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Effect of Diesel Exhaust Particulate on Bacillus Calmette-Guerin Lung Infection in Mice and Attendant Changes in Lung Interstitial Lymphoid Subpopulations and IFNγ Response

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The effect of exposure to diesel exhaust particulate (DEP) on bacillus Calmette-Guerin (BCG) lung infection in mice was studied. C57Bl/6J female mice were infected with BCG (2.5×10^4 bacteria/mouse) by intrapulmonary instillation, with or without coadministration of DEP (100 μg/mouse). Five weeks later, mice exposed to DEP + BCG had about a four-fold higher BCG load in the lungs than mice exposed only to BCG ($p < 0.05$). DEP treatment alone had no effect on the total number of lung lymphocytes or numbers of T, B, or NK cells recovered from lungs. In contrast, BCG infection significantly increased ($p < 0.05$) recovery levels of all types of lymphocytes from lungs. Coexposure to DEP + BCG further increased the recovery of lymphocytes from lungs of BCG-infected mice. The pulmonary lymphocyte subpopulation expressing the greatest levels of mRNA for IFNγ after BCG infection was CD4+ T cells. Expression levels were similar in mice exposed to BCG or BCG + DEP and were elevated as compared to noninfected mice and mice treated with DEP alone. Recovery of IFNγ-secreting lymphocytes and IFNγ-secreting T cells was significantly higher ($p < 0.05$) from lungs of BCG-infected mice as compared to control or DEP-exposed mice. BCG and BCG + DEP groups of mice did not differ significantly in the numbers of IFNγ-secreting lymphocytes in lungs. Taken together, these results indicated that coexposure to DEP + BCG did not significantly affect the level of IFNγ response of mice to BCG infection. However, DEP treatment was found to inhibit IFNγ-induced nitric oxide (NO) production by mouse alveolar macrophages *in vitro*. Our results indicate that DEP exposure did not alter the IFNγ response to BCG infection, but reduced responsiveness of alveolar macrophages to IFNγ. Reduced sensitivity of DEP-exposed alveolar macrophages to IFNγ may contribute to a greater load of BCG in the lungs of BCG-infected mice given DEP.

Key Words: **diesel exhaust; BCG; interferon; T cells; NK cells; macrophages; nitric oxide; lung; infection.**

It is estimated that one-third of the human population is infected with *Mycobacterium tuberculosis* (Bleed *et al.*, 2000). Most infected persons, however, contain the infection and remain free of tuberculosis. In some infected persons with compromised immunity, the dormant infection may flare up in the form of active tuberculosis. Pulmonary tuberculosis is the most common form of the disease and is spread by inhaling an aerosol containing *M. tuberculosis*. Inhaled mycobacteria are taken up by macrophages and survive intracellularly in these cells. An important component of the protective immune response to mycobacterial infection is the release of IFNγ by sensitized T cells, which can inhibit the growth of intercellular mycobacteria in macrophages (Flynn *et al.*, 1993; Mogues *et al.*, 2001; Xing *et al.*, 2001).

Diesel exhaust constitutes an important component of urban air pollution and is associated with a variety of lung diseases (McClellan 1987; Sydbom *et al.*, 2001). Diesel exhaust particulate (DEP) contained in diesel exhaust accumulates in the lungs of people living in areas where the air is polluted with diesel exhaust. It has been reported that treatment with DEP may inhibit the macrophage function (Yang *et al.*, 1999, 2001). Since *M. tuberculosis* resides in macrophages, and protective immunity to tuberculosis is dependent upon macrophage activation, it is important to understand if exposure to DEP may influence the course of *M. tuberculosis* infection. In the present study, we have examined the hypothesis that exposure to DEP may alter susceptibility to mycobacterial infections, perhaps by interfering with macrophage function and/or local immune responses in lungs.

