Effect of Exposure to Diesel Exhaust Particles on the Susceptibility of the Lung to Infection

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There are at least three mechanisms by which alveolar macrophages play a critical role in protecting the lung from bacterial or viral infections: production of inflammatory cytokines that recruit and activate lung phagocytes, production of antimicrobial reactive oxidant species, and production of interferon (an antiviral agent). In this article we summarize data concerning the effect of exposure to diesel exhaust particles on these alveolar macrophage functions and the role of adsorbed organic chemicals compared to the carbonaceous core in the toxicity of diesel particles. *In vitro* exposure of rat alveolar macrophages to diesel exhaust particles decreased the ability of lipopolysaccharide (LPS), a bacterial product] to stimulate the production of inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). Methanol extract exhibited this potential but methanol-washed diesel particles did not. Exposure of rats to diesel exhaust particles by intratracheal instillation also decreased LPS-induced TNF-α and IL-1 production from alveolar macrophages. In contrast, carbon black did not exhibit this inhibitory effect. Exposure of rats to diesel exhaust particles by inhalation decreased the ability of alveolar macrophages to produce antimicrobial reactive oxidant species in response to zymosan (a fungal component). In contrast, exposure to coal dust increased zymosan-stimulated oxidant production. *In vivo* exposure to diesel exhaust particles but not to carbon black decreased the ability of the lungs to clear bacteria. Inhalation exposure of mice to diesel exhaust particles but not to coal dust depressed the ability of the lung to produce the antiviral agent interferon and increased viral multiplication in the lung. These results support the hypothesis that exposure to diesel exhaust particles increases the susceptibility of the lung to infection by depressing the antimicrobial potential of alveolar macrophages. This inhibitory effect appears to be due to adsorbed organic chemicals rather than the carbonaceous core of the diesel particles. *Key words*: alveolar macrophages, ambient particulate matter, antimicrobial activity, cytokines, interferon, occupational exposure, reactive oxygen species. — *Environ Health Perspect* 109(suppl 4):609–612 (2001). *http://ehpnet1.niehs.nih.gov/docs/2001/suppl-4/609-612castranova/abstract.html*

Heavy-duty diesel engines emit 30–100 times as many particles as do gasoline engines. Diesel exhaust particles (DEPs) range in diameter from 0.02 to 0.2 μm. These respirable particles can adsorb over 450 different organic compounds, including mutagenic and carcinogenic polycyclic aromatic hydrocarbons (*1*). Because of their fine to ultrafine size, DEP readily deposit in the gas exchange regions of the lung (*2*). For these reasons, exposure of truckers, railroad and construction workers, and engine mechanics to DEPs is an occupational health concern. Of particular concern is the use of diesel equipment in underground mines where DEP levels up to 2000 μg/m^3 have been reported (*3*).

Epidemiologic studies have shown a consistent association between elevated levels of particulate matter (PM) in ambient air and increased respiratory mortality and morbidity in high-risk groups (*4–7*). Associations between ambient PM and the incidence of pulmonary infections have also been reported (*8*). DEPs are a major component of particulate air pollution in most urban areas. Therefore, the effects of exposure to DEPs on the susceptibility to pulmonary infections is of environmental and occupational concern.

Alveolar macrophages (AMs) play a key role in defense against pulmonary infection (*9*). At least three properties of AMs play key antimicrobial roles, i.e., the production of inflammatory cytokines, reactive oxidant species (ROS), and interferon (*10–12*). In the present article we summarize data from our laboratory indicating that exposure of AMs to DEPs depresses the ability of these pulmonary phagocytes to produce inflammatory cytokines and ROS in response to the bacterial product lipopolysaccharide (LPS) or the fungal product β-glucan (zymosan). Furthermore, DEPs inhibit the pulmonary production of interferon in response to viral exposure. These suppressive effects of DEPs on the production of antimicrobial agents result in pulmonary susceptibility to both viral and bacterial infection, as demonstrated in animal models exposed to DEPs.

Methods

Effects of in vitro *exposure of AMs to DEPs on LPS-stimulated cytokine production.* Methods for these *in vitro* studies have been described previously (*13*). Briefly, we harvested AMs from naive, male, specific pathogen-free, Sprague-Dawley rats (Hilltop, Scottsdale, PA, USA) by bronchoalveolar lavage (BAL). AMs (1×10^6) were pretreated with vehicle, 50 μg/mL DEPs, methanol-washed DEPs, or the equivalent concentration of methanol extract for 2 hr at 37°C. After pretreatment, AMs were challenged with 0.1 μg/mL LPS at 37°C for 22 hr, and the production of tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1) was determined by monitoring cytokine levels of the supernate using a fibroblast killing or a thymocyte proliferation bioassay, respectively.

