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EFFECT OF DIESEL EXHAUST PARTICULATE (DEP) ON IMMUNE RESPONSES: CONTRIBUTIONS OF PARTICULATE VERSUS ORGANIC SOLUBLE COMPONENTS

Paul D. Siegel,[1] Rajiv K. Saxena,[2] Q. B. Saxena,[3] Joseph K. H. Ma,[4] Jane Y. C. Ma,[1] Xue-Jun Yin,[4] Vincent Castranova,[1] Nabil Al-Humadi,[1] Daniel M. Lewis[1]

[1]HELD, NIOSH, Morgantown, West Virginia, USA
[2]HELD, NIOSH, Morgantown, West Virginia, USA, and Jawaharlal Nehru University, New Delhi, India
[3]HELD, NIOSH, West Virginia, USA, and Indian Council of Medical Research, New Delhi, India
[4]School of Pharmacy, West Virginia University, Morgantown, West Virginia, USA

The effect of diesel exhaust particulate (DEP) exposure on innate, cellular and humoral pulmonary immunity was studied using high-dose, acute-exposure rat, mouse, and cell culture models. DEP consists of a complex mixture of petrochemical-derived organics adsorbed onto elemental carbon particles. DEP is a major component of particulate urban air pollution and a health concern in both urban and occupational environments. The alveolar macrophage is considered a key cellular component in pulmonary innate immunity. DEP and DEP organic extracts have been found to suppress alveolar macrophage function as demonstrated by reduced production of cytokines (interleukin-1 [IL-1], tumor necrosis factor-α [TNF-α]) and reactive oxygen species (ROS) in response to a variety of agents, including lipopolysaccharide (LPS), interferon-γ (IFN-γ), and bacteria. Fractionation of DEP organic extract suggests that this activity was predominately in polyaromatic-containing and more polar (resin) fractions. Organic-stripped DEP did not alter these innate pulmonary immune responses. DEP also depressed pulmonary clearance of Listeria monocytogenes *and* Bacillus Calmette-Guerin *(BCG). The contribution of the organic component of DEP is less well defined with respect to acquired and humoral immunity. Indeed, both DEP and carbon black enhanced humoral immune responses (specific immunoglobulin [Ig] E and IgG) in an ovalbumin-sensitized rat model. It is concluded that both the particulate and adsorbed organics may contribute to DEP-mediated immune alterations.*

Diesel exhaust particulate (DEP) effects on health have become a major concern in both general and occupational environments. DEP has been associated with a variety of adverse health outcomes involving potential immune mechanisms, including acute pulmonary inflammation, altered allergic sensitization, and exacerbation of asthma and respiratory infections (Sydbom et al., 2001).

Address correspondence to Paul D. Siegel, PhD, Team Leader, Analytical Services Branch, HELD, National Institute for Occupational Safety and Health, 1095 Willowdale Rd., Morgantown, WV 26505, USA. E-mail: psiegel@cdc.gov

Although it has been noted that DEPs are a major contributor to the total urban air pollution particulate, association of DEP to specific health outcomes is difficult due to simultaneous exposure to toxic volatile pollutants, including hydrocarbons, ozone, nitrogen oxides, formaldehyde, and carbon monoxide. The particulate generated from diesel engines is itself a complex mixture of an estimated 18,000 combustion chemical products adsorbed onto a carbon core (Weisenberger, 1981). Exposure levels to DEP have been reported as high as 2 mg/m^3 in mines (Mine Safety and Health Administration,1998). High-dose, acute exposure animal models have therefore been employed to study the potential mechanisms of DEP mediated toxicities.

This article is a review of research performed in, or in collaboration with, our laboratories studying the mechanisms of DEP toxicities on the immune system using high-dose, acute-exposure mouse and rat models. The effects of DEP on innate immunity, including pulmonary inflammation and alveolar macrophage function, and on adaptive/acquired immunity, using both infection and allergic sensitization models, are presented. DEP chemical fraction experiments are also reviewed that suggest potential roles of various components in DEP's effects.