As is the case in human *M. tuberculosis* infection, mice contain, but do not eliminate, pulmonary BCG infection. The BCG mouse infection model has therefore been used extensively to study the molecular and cellular basis of protective immunity to mycobacterial infection (Erb *et al.*, 1999; Fulton *et al.*, 2000; Ibsen *et al.*, 1997; Saxena *et al.*, 2002a,b; Wakeham *et al.*, 1998). In previous studies using this model, we found the pulmonary clearance of BCG from the mouse lung

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was associated with IFNγ production by T cells (Saxena, 2002b). In the present study, we have used this model to test the hypothesis of a possible relationship between DEP exposure and mycobacterial infection. In particular, we examined the effects of DEP on INFγ production and the INFγ-induced response of macrophages.

MATERIALS AND METHODS

Animals. C57Bl/6J female mice 12 to 15 weeks of age were obtained from Jackson Laboratories, Bar Harbor, ME, and used between 18 and 30 weeks of age. Mice were maintained in filter-topped cages in the animal facilities at NIOSH. All studies were reviewed and approved by the NIOSH Institutional Animal Care and Use Committee.

Reagents. Standard DEP (reference material 1650) was obtained from the National Institute of Standards and Technology, Gaithersburg, MD. Monoclonal antibodies (Mabs) labeled with FITC or PE (anti-CD3 [Clone 17A2], anti-NK1.1 [clone PK136], anti-CD4 [clone GK1.5], anti-CD8 [(clone 53–6.6], anti-CD16/32 [Fc-block, clone 2.4G2], and antimouse IFNγ [clone XMG1.2)], and their respective isotypic controls were obtained from Pharmingen (Pharmingen/Becton Dickinson, San Diego, CA). Unless otherwise specified, all other analytical reagents and culture media were obtained from Sigma (St. Louis, MO).

BCG and IT instillation. A seed culture of BCG (*M. bovis* Pasteur, TMCC # 1011) was kindly provided by Professor Ian Orme of the Microbiology Department, Colorado State University, Fort Collins, CO. BCG was grown in Middlebrook 7H9 culture medium supplemented with OADC® (oleic acid-albumin-dextrose-catalase) and 0.05% Tween 80. Viable BCG were counted by plating bacterial suspensions at different dilutions on Middlebrook 7H10 agar plates supplemented with OADC® and counting colonies after two weeks. Intrapulmonary instillation of BCG and/or DEP (50 μl bacterial suspension containing 2.5×10^4 bacteria, with or without 100 μg DEP per mouse) was carried out as previously described (Keane-Myers *et al.*, 1998). Control mice received 50 μl of sterile 0.1-M phosphate buffered saline (pH 7.4, PBS) by the same procedure.

Processing of tissues. Mice were sacrificed by pentobarbital overdose. Isolation of lung interstitial cells was done by enzymatic digestion of lung tissue, mechanical dispersion, and separation on a discontinuous Percoll gradient, as previously described (Saxena *et al.*, 2002a). The BCG load in different organs was determined by plating different dilutions of tissue homogenates on Middlebrook agar 7H10 supplemented with OADC® and counting the bacterial colonies two weeks later.

Flow cytometry. Cells derived from lungs were stained with Mabs against several membrane markers as well as against IFNγ, to detect cytoplasmic IFNγ. Cell suspensions of 2 to 3×10^6 cells per ml were distributed 0.1 ml per well in a deep 96-well plate. Cells were washed two times with staining buffer (1% FCS and 0.1% sodium azide in PBS) and suspended in 20 μl of staining buffer containing 1 μg of anti-CD16/32 mab (Fc-Block) and incubated at room temperature for 20 min. Staining Mabs (1 μg in 20 μl staining buffer) were then added and incubation continued for an additional 30 min at room temperature in the dark. Cells were washed twice with staining buffer and fixed by adding 0.1 ml of 0.4% paraformaldehyde solution to the loosened pellets of stained cells. For cytoplasmic staining for IFNγ, $0.3–0.5 \times 10^6$ cells in 200 μl RPMI-1640 media containing 10% fetal calf serum were cultured for 4 h with 50 ng/ml of phorbol myristic acid (PMA) and 500 ng/ml of ionomycin. Fixing/permeabalization and staining of cells for cytoplasmic IFNγ and membrane markers for T or NK cells was done by using a kit and the protocol recommended by the manufacturer (Pharmingen, San Diego, CA). Cells were analyzed on a Becton Dickinson FACSCalibur flow cytometer. Cells stained with isotypic control antibodies were used to demarcate the lymphocyte window as well as to set gates for discrimination between cells, with or without specific stain.

