occupational settings such as in certain underground mining sites, the air DEP concentration has reached as high as 3.65 mg/m^3 (Department of Labor, Mine Safety, and Health Administration, 1998). Even at 1 mg/m^3, the daily deposit of DEP would be 2400 μg (percentage of deposit, 25%; minute ventilation, 20 l/min). At this rate, the accumulated lung deposit of DEP would reach the value of 97,000 μg in 40 working days. These calculations demonstrate that the lung deposits of DEP from doses used in the current 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 immunological reactions such as pulmonary allergic sensitization. These rats exhibited very high resistance to *Listeria* infection and survived at initial inoculation doses as high as 600,000 CFU of *Listeria*/rat (data not shown). In the present study, rats exposed to DEP or clean air for 5 days were inoculated with 100,000 CFU of bacteria and maintained for up to 10 days. All rats, including those exposed to DEP and showed elevated lung burden of bacteria (42.96×10^6 CFU of *Listeria*/lung) at 3 days postinfection, survived without marked symptoms during the entire experimental period. The repeated exposure protocol resulted in a significant retardation of *Listeria* clearance up to 7 days postinfection. In comparison, rats exposed to 100 mg/m^3 for 4 h showed strongly aggravated infection at day 3, but the bacteria count returned to control level at 7 days postinfection (Yin *et al*., 2002). This indicates the dynamic nature of pulmonary responses with respect to the severity of the toxic stimulation and the potential of a delayed effect by the accumulation of inhaled DEP on the pulmonary immune system. For this reason, we have examined the effect of repeated DEP exposure on AM production of cytokines and the development of T cell–mediated immune responses against *Listeria* infection.

Numerous AM-derived cytokines are known to be necessary for the generation of a protective immune response against *Listeria* (Bancroft *et al*., 1989; Czuprynski *et al*., 1992). Both IL-1β and TNF-α activate NK cells to release IFN-γ, which activates macrophages to kill the bacteria. These cytokines are also T cell activators (Akira *et al*., 1990; Hsieh *et al*., 1993).

IL-10, on the other hand, is a potent immunosuppressive factor that downregulates macrophage bactericidal activity (Fleming *et al*., 1999). The effect of DEP exposure on the production of IL-10 by AM is of interest because some intracellular pathogens, including *Listeria*, specifically target macrophages for infection and use IL-10 to dampen the host immune response and, thus, prolong their survival (Redpath *et al*., 2001). Also, IL-12 has been shown to play a key role in the initiation of T cell–mediated immunity (Trinchieri, 1995, 1998). This cytokine is produced rapidly by AM following infection to initiate the development of Th1 responses (Park and Scott, 2001). Our study shows that the repeated DEP exposure resulted in a diminished ability of *Listeria*-infected AM to secret IL-1β, TNF-α, and IL-12 up to 7 days postinfection. These results are consistent with those obtained from a single dose exposure at 100 mg/m^3 (Yin *et al*., 2002). Furthermore, we showed that DEP strongly augmented *Listeria*-induced IL-10 production by AM up to 10 days postinfection. This clearly indicates a prolonged effect of the inhaled DEP on macrophage function and suggests that the inhibition of the innate immune responses is responsible for the impairment of early bacterial clearance.

Although the innate immunity is efficient in limiting the initial spread of infection, sterilization of *Listeria* infection depends on the later development of acquired T cell responses involving CD4$^+$ Th1 and CD8$^+$ cells, (Kaufmann, 1993; Unanue, 1997; Shen *et al*., 1998). In this aspect, the results of our study demonstrated a striking difference between the effects of repeated DEP exposure at a lower dose and the single high-dose exposure (with the same total lung burden of DEP) on the development of T cell–mediated immune responses. Under repeated low-dose exposure, DEP was found to suppress the development of bacteria-induced T lymphocytes in LDLN at 7 and 10 days postinfection (Fig. 3) and markedly inhibit the development of the CD8$^+$ T cell subset that is closely associated with *Listeria*-induced T cell responses in the Brown Norway rats (Yin *et al*., 2003). The effect of repeated DEP exposure on the pattern of cytokine secretion by lymphocytes also indicates a diminished T cell–mediated immune responses. For up to 10 days postinfection, DEP exposure inhibited the *Listeria*-induced lymphocyte secretion of IL-2, which promotes T cell proliferation, and of IFN-γ, a key cytokine in cell-mediated immunity for bacterial defense (Fig. 4). Although lymphocytes from the combined DEP and *Listeria* exposure showed a moderate increase in IL-6 production, they were significantly less responsive to HKLM in the production of IL-10 (Fig. 5). In rats exposed to 100 mg/m^3, the DEP exposure augmented the development of T cell–mediated immune responses (characterized by an elevation of the CD4$^+$ and CD8$^+$ cell counts and the CD8$^+$/CD4$^+$ ratio), increased IL-2 responsiveness, and increased lymphocyte production of IL-2 and IFN-γ (Yin *et al*., 2003).