METHODS

Exposure to DEP and Bacteria

Pulmonary exposure to DEP (standard reference material 1650 from the National Institute of Standards and Technology, Gaithersburg, MD) was used for all studies. DEP aerosol was generated as described by Yin et al. (2002). Briefly, DEP was suspended in distilled water by sonication. The suspension was aerosolized by a nebulizer (TSI model 3076, TSI, Inc., St Paul, MN) through a diffusion dryer, combined with humidified dilution air and introduced into a nose-only inhalation chamber. Intratracheal (IT) instillation of 500 µl DEP suspension into rats (Sprague-Dawley, Hilltop Laboratories, Scottsdale, PA, or Brown Norway rats, Charles Rivers Laboratories, Stoneridge, NY) was done according to Yang et al. (1999) and for mice (50 µl suspension, IT) according to Saxena et al. (2003). In vitro DEP exposure studies employed RAW 264.7 macrophages obtained from American Type Culture Collection (Manassas, VA). Cells were cultured and exposed to the macrophage agonists ± DEP as reported by Saxena et al. (2003). In a separate study, Brown Norway rats (Harlan Laboratories, Indianapolis, IN) were inoculated, intratracheally, with 10^5 *Listeria monocytogenes*/500 µl saline, according to the method of Antonini et al. (2000), 1 d following inhalation exposure to DEP or air . Female mice, C57Bl/6 (Jackson Laboratories, Bar Harbor, ME), were given 2.5×10^4 *Mycobacteria bovis* (BCG, TMCC number 1011) in 50 µl phosphate-buffered saline (PBS), intratracheally, with or without DEP (Saxena et al., 2003).

TABLE 1. Parameters Measured to Evaluate the Effects of DEP

Endpoint	Method	Reference
BAL granulocyte count	Coulter cell counter with a sizing unit (Coulter Electronics, Hialeah, FL)	Castranova et al. (1990)
BAL albumin and total Protein	Cobas Fara II analyzer and methods (Roche Diagnostics Systems, Montclair, NJ)	Porter et al. (2002)
BAL lactic dehydrogenase (LDH)	Sigma Diagnostic Reagents and procedures (Sigma Chemical Co., St. Louis, MO)	Porter et al. (2002)
IL-1β, IL-12, TNF-α, and IFN-γ in BAL and cell culture supernatant fluid	ELISA (Biosource International, Inc. Camarillo, CA)	Yin et al. (2002)
macrophage nitric oxide	Culture fluid supernatant nitrite measurement using Greiss reagents	Saxena et al. (2003)
Macrophage phagocytosis index	Phagocytosis of carboxylated-modified yellow-green FluoSpheres stained with fluorochrome nile red (Molecular Probes, Eugene, OR) and enumerated using confocal fluorescent microscopy	Yin et al. (2002)
Pulmonary Lymphocyte, lymphocyte subset, and IFN-γ-positive cells	Flow cytometry following appropriate antibody staining	Saxena et al. (2003)
IFN-γ mRNA in lymphocytes and lymphocyte subsets	Cell isolation by negative selection using monoclonal antibody bound beads (Stem Cell Technologies, Inc., Vancouver, Canada) followed by real-time PCR IFN-γ mRNA measurement	Saxena et al. (2003)
OVA-specific IgE and IgG	ELISA	Al-Humadi et al. (2002)
Bacterial load	Culture and enumeration of bacterial colonies from tissue	Yin et al. (2002) (*Listeria*) Saxena et al. (2003) (BCG)

Inflammatory, Immunological, and Infectious Disease Endpoints

Multiple endpoints were measured throughout the various studies. Table 1 summarizes the parameters measured and briefly states the methods used.

RESULTS AND DISCUSSION

The results reported in the literature from epidemiologic studies that have examined associations between DEP and infections are mixed. Wjst et al. (1993), in a cross-sectional study, found no association between traffic density and respiratory infection in children. Rossi et al. (1999) reported an association between total suspended particulate and respiratory infection deaths on the

concurrent day. Particulate levels of 100 μg/m^3 air resulted in an 11% increase in deaths due to pneumonia in Milan, Italy. Similarily, Ransom and Pope (1992) reported an association between PM10 concentration (7 to 251 μg/m^3) and school absenteeism. Pope and Dockery (1992) reported acute health effects of respirable particulate, especially cough. Alteration of immune function by DEP can not be inferred by these studies, as acute non-immune-related effects could possibly contribute to both the increase in school absenteeism and pneumonia mortality observed in the respective studies. Due to the limitations of epidemiological studies to assess immunotoxicity, laboratory studies have been employed to investigate DEP's potential to alter the immune system and the course of or susceptibility to infectious diseases.