Isolation of pure lymphoid subpopulations and real-time PCR. Pure lung-derived lymphocyte subpopulations were isolated by negative selection following treatment of cells with appropriate monoclonal antibodies coupled with magnetic beads. Equipment, reagents, and kits for this purpose were obtained from Stem Cell Technologies, Inc., Vancouver, WA, and detailed protocols for cell separation, supplied by the manufacturer, were followed. Purity of CD4+ T cells, CD8+ T cells, and NK cells, isolated by using these kits, was above 95% in all cases as determined by flow cytometric analysis.

Purified cell preparations were stored in RNALater® (Ambion, Austin, TX) at –20°C until used for RNA isolation. Prior to preparation of total RNA, RNALater was diluted by 50% with PBS and cells ($1–2 \times 10^6$) pelleted by centrifugation at 4000 × g for 10 min. The supernatant was removed and total RNA extracted from the cell pellet using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was diluted in 20 μl DEPC-treated H_2O and subjected to DNase treatment (DNA-Free kit, Ambion, Austin, TX). Reverse transcription of extracted RNA was performed using random hexamers (Taqman Reverse Transcription Reagents kit, Applied Biosystems, Foster City, CA). Conditions for the reverse transcription reaction were as follows: 10 min at 25°C, 60 min at 48°C, and 5 min at 95°C. Ten μl per reaction of the resulting cDNA was used to perform separate Taqman real-time polymerase chain reactions to amplify cDNA encoding IFNγ and G3PDH. Taqman primers, probes, and PCR master mix were obtained from a commercial source (Applied Biosystems, Foster City, CA). Reaction mixtures were incubated in the thermal cycler (iCycler, BioRad, Hercules, CA) for two min at 50°C and then for ten min at 95°C. Thermal cycling was conducted for 60 cycles of 15 s at 95°C and 1 min at 60°C, with measurement of fluorescence done during the 60°C step. Abundance of mRNA was determined as cycle threshold (CT), defined as the cycle at which fluorescence exceeded baseline plus 10 standard deviations. CT is inversely related to mRNA abundance; the greater the initial amount of mRNA present, the lower the CT value. Due to the exponential nature of PCR, a difference in CT value of one (1) represents a two-fold difference in mRNA abundance. Based on these principles, IFNγ mRNA abundance in each sample is expressed as a percentage of G3PDH mRNA abundance according to the following formula:

Abundance of IFNγ message = $(100) * (2^{-\Delta CT})$, where $\Delta CT = (CT_{IFN}) - (CT_{G3PDH})$.

Nitric oxide (NO) assay. Alveolar macrophages from normal mice were obtained by bronchoalveolar lavage and cultured in RPMI1640 culture medium supplemented with 10% fetal calf serum, 300 μg/ml glutamine, and 60 μg/ml gentamycin. NO production was measured by determining the nitrite levels in cell culture supernatants using modified Griess reagent (Sigma, catalog no. G4410). Culture supernatants were mixed 1:1 (v/v) with Griess reagent and the color generated after a 15-min incubation at room temperature was read at 540 nm.

Statistical analysis. Two-way comparisons were performed by the Student's *t*-test using computer software Sigmastat (SPSS, Chicago, IL). Comparisons were considered significantly different at a level of $p \leq 0.05$.