The difference in pulmonary responses observed between the single and repeated exposure protocols may be attributed to the competing effect of *Listeria* induction of T cell–mediated immunity and the inhibition of the innate immunity by DEP. Exposure to a high dose of DEP can strongly inhibit the early defense mechanism, resulting in severely aggravated infection. In this case, pulmonary response may be dominated by an increase in T cell–mediated immunity. The Brown Norway rats appear to be very effective in mounting a CD8$^+$ T cell–mediated immunity against *Listeria*. Under repeated or chronic exposure to DEP at a low dose, the suppression of the initial immune responses can be moderate, and the development of T

cell–mediated immune responses will likely reflect the pharmacological effect of DEP. The mechanism through which DEP may alter production of cytokines by AM has not been reported. Studies have shown that the organic component of DEP was able to induce the generation of intracellular reactive oxygen species (ROS) in AM through, at least in part, increased expression of cytochrome P450 1A1 and the interaction of the organic compounds with the microsomal enzymes (Bonvallot *et al*., 2001; Takano *et al*., 2002; Ma and Ma, 2002). The ROS-mediated oxidative stress was, in turn, shown to induce an increased expression of heme oxygenase-1 (HO-1; Rensing *et al*., 1999), a stress-responsive protein that has been shown to enhance cellular production of IL-10 but downregulate TNF-α (Inoue *et al*., 2001; Tullius *et al*., 2002). Preliminary studies from our laboratory showed that the DEP-mediated changes in the production of IL-12, TNF-α, and IL-10 by *Listeria*-infected AM were regulated through ROS generation and HO-1 activity, and the same results can be replicated in cells directly stimulated with superoxide anion.

It should be noted that despite the suppressive effect of DEP on the immune responses, substantial clearance of the bacteria from lung tissue occurred at 7 and 10 days postinfection. It is possible that the cell-mediated immunity to *Listeria* developed by the Brown Norway rats was strong and eventually overcame the suppressive effect of DEP. *Listeria*, as an intracellular pathogen, may also find a home in AM and lymphocytes by inducing cellular production of IL-10 to prolong their survival. As can be seen from Figures 2 and 5, the *Listeria* infection had an inductive effect on the production of IL-10 by AM and lymphocytes up to 10 days postinfection. DEP exposure strongly enhanced the *Listeria*-induced IL-10 production by AM. Interestingly, the combined DEP and *Listeria* exposure yielded lymphocytes that secreted less IL-10 in response to HKLM than cells from *Listeria*-infected rats. The reason for this effect is not yet clear. One possibility is that the number or percentage of *Listeria*-specific T cells in lymphocytes from the combined exposure was much reduced compared to cells from rats exposed to *Listeria* only. Consequently, lymphocytes from the combined exposure are less responsive to HKLM. DEP moderately enhanced lymphocyte production of IL-6 at 3 and 7 days postexposure, which may have enhanced T cell proliferation. This cytokine is known to enhance IL-2 responsiveness and IL-2 secretion by lymphocytes (Akira *et al*., 1990; Ford *et al*., 1991).

In summary, this study demonstrates that DEP, following a repeated exposure protocol, suppressed the host defense against *Listeria* by downregulating the innate and bacteria-induced T lymphocyte responses in Brown Norway rats. The results on T cell development are strikingly different than those obtained from rats receiving a single high-dose exposure, suggesting that pulmonary responses to DEP may vary depending on the severity of exposure and that inhaled DEP and their accumulation may have a delayed effect on the pulmonary immune system.

ACKNOWLEDGMENTS

This research was supported in part by grant NIH RO1 HL 62630 from the National Heart, Lung, and Blood Institute.

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