Effects on Innate Immunity

Pulmonary Inflammaton The specific role of DEP in the acute pulmonary inflammatory response noted following exposure to diesel exhaust (DE) has not been established. Acute human exposure studies have demonstrated that DE, which includes particles, vapor, and gases, can cause pulmonary inflammation. Salvi et al. (1999) noted that bronchoalveolar lavage (BAL) neutrophils increased an average of 326% over control 6 h after a 1-h exposure of volunteers to 300 μg/m^3 diesel exhaust. Rudell et al. (1999) exposed healthy volunteers to DE and filtered DE (particulate reduced by 50%). DE caused a 4 fold increase in bronchioalveolar lavage (BAL) neutrophils. Filtration of DE did not significantly lower the pulmonary neutrophilia resulting from DE exposure.

A 4-h exposure to DEP reentrained at 17, 50, and 100 mg/m^3 air to rats increased BAL granulocytes 3 d postexposure by 8%, 104%, and 586%, respectively. Only exposure to the two higher concentrations (50 and 100 mg/m^3) gave results significantly different from control. Rats exposed to DEP by IT instillation displayed a dose-dependent pulmonary inflammation, as noted by increase in BAL granulocytes. Lactate dehydrogenase (LDH) and albumin were also dose-dependently elevated in these animals (Yang et al., 1999). Carbon black produced a very similar pulmonary inflammatory effect to that of DEP (Figure 1). This suggests that pulmonary inflammation induced by DEP is, at least in part, mediated by a particulate effect. The large DEP difference in exposure dose to obtain similar pulmonary inflammation in the rat and human may be due to one or a combination of factors. Humans may be more sensitive, and/or the volatile/gaseous component of DE may play an important role in DEP induced pulmonary inflammation.

Alveolar Macrophage Function Alveolar macrophages (AM) are a key cell in pulmonary innate immune defenses. Castranova et al. (1985) found that AM from rats exposed chronically to 2 mg/m^3 DE, 7 h/d, 5 d/wk, for 2 yr had an altered surface morphology and had a greatly diminished capacity to produce reactive oxygen species when stimulated ex vivo with zymosan. Rudell et al. (1999) reported a reduction in macrophage phagocytic index from 92% to 85% from nonsmoker human subjects exposed to DE for 1 h. Both acute in vivo and in vitro DEP exposure studies were employed to examine the potential

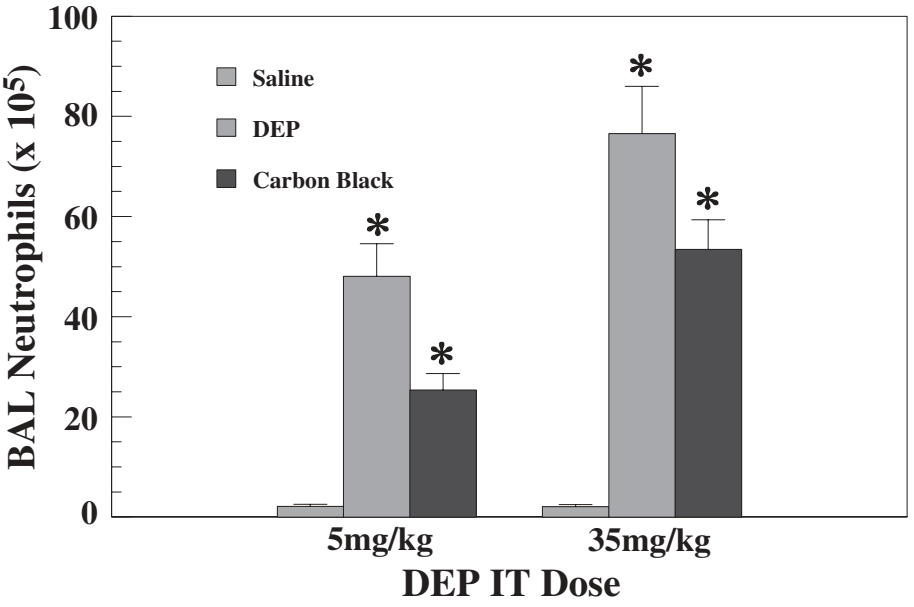

FIGURE 1. Pulmonary cellular inflammatory response to DEP and carbon black. Cells were harvested by BAL from the lungs of Sprague-Dawley rats 3 d following IT instillation of saline, DEP/saline, or carbon black/saline. Data are modified from Yang et al. (1999) and represent the means ±S.E. Asterisic indicates significantly different from saline control, $p < .05$.