RESULTS

Effect of DEP on BCG load in lungs of BCG-infected mice. We have previously standardized a mouse model for studying the course of BCG lung infection in mice after intrapulmonary instillation of BCG and the attendant changes in various lymphoid subpopulations in lung interstitium (Saxena *et al.*, 2002a). In this model, BCG load in lungs of infected mice peaked at five weeks and fell thereafter. In order to study the effect of DEP treatment on BCG peak lung load, C57Bl/6J mice were administered BCG with or without DEP, and BCG load was assessed at the 5-week time point. Results in Table 1 indicate that the lungs of mice infected with BCG in the

TABLE 1
Bacterial Load in Organs of C57Bl/6J Mice Infected with BCG in the Presence or Absence of Diesel Exhaust Particulate

Organ	and BCG Load (colonies/organ) Control	+DEP	Significance of difference
Lung	32140 ± 26845	139200 ± 53629	$p < 0.05$
Spleen	14316 ± 14588	27166 ± 15887	NS
Liver	120 ± 201	1772 ± 1228	$p < 0.05$
Lymph node	1832 ± 389	4202 ± 2123	$p < 0.05$

Note. DEP, diesel exhaust particulate. Intrapulmonary instillation of BCG was performed in C57Bl/6J female mice (2.5×10^4 bacteria ± 100 μg DEP in 50 μl PBS). Five weeks later, BCG load in lungs, spleens, livers, and lymph nodes from infected mice was determined as described in Materials and Methods. Each value represents a mean ± SD of five observations. NS, not significant.

presence of DEP had four times the BCG load of lungs from mice infected with BCG in the absence of DEP ($p < 0.05$). Although BCG load in other organs of the mice was significantly lower than in lungs, a significantly greater BCG load was seen in DEP-treated mice (Table 1). Thus, coexposure to DEP was associated with both an increase in the peak load of BCG in the lungs and increased systemic dissemination of infection.

Lymphocyte subpopulations in lung interstitium of mice infected with BCG in presence and absence of DEP. BCG infection results in an increase in the number of pulmonary T cells, which peaks at five weeks post infection (Saxena *et al.*, 2002b). In order to assess the effect of DEP on changes in lymphoid populations associated with BCG infection, C57Bl/6J mice were infected with BCG in the presence or absence of DEP, and lung interstitial lymphoid populations were isolated five weeks after the infection. These cell preparations were stained with monoclonal antibodies recognizing various phenotypic markers, and analyzed on a flow cytometer. Total recoveries of various lymphoid populations from mice infected with BCG, with or without DEP, are summarized in Table 2. These results show that DEP by itself had no significant effect on the total lymphocyte recovery or the recoveries of NK cells and CD4 and CD8 T cells. Total recovery of interstitial lymphocytes as well as the T, B, and NK cells from lungs of BCG infected mice were substantially greater than the corresponding recoveries from controls or mice treated with DEP alone. Cell recoveries from BCG infected and DEP treated mice were further increased over BCG alone group (Table 2).

IFNγ gene expression in various lymphocyte subpopulations in control and DEP-treated mice infected with BCG. In order to assess IFNγ gene expression in different lymphocyte subpopulations, $NK1.1^+$, $CD4^+$, and $CD8^+$ T cells were isolated from control, +DEP, +BCG, and +DEP + BCG groups of mice. cDNA was prepared from lymphocytes total RNA by reverse transcription. Real-time PCR was used to determine the relative abundance of IFNγ and G3PDH (glyceraldehyde-3-phosphate dehydrogenase) message in unfractionated lung-derived lymphocytes, and purified $NK1.1^+$, $CD4^+$, and $CD8^+$ T-cell preparations. Results in Figure 1 show IFNγ message abundance expressed as a percentage of G3PDH message. These results indicate that DEP treatment by itself had no effect on IFNγ gene expression in CD4 or CD8 T cells. However, there seems to be some decline in IFNγ expression in NK cells from DEP-treated mice. In BCG infected lungs, the concentration of IFNγ mRNA increased most markedly in $CD4^+$ T cells. In general, the increase in IFNγ message in lymphocytes derived from lungs of mice infected with BCG, in the presence or absence of DEP, were not different, with the exception of CD8 cells, where some increase in IFNγ message was noted in the BCG + DEP group (Fig. 1).