Effects of intratracheally instilled DEPs on the ex vivo *production of cytokines from AMs in response to LPS.* Methods for these studies have been described previously (*14*). Briefly, male, specific pathogen-free, Sprague-Dawley rats were intratracheally instilled with vehicle, DEPs [5 mg/kg body weight (bw)], or carbon black (5 mg/kg bw). We harvested AMs from these treated rats by BAL 3 days postexposure. We allowed AMs (1×10^6) to adhere to tissue culture plates, and then exposed them to LPS (0.1 μg/mL). After a 24-hr *ex vivo* exposure to LPS, we collected supernates for measurement of IL-1 and TNF-α using a thymocyte proliferation or a fibroblast killing bioassay, respectively.

Effects of intratracheally instilled DEPs on TNF-α production by AMs after in vivo *LPS treatment.* Methods for these *in vivo* studies have been described previously (*14*). Briefly, we exposed male, specific pathogen-free, Sprague-Dawley rats to vehicle or DEPs (5 mg/kg bw) by intratracheal instillation. Three days after DEP exposure, the rats were intratracheally instilled with vehicle or LPS (1 mg/kg bw). AMs were harvested by BAL 3 hr after LPS treatment and cultured (1×10^6 adherent AMs) for 18 hr at 37°C. After this culture period, we collected supernates and monitored TNF-α using a fibroblast killing bioassay.

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Effects of inhalation of DEPs on ex vivo *zymosan-stimulated production of reactive oxidant species and surface morphology of AMs.* Methods for these studies have been described previously (*15*). Briefly, we exposed specific pathogen-free, male, Fischer 344 rats (Charles River, Wilmington, MA, USA) by inhalation to filtered air, DEP (2 mg/m^3), or coal dust (2 mg/m^3) 7 hr/day, 5 days/week, for 2 years. After a 2-year exposure, AMs were harvested by BAL. We exposed AMs (4×10^6) *ex vivo* to unopsonized zymosan (6 mg/mL) in the presence of luminol (10^{-8} M) and determined production of ROS by measuring chemiluminescence in a liquid scintillation counter operated in the out-of-coincidence mode.

We determined the surface morphology of AMs harvested for air or DEP-exposed rats using an ETEC autoscan scanning electron microscope (Jeol, Peabody, MA, USA). Cellular spreading and surface ruffling of micrographs were scored by three independent observers.

Effects of inhalation of DEPs on the pulmonary response to viral infection. Methods for these studies were published previously (*16*). Briefly, we exposed female, CD-1, white, Swiss mice (Charles River, Wilmington, MA, USA) by inhalation to filtered air, DEPs (2 mg/m^3), or coal dust (2 mg/m^3) 7 hr/day, 5 days/week, for 6 months. Twenty-four hours after this inhalation exposure, the mice were exposed intranasally to *Influenza* virus. Four days after viral inoculation, viral multiplication was determined from homogenized lung tissue by immune fluorescence cell counting. Pulmonary interferon production was determined by monitoring the ability of lung suspensions to cause a 50% killing of a Sendai virus culture.

Effects of intratracheally instilled DEPs on the pulmonary response to bacterial infection. Specific pathogen-free, male, Sprague-Dawley rats were intratracheally instilled with vehicle, DEPs (5 mg/kg bw), or carbon black (5 mg/kg bw). Three days after exposure, the rats were inoculated by intratracheal instillation of 5,000 *Listeria monocytogenes* bacteria. Seven days postinoculation, the rats were sacrificed, the lungs homogenized, the homogenates plated on brain-heart infusion broth agar plates at 37°C overnight, and colony-forming units counted.

Results

The effects of *in vitro* exposure of AMs to DEPs, methanol-washed DEPs, or the methanol extract of DEPs on LPS-stimulated cytokine production are shown in Table 1. A 2-hr pretreatment of AMs with DEPs (50 µg/mL) significantly decreased (70%) the ability of AMs to produce IL-1 in response to stimulation with the bacterial product LPS. Similarly, DEP pretreatment decreased LPS-induced TNF-α production by AMs by 34%. This inhibitory effect of DEPs on subsequent AM responsiveness to LPS appears to be due primarily to the adsorbed organic compounds associated with DEPs, as methanol-washed DEPs failed to significantly inhibit LPS-induced IL-1 or TNF-α production by AMs. On the other hand, the methanol extract was as effective as DEPs, causing a 66 or 43% inhibition of LPS-induced IL-1 or TNF-α production, respectively.