role of DEP in DE-mediated alteration of AM function. Table 2 summarizes the results of in vivo experiments. Dose-dependent decreases of 25% and 39% in AM phagocytosis were noted from rats exposed for 4 h to 50 or 100 mg/m^3 DEP, respectively. At the higher exposure, basal levels of interleukin (IL)-1β from both BAL and AM culture supernatant fluid were elevated (data not shown). IL-12 levels were increased only in BAL fluid, while tumor necrosis factor (TNF)-α levels were not significantly different from control. The pattern of AM cytokine responses to DEP was different from rats infected with *Listeria monocytogenes* or following ex vivo stimulation of macrophages with endotoxin (lipopolysaccharide, LPS). *Listeria* infection, alone, induced AM to produce high levels of IL-1β, IL-12, and TNF-α (data not shown). DEP exposure of *Listeria*-infected rats dose-dependently suppressed IL-1β and IL-12 levels in BAL and/or AM (basal secretion) culture supernatant fluid (Yin et al., 2002). DEP-induced suppression of macrophage cytokine production was evident when these cells were challenged with LPS, ex vivo, in both bacterial-infected and noninfected animals (Table 2).

A series of in vitro studies were conducted to explore the relative roles of the organic fraction and carbonaceous core of DEP in suppressing the AMs' ability to respond to infection/stimuli. DEP-decreased lipopolysacchride (LPS) stimulated IL-1 and TNF-α production by 70% and 34%, respectively, when

TABLE 2. Effect of DEP ± *Listeria* Exposure to Brown Norway Rats on Alveolar Macrophage Phagocytic Activity and Macrophage-Derived Cytokines

	−*Listeria*				+*Listeria*			
	50 mg/m^3 DEP		100 mg/m^3 DEP		50 mg/m^3 DEP		100 mg/m^3 DEP	
	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
AM WPI[a]	75 ± 7[c]	82 ± 10[c]	61 ± 5[c]	69 ± 6[c]	80 ± 7	84 ± 10	57 ± 5[d]	53 ± 8[c]
BAL IL-1	114 ± 13	117 ± 14	255 ± 35[c]	148 ± 21	88 ± 8	119 ± 14	53 ± 8[d]	109 ± 13
BAL TNF-α	72 ± 17	76 ± 19	55 ± 10	114 ± 19	84 ± 19	75 ± 13	111 ± 23	79 ± 14
BAL IL-12	140 ± 19	85 ± 8	247 ± 65[c]	61 ± 9	59 ± 4[d]	69 ± 8	51 ± 6[d]	57 ± 4
AM[b] IL-1	103 ± 12	97 ± 15	56 ± 15[c]	37 ± 13[c]	56 ± 6[c]	42 ± 5[d]	53 ± 10[c]	43 ± 6[c]
AM[b] TNF-α	46 ± 8	69 ± 14	27 ± 7[c]	37 ± 13[c]	70 ± 6[d]	79 ± 18	44 ± 9[d]	25 ± 9[d]
AM[b] IL-12	105 ± 10	103 ± 19	102 ± 136	126 ± 1120	92 ± 6	62 ± 10	65 ± 7[d]	69 ± 10[d]

Note. Data represent percent of air exposed non-*Listeria*- and *Listeria*-infected controls (mean ± SE), respectively. Data are modified from Yin et al. (2002).

[a] WPI, weighted phagocytic index.

[b] Brown Norway rats were exposed to DEP for 4 h. Alveolar macrophages harvested by BAL and challenged with LPS (0.1 μg/ml) at 37 °C for 24 h. Culture supernant fluids were recovered and assayed for cytokine levels.

[c] Different from air-exposed (noninfected) controls, ANOVA, $p < .05$.