TABLE 2
Recoveries of Total Lymphocytes, and T, B, and NK Cells from Lungs of C57Bl/6J Mice Infected with BCG in the Presence or Absence of DEP

Treatment	Cell recovery ($\times 10^6$/lung) ± SD All lymphocytes	T cells	B cells	NK cells
Control	5.64 ± 0.94	1.49 ± 0.37	2.61 ± 0.46	0.84 ± 0.13
+DEP	5.65 ± 0.93	1.49 ± 0.26	2.09 ± 0.23	0.76 ± 0.18
+BCG	13.90 ± 3.83*	3.83 ± 1.02*	6.27 ± 1.65*	1.37 ± 0.37*
DEP + BCG	19.87 ± 5.81**	6.31 ± 2.52	8.10 ± 1.70**	1.70 ± 0.44**

Note. Intrapulmonary instillation of BCG was performed in C57Bl/6J female mice (2.5×10^4 bacteria ± 100 μg DEP in 50 μl PBS to each mouse). Five weeks later, interstitial lung lymphocytes were isolated and counted. Cell preparations were stained with monoclonal antibodies and analyzed on a flow cytometer. Knowing the total lymphocyte recovery from lungs, and the proportions of different subpopulations, total recoveries of T, B, and NK cells from lungs were calculated. Each value of cell recovery is a mean ± SD of five observations.

*$p < 0.05$, effect of BCG over control and +DEP groups.

**$p < 0.05$, difference between BCG and BCG + DEP groups.

Lymphocyte IFNγ responses were also examined at the protein level using flow cytometry. Results in Figure 2 indicate that DEP by itself had no effect on the total number of IFNγ positive lymphocytes and IFNγ positive T cells in mouse lungs. Five weeks after BCG infection, there was a marked (about fourfold) increase in number of IFNγ-positive lymphocytes. The increase was more pronounced (about sixfold) in number of $CD3^+$ T cells making IFNγ (Fig. 2). In mice infected with BCG in the presence of DEP, IFNγ response was not significantly different from the BCG-alone group (Fig. 2).