The effects of intratracheal instillation of DEPs on the *ex vivo* production of cytokines from AMs in response to LPS are shown in Table 2. *In vivo* exposure to DEPs (5 mg/kg bw) caused AMs to be significantly less responsive to *ex vivo* stimulation with the bacterial product LPS. Indeed, LPS-stimulated IL-1 production was 42% less than anticipated, whereas TNF-α production was 90% less than anticipated from the sum of the separate effects of *in vivo* DEPs and *in vitro* LPS alone. This inhibitory effect of DEPs appears to be a property of the adsorbed organic compounds on DEPs, as intratracheal instillation of carbon black did not inhibit the subsequent response to *ex vivo* LPS treatment. In fact, preexposure to carbon black made harvested AMs more responsive to *in vitro* LPS treatment.

The effects of intracheally instilled DEPs on TNF-α production by AMs after *in vivo* LPS treatment are shown in Table 3. AMs harvested from control rats, i.e., intratracheally instilled twice with saline, produced a low level of TNF-α. *In vivo* exposure to DEPs (5 mg/kg bw) followed by saline caused TNF-α production by harvested AMs to increase by 133% above the control level. *In vivo* exposure to saline followed by LPS (1 mg/kg bw intratracheally) caused AM production of TNF-α to increase by 473% above the control. However, *in vivo* LPS treatment of DEP-exposed rats yielded AMs in which TNF-α production was only 20% above control. These data indicate that preexposure to DEPs significantly inhibited the ability of AMs to be stimulated *in vivo* with the bacterial product LPS.

The effects of inhalation of DEPs on the *ex vivo* production of ROS by AMs in response to the fungal β-glucan zymosan are shown in Table 4. AMs harvested from rats exposed to DEPs (2 mg/m^3 for 2 years) were less responsive to *in vitro* stimulation by zymosan than AMs from filtered air controls. Indeed, zymosan-stimulated ROS production, monitored as chemiluminescence (CL), was decreased by 62% in DEP-exposed AMs. The depressive effect of DEPs appears to be associated with the adsorbed organics on DEPs, as inhalation of coal dust (carbon-based particles as are DEPs) significantly enhanced zymosan-stimulated CL from AMs by 22%. Depression of phagocytic activity of AMs following inhalation of DEPs for 2 years was also inferred from observation of the surface features of harvested AMs by scanning electron microscopy. As shown in Figure 1, AMs from DEP-exposed rats had a smooth surface, whereas AMs from the filtered air rats exhibited a ruffled surface. In contrast to DEP-exposed cells, AMs from rats after inhalation of coal dust (2 mg/m^3 for 2 years) were significantly more ruffled and exhibited more surface spreading than controls (*15*).

Inhalation of DEPs (2 mg/m^3 for 6 months) also depressed the lung's ability to produce interferon in response to viral infection. Indeed, DEP inhalation decreased viral-induced pulmonary production of interferon

Table 1. LPS-induced cytokine production by AMs: inhibition by *in vitro* exposure to DEPs.[a]

Treatment	Percent inhibition of the LPS response	
	IL-1	TNF-α
DEP	70 ± 8*	34 ± 10*
Methanol-washed DEPs	31 ± 8	20 ± 11
Methanol extract	66 ± 8*	43 ± 9*

[a]AMs were pretreated at 37°C for 2 hr with saline (control), 50 µg/mL DEPs, methanol-washed DEPs, or methanol extract prior to challenge with 0.1 µg/mL LPS at 37°C for 22 hr. Values are means ± SE of 3–6 experiments. Data modified from Yang et al. (*13*). *Significant inhibition from LPS after saline (ANOVA analysis; $p < 0.05$).

Table 2. *Ex vivo* LPS-induced cytokine production by AMs: inhibition by *in vivo* exposure to DEPs.[a]

Treatment	Percent of the sums of the separate responses to DEPs and LPS	
	IL-1	TNF-α
DEP	58 ± 10*	10 ± 1*
Carbon black	162 ± 40	250 ± 41

[a]Rats were intratracheally instilled with vehicle (control), DEPs (5 mg/kg bw), or carbon black (5 mg/kg bw). AMs were harvested by BAL 3 days postinstillation and challenged with LPS (0.1 µg/mL) at 37°C for 24 hr. Values are means ± SE of 5 experiments. Data modified from Yang et al. (*14*). *LPS response with AMs harvested from DEP-treated rats was significantly decreased from the sum of the separate responses to *in vitro* LPS and *in vivo* DEPs (ANOVA analysis; $p < 0.05$).