[d] Different from air-exposed *Listeria*-infected controls, ANOVA, $p < .05$.

added directly to primary rat AM (Yang et al., 1999). Similarly, DEP suppressed by >60% the IFNγ-stimulated nitric oxide (NO) release from primary murine AM (Saxena et al., 2003) and caused approximately 98% reduction in *Bacillus Calmette Guerin* (BCG)-stimulated NO production from the RAW264.7 murine macrophages (Saxena et al., 2003). Methanol and dichloromethane extracts of DEP, but not the insoluble carbon core, potently inhibited the ability of the macrophage to respond to LPS or BCG, to a similar degree as intact DEP.

Methanol is a very polar organic solvent. The observation that the inhibitory activity was found in the methanol wash would suggest that these inhibitory constituents were themselves relatively polar. An organic chemical fractionation experiment to substantiate this observation was conducted (Table 3). Dichloromethane DEP extracts were separated into six different fractions using a modification of the method of Blevins et al. (1993). BCG-stimulated RAW 264.7 murine macrophage NO production was suppressed by 98% and 83% in culture by 10 μg/ml DEP and DEP organic extract, respectively. The three most polar fractions, designated as the more polar aromatic, resin, and residual (also containing resins) fractions, were found to significantly suppress BCG-stimulated NO production from macrophages. None of the active fractions were as potent as the crude DEP extract, displaying inhibitory activities ranging from 29% to 37% decrease in observed NO production. The reason for the greater potency of the intact organic extract is not known. It was noted previously that this extract probably contains thousands of chemicals (Weisenberger,

TABLE 3. Inhibitory Activity of DEP and DEP Organic Fractions on BCG-Stimulated NO Production From RAW264.7 Macrophages

	Treatment	Nitrite oxide production (μ*M* nitrite)
Increasing polarity ↓	None	29.84 ±1.78
	DEP	0.63 ±0.36[a]
	Crude DEP extract	2.49 ±1.23[a]
	Asphaltenes fraction	31.92 ±1.06
	Saturated hydrocarbon fraction	34.04 ±1.58
	Less polar aromatic hydrocarbon fraction	29.61 ±1.34
	More polar aromatic hydrocarbon fraction	21.28 ±0.36[a]
	Resins fraction	20.10 ±2.10[a]
	Residual (resins) fraction	18.92 ±0.95[a]

Note. RAW264.7 cells were exposed to BCG (1×10^6/ml) followed by various DEP fractions (10 μg/ml). All values represent the mean ± SE of three replicates. Data modified from Saxena et al. (2003).

[a] Significant, ANOVA, $p < .05$.

1981). Permissive or synergistic effects on macrophages by different chemical constituents may exist and be lost with fractionation.

It is concluded that DEP can suppress alveolar macrophage response to infection or to cytokine activation. The extraction and fractionation experiments further implicate the organic constituents in DEP induced alveolar macrophage suppression, independent of the particulate itself. These studies suggest DEP may be important in DE-induced alteration of alveolar macrophage function, but potential interactions of DEP with DE volatiles in alteration of innate immunity have yet to be explored.

Effects on the Cellular Immune System

Systemic immunosuppression by short-term exposure to DEP was reported by Yang et al. (2003). Transient suppression of the number of splenic anti-sheep red blood cell IgM antibody-forming cells (AFC) and a decrease in concanavalin A (Con A)-stimulated production of IL-2 and IFN-γ from splenic cells following IT exposure to DEP was demonstrated in mice.

The effects of DEP on the cellular immune system were further explored using two different infectivity models: an acute pulmonary infection model using *Listeria* infected rats, and a more chronic pulmonary/systemic BCG murine infection model. Brown Norway rats were exposed for 4 h to DEP at 0, 50, or 100 mg/m^3, and then to 100,000 *Listeria* (by IT instillation) 2 h post DEP inhalation. Pulmonary bacterial load was examined at both 3 and 7 d following exposure (Yin et al., 2002). The chronic infection model entailed IT exposure of C57Bl/6 mice to BCG (2.5×10^4 bacteria)±DEP (100 μg; Saxena et al., 2003). Bacterial load was examined in lung, spleen, liver, and lymph nodes.