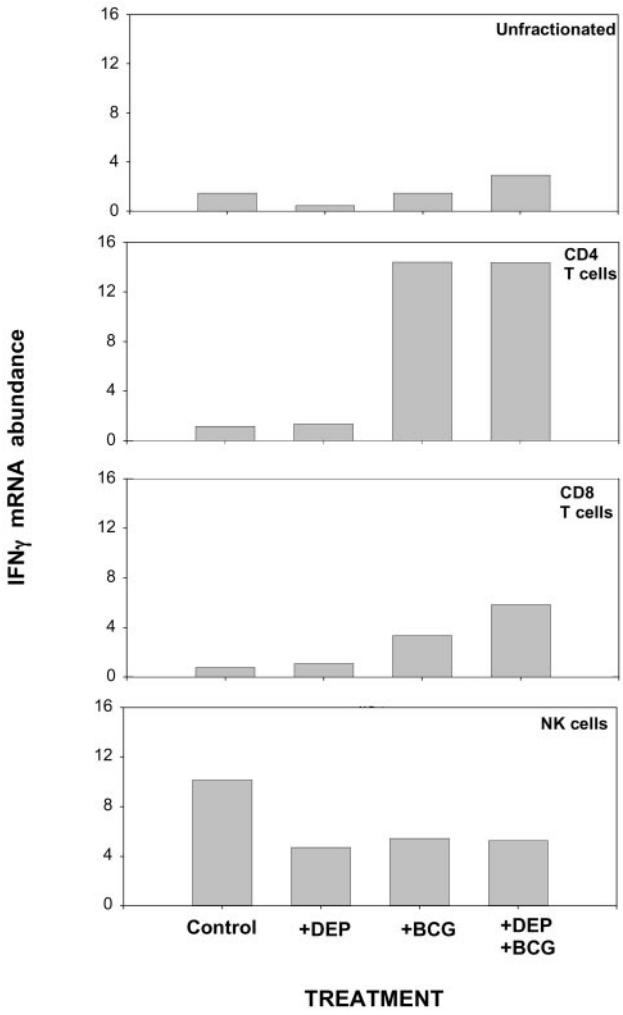

FIG. 1. Effect of exposure to DEP on BCG-induced IFNγ gene transcription in lymphocyte subpopulations derived from lung interstitium. Groups of mice were instilled with 50 μl PBS (control group) or PBS containing DEP (100 μg), BCG (2.5×10^4) and both DEP + BCG. After five weeks, lungs of 5 mice from each group were pooled and interstitial mononuclear cells isolated as previously described (Saxena *et al.*, 2002a). Cells from each group were used to isolate pure CD4 T cells, CD8 T cells, and NK cells. Total RNA was isolated from unfractionated cells as well as purified cell fractions from each group of mice. Reverse transcription was used to make cDNA, and Taqman real-time PCR was done using primers for IFNγ and G3PDH to determine the relative levels of IFNγ mRNA, as described in Materials and Methods. Each bar represents the relative IFNγ mRNA abundance in the given cell preparation as % of abundance of mRNA encoding the housekeeping gene G3PDH.

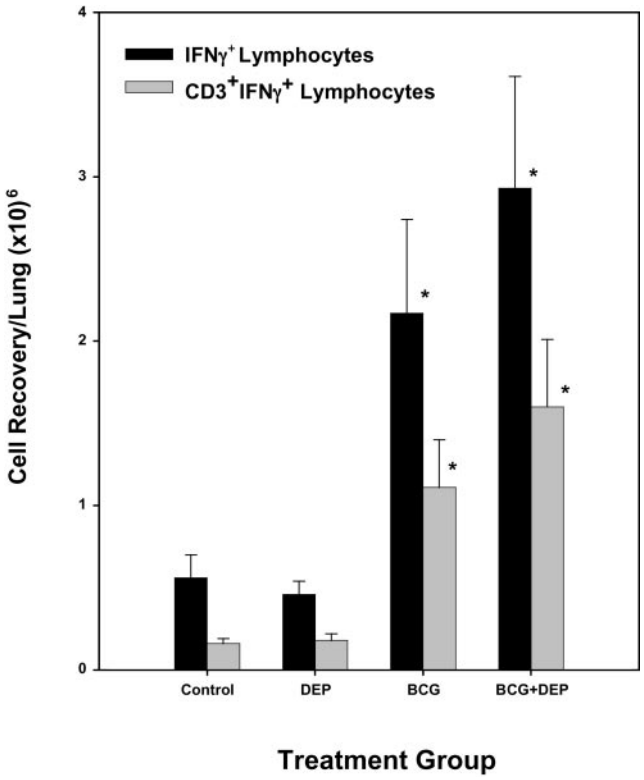

FIG. 2. Recoveries of total IFNγ-producing lymphocytes and T cells from lungs of control and DEP-exposed mice infected with BCG. Groups of mice were instilled with 50 μl PBS (control group) or PBS containing DEP (100 μg), BCG (2.5×10^4), or both DEP + BCG. After five weeks, interstitial lymphocytes were derived from lungs of individual mice by the procedure described previously (Saxena *et al.*, 2002a), and cells were stained for cytoplasmic IFNγ and membrane CD3. The % of cells expressing the marker was determined by flow cytometry and recoveries of different IFNγ-producing cell populations calculated from the total cell recoveries for individual lungs. Each bar represents the mean of values obtained from five mice ± SD; $*p < 0.05$ different from control and DEP groups.