Table 3. Effect of *in vivo* exposure to DEPs on the production of TNF-α from AMs in response to subsequent *in vivo* exposure to LPS.[a]

Treatment	TNF-α (ng/10^6 AM)
Saline then saline	1.5 ± 0.2
DEPs then saline	3.5 ± 0.3
Saline then LPS	8.6 ± 1.7
DEPs then LPS	1.8 ± 0.2*

[a]Rats were intratracheally instilled with saline or DEPs (5 mg/kg bw) 3 days prior to a second instillation with saline or LPS (1 mg/kg bw). AMs were harvested by BAL 3 hr after the second instillation and production of TNF-α was measured after culturing the cells for 18 hr at 37°C. Values are means ± SE of 5 experiments. Data modified from Yang et al. (*14*). *TNF-α production from AMs harvested from rats exposed to DEPs and then to LPS was significantly decreased from the level anticipated from the sum of TNF-α production from AMs after *in vivo* exposure to DEPs plus saline or saline plus LPS (ANOVA analysis; $p < 0.05$).

by 78% (*16*). In contrast, inhalation of coal dust (a carbon particle lacking adsorbed organic chemicals) exhibited nearly normal interferon production following pulmonary inoculation with *Influenza* virus (*16*). As expected from the interferon production results, DEP inhalation increased the pulmonary susceptibility to viral infection, as noted by a significant increase (58%) in macroscopic lung consolidation in Influenza-infected mice exposed to DEPs compared to filtered air controls (*16*). In contrast, coal-exposed mice exhibited viral susceptibility that was not different from the filtered air controls. *In vivo* viral killing was also depressed in lungs exposed to DEPs (2 mg/m^3 for 6 months), i.e., pulmonary viral multiplication was 78% higher in DEP-exposed lungs than in controls (*16*). In contrast, exposure to coal dust did not significantly affect pulmonary viral multiplication after *Influenza* inoculation.

Intratracheal instillation of DEPs (5 mg/kg bw) also depressed the ability of rat lungs to kill *Listeria* bacteria. Indeed, the number of viable bacterial colony-forming units 7 days after inoculation were significantly (420%) higher in DEP-exposed lungs than controls (*17*). In contrast, lungs instilled with carbon black (5 mg/kg bw) were not significantly different from controls in the ability to clear *Listeria* bacteria (*17*).

Discussion

AMs represent the lung's first line of defense against inhaled microbes (*18*). AMs protect the organism from pulmonary infections by three main mechanisms: the production of inflammatory cytokines, the production of reactive oxidant species, and the production of interferon. In the present article, we present data indicating that exposure to DEPs depresses the ability of AM to perform these three key functions in response to bacterial, fungal, or viral exposure. The data support the hypothesis that excessive exposure to DEPs may increase the susceptibility to pulmonary infection. This may partly explain the increased incidence of pneumonia on days with high ambient particulate levels (*8*).

Table 4. Effect of inhalation of DEPs on the production of ROS by AM-stimulated *ex vivo* with β-glucan.[a]

Treatment	Zymosan-stimulated CL (peak cpm/4 × 10^6 AM)
Air	58,000
DEPs	22,000*
Coal	71,000**

[a]Rats inhaled filtered air, DEPs (2 mg/m^3), or coal dust (2 mg/m^3) for 2 years, 7 hr/day, 5 days/week. After inhalation exposure, AMs were harvested by BAL, and CL in response to zymosan was measured. Values are means of 5 experiments. Data modified from Castranova et al. (*15*). *Significant decrease from control. **Significant increase from control ($p < 0.05$).

LPS, a product of gram-negative bacteria, is a potent stimulant of IL-1 and TNF-α from AMs (*13,14*). IL-1 and TNF-α are proinflammatory cytokines involved in the recruitment of inflammatory cells, activation of neutrophils, the enhancement of endothelial permeability, stimulation of eicosanoid biosynthesis, and induction of adhesion molecule expression (*19*). TNF-α also stimulates AMs to secrete various chemokines, such as macrophage inflammatory proteins 1 and 2 and cytokine-induced neutrophil chemoattractant (*20*). Furthermore, IL-1 and TNF-α are potent T-cell activators (*21,22*). Activated T-cells secrete interferon, which in turn activates the production of antimicrobial oxidants from AMs (*23*). As a result of these properties, TNF-α and IL-1 play an important role in the ability of the lung to respond to bacterial infections (*10,24,25*). Data reviewed in this manuscript indicate that either *in vitro* or *in vivo* exposure of AMs to DEPs depresses the ability of AM to produce these proinflammatory cytokines in response to either *in vivo* or *in vitro* treatment with LPS. Adsorbed organic constituents of DEPs appear critical to this inhibitory activity, as methanol-washed DEPs or carbon black failed to significantly inhibit LPS-stimulated IL-1 or TNF-α production from AMs.