Inhibition of IFNγ induced NO production in mouse alveolar macrophages by DEP: The BCG load was significantly higher in lungs of DEP-exposed mice even though the IFNγ response was not inhibited by DEP. One possible explanation of these observations could be that DEP treatment reduced the responsiveness of lung macrophages to IFNγ. To test this possibility, mouse alveolar macrophages were stimulated *in vitro* by IFNγ in the presence or absence of DEP. Release of nitric oxide was assessed as a parameter of macrophage activation by measuring nitrite levels in culture supernatants. Results in Figure 3 show that levels of nitrite increased in a time dependent manner in culture supernatants of IFNγ-treated mouse alveolar macrophages. By itself, DEP caused no release of NO by alveolar macrophages, but the release of NO in response to IFNγ was significantly inhibited by DEP (Fig. 3).

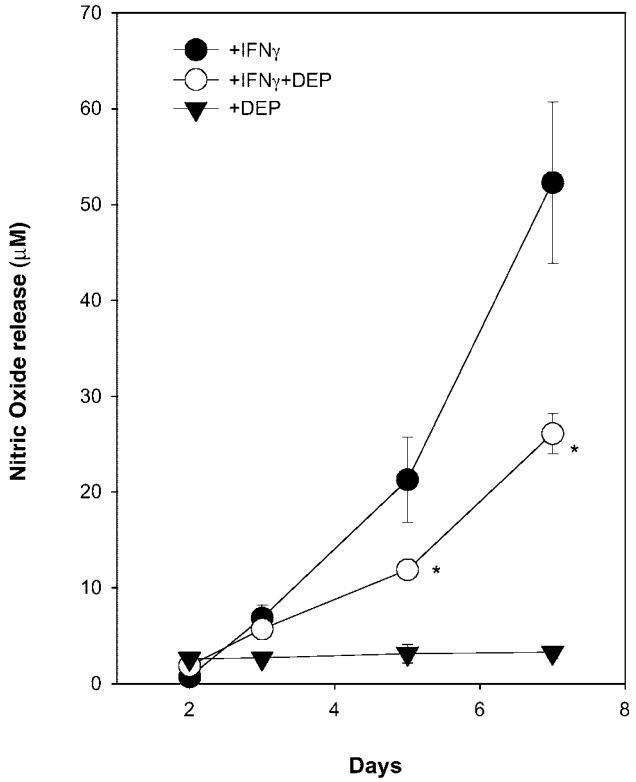

FIG. 3. Effect of DEP on IFNγ-induced nitric oxide (NO) secretion in mouse alveolar macrophages in culture. Mouse alveolar macrophages obtained by bronchoalveolar lavage were cultured in 48-well micro test plates (10^6/ml) in the presence of mouse IFNγ (5 ng/ml), DEP (10 μg/ml), or both. NO_x was measured in culture supernatants at the given time points. Each value is a mean ≤ SD of three replicate observations; $*p < 0.05$ inhibition due to DEP.

DISCUSSION

There is evidence that exposure to DEP can deviate the immune response towards a Th2 type, which is conducive to the development of an IgE response and allergies (Diaz-Sanchez *et al.*, 1994, Fujimaki *et al.*, 1997; Miyabara *et al.*, 1998). Since a Th1 type of immune response is needed for protective immunity to mycobacterial infections (Flynn *et al* 1993), exposure to diesel exhaust pollution might increase the susceptibility to tuberculosis infection. The present study was designed to test this hypothesis. Effects of intratracheal administration of DEP on the function of alveolar macrophages and on susceptibility to another intracellular pathogen, *Listeria monocytogenes*, have been reported (Yang *et al.*, 1999, 2001). In these studies, a DEP dose range of 5 to 35 mg/kg body weight was used in a rat model. The lowest dose of 5 mg/kg body weight induced significant inhibition of IL-1 production by alveolar macrophages and enhanced susceptibility to *L. monocytogenes* infection in rats (Yang *et al.*, 2001). This dose, which corresponded to about 100 μg/mouse (body weight about 20 g), was adopted for the present study. Our results show that, at this dose, DEP exposure increased the bacterial load of murine experimental BCG infection.

IFNγ plays a crucial role in imparting protection from mycobacterial infection (Flynn *et al.*, 1993). IFNγ gene-knockout mice infected with *M. tuberculosis* have disseminated disease and succumbed to the infection (Cooper *et al.*, 1993). We have previously shown that in mice T cells as well as NK cells are important sources of IFNγ in lung interstitium (Saxena *et al.*, 2002b). After initiation of BCG lung infection in mice, IFNγ-producing cells accumulate in lung interstitium. Bacterial load, as well as the number of IFNγ-producing cells, attain maxima five weeks post-infection and subside thereafter (Saxena *et al.*, 2002b). Both T cells and NK cells contribute to the IFNγ response in BCG-infected lungs (Saxena *et al.*, 2002b). Results of the present study indicate that (1) the accumulation of T and NK cells in BCG-infected lungs was not inhibited in DEP-exposed mice, (2) expression levels of IFNγ mRNA in pulmonary NK cells, CD4 T cells, and CD8 T cells isolated after BCG infection were not affected by coexposure to DEP, and (3) accumulation of IFNγ-producing lymphocytes in the lungs after BCG infection was not decreased by coexposure to DEP. Taken together, these results suggest that the development of local IFNγ responses in the lungs of BCG-infected mice was not reduced as a result of exposure to DEP. A defective IFNγ response is therefore unlikely to be the cause of impaired BCG clearance from DEP-exposed mice.

IFNγ activates macrophages to kill or stop the intracellular growth of mycobacteria. NO generated in macrophages in response to IFNγ signal is a crucial mediator of antibacterial action of activated macrophages (Ehrt *et al.*, 2001; Xing *et al.*, 2001). Even if IFNγ response was not altered as a result of DEP exposure, an alternate cause for impaired BCG clearance could be that the pulmonary macrophages from DEP-treated mice could not properly respond to the activation signal of IFNγ. This possibility was examined by assessing IFNγ-induced NO release by control and DEP-treated mouse alveolar macrophages. Our results indicated that DEP-exposed alveolar macrophages released significantly lower amounts of NO in response to IFNγ. This result is compatible with the hypothesis that DEP exposure impairs BCG clearance, at least in part by impairing the ability of pulmonary macrophages to mount response critical to host defense, such as NO production after stimulation with IFNγ. DEP particles have a variety of adsorbed bioactive organic molecules, some of which are known to have an inhibitory effect on macrophage function (Yang *et al.*, 1999, 2001). It has been proposed that a key mechanism of action for these toxicants is induction of intracellular oxidative stress (Al-Humadi *et al.*, 2002; Whitekus *et al.*, 2002). Although only NO production was evaluated in this study, a variety of other macrophage functions relevant to host defense might also be affected by DEP exposure and contribute to the enhanced *in vivo* load of BCG infection in the mouse model. It should also be noted that DEP from a single source was used throughout these studies, and that DEP samples from other sources may have different toxicities or potencies.

In conclusion, DEP exposure appears to impair clearance of

murine experimental pulmonary BCG infection. Impaired clearance is not the result of impairment in pulmonary IFNγ-producing lymphocytes or T-cell responses. Rather it appears to result, at least in part, from impaired ability of pulmonary macrophages to respond to IFNγ and engage in functions critical to host defense, such as production of NO.

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