β-Glucans, such as zymosan, are fungal products that are potent stimulants of the production of ROS by AMs (*15*). ROS produced by AMs include superoxide anion, hydrogen peroxide, and hydroxyl radical (*26*). These ROS play an important role in bacterial killing (*11,27*). ROS production by AMs can be monitored by measuring the generation of CL (*28*). Data presented here indicate that inhalation of DEPs significantly decreased the ability of AMs to generate CL in response to zymosan. This depression of AM function is also demonstrated by the absence of surface ruffling of AMs harvested from DEP-exposed rats. Indeed, Davis and colleagues found that depression of surface ruffling appears to be an indication of compromised phagocytic activity (*29*). In contrast to DEPs, inhalation of coal dust, a carbonaceous particle lacking adsorbed organic compounds associated with DEPs, enhanced zymosan-stimulated CL and surface ruffling.

Such compromised phagocytic activity and decreased ROS production could make the lung more susceptible to pulmonary infection. Indeed, data presented here indicate that the ability of the lung to clear *Listeria* bacteria was decreased in lungs exposed to DEPs. AMs from these lungs were less able to exhibit an increase in ROS production after *Listeria* infection (*17*). Evidence indicates that adsorbed organic components play an important role in the depression of bacterial killing, as intratracheal instillation of carbon black failed to decrease bacterial clearance or bacterial-induced ROS production by AMs (*17*).

Interferon has been shown to exhibit potent antiviral activity (*30*). AMs and alveolar type II cells have been shown to produce interferon in response to exposure to virus (*12*). Data reviewed in this manuscript indicate that inhalation of DEPs depressed the lung's ability to produce interferon in response to viral infection. This deficiency resulted in enhanced viral multiplication in DEP-exposed lungs. Coal dust, which lacks the adsorbed organic compounds associated with DEPs, failed to significantly decrease interferon production or viral killing.

In summary, exposure to DEPs decreased proinflammatory cytokine production from LPS-exposed AMs, decreased ROS production from zymosan-exposed AMs, and

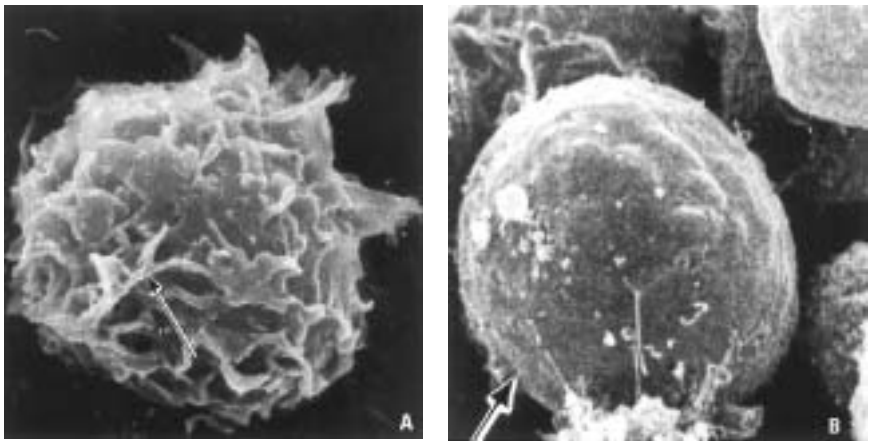

Figure 1. Effect of *in vivo* exposure to DEPs on the surface activity of AMs. Rats were exposed by inhalation to filtered air or DEPs (2 mg/m^3, 7 hr/day, 5 days/week, for 2 years). AMs were then harvested by BAL and surface morphology was monitored by scanning electron microscopy. (*A*) Filtered air. (*B*) DEPs. Arrows indicate the presence or absence of surface ruffling (original magnification ×3,500).

decreased interferon production in viral-exposed lungs. The depression of the production of antimicrobial agents after DEP exposure was associated with enhanced bacterial and viral multiplication in the lungs of animals exposed to DEPs. Evidence indicates that the adsorbed organic components of DEPs were critical to this suppression of antimicrobial activity. These data suggest that occupational or environmental exposure to DEPs may be associated with increased susceptibility to pulmonary infection.

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