Listeria infection is usually localized to the lung in this model and is cleared by >95% within 1 wk following infection. Bacterial load was almost 10 times greater than infected control levels in DEP exposed rats at 3 d postinfection.

At this early time point the major defense against infection is by innate immunity. By d 7 postinfection, specific T-cell immunity has developed and bacteria were nearly completely cleared from the lung (Table 4). Production of IFN-γ by pulmonary associated lymph node cells from *Listeria*-infected rats, stimulated ex vivo with either Con A or heat-killed *Listeria*, was suppressed 3 d following exposure to 100 mg/m^3 DEP, but was greatly augmented by 7 d postexposure (Yin et al., 2003). It must be noted that IFN-γ production by the lymph node cells 3 d following infection is only about 10% that observed on d 7.

BCG is a mycobacterium that produces a systemic infection when instilled into the lungs of mice. It is very slowly cleared from the body and thus represents a more chronic, persistent infection. At 5 wk following infection, immunosuppression from acute exposure to DEP was still evident. Bacterial loads were significantly increased in all organs examined (Table 4), except the spleen. Pulmonary T-lymphocyte-dependent immunity was evaluated by (1) recoveries of total pulmonary lymphocytes and T-, B-, and natural killer (NK)-cell subsets, and (2) IFN-γ production by pulmonary lymphocytes. BCG infection produced significant increases in total pulmonary lymphocytes and T, B, and NK cells of 2.5-, 2.5-, 2.4-, and 1.6-fold over saline controls, respectively. DEP exposure itself did not alter pulmonary lymphocyte populations, but caused an enhancement of the BCG-induced increase of pulmonary lymphocyte numbers of 3.5-, 1.2-, 3.1-, and 2.0-fold increase relative to saline controls for total pulmonary lymphocytes and T, B, and NK cells, respectively. All increases, except that for T cells, reached statistical significance ($p < .05$) over BCG-infected mice (Saxena et al., 2003). DEP did not alter the IFN-γ message or the number of pulmonary lymphocytes producing IFN-γ from BCG-infected mice.

These studies suggest that DEP may produce a transient T-cell-dependent immunosuppression as evidenced by a decrease in production of IFN-γ following ex vivo Con A stimulation. The early increase in bacterial load observed in *Listeria*-infected rats is probably fully attributable to suppression of innate immune parameters, especially alveolar macrophage function. The increased BCG systemic

TABLE 4. Effect of DEP on Bacterial Load in Acute (*Listeria*) and Persistent (BCG) Infection Models

	Listeria pulmonary load			
DEP exposure	3 d[a]	Percent cleared at 7 d[b]	BCG load[c]	
0 mg/m^3	100 ± 27	95	Lung	433 ± 167[d]
50 mg/m^3	221 ± 72[d]	98	Spleen	192 ± 111
100 mg/m^3	981 ± 177[d]	99	Liver	1477 ± 1023[d]
			Lymph node	229 ± 116[d]

[a] Data reported as mean ± SE percent of pulmonary bacterial load in *Listeria*-infected air exposed controls 3 d postexposure in Brown Norway male rats. Data modified from Yin et al. (2002).

[b] Percent of 3-d *Listeria* pulmonary load (± respective DEP exposure) cleared by 7 d postinfection.

[c] Data reported as mean ± SE percent of bacterial load in BCG infected controls 5 wk following IT instillation of 2.5×10^4 BCG +100 μg DEP into C57Bl/6 female mice. Data modified from Saxena et al. (2003).

[d] Significantly different from respective control organ bacterial load following infection, $p < .05$.

load in the persistent infection model may also be related directly to DEP's alteration of macrophage function. Both T cells and NK cells produce IFN-γ in response to BCG infection (Saxena et al., 2003), which activates macrophages to kill or stop intracellular growth of mycobacteria. DEP impaired clearance of BCG, in the presence of normal production of IFN-γ by T cells and NK cells, may be mediated by suppression of macrophage activation by both bacteria and IFN-γ. This suppression of macrophage activation may thus result in impaired ability to mount responses critical to host defenses, such as production of NO, IL-1, and TNF. Thus, enhanced bacterial load at 5 wk following BCG infection may be related to the decreased NO production by macrophages.

Effects on Humoral Immunity

A multitude of studies have been conducted and reviews written examining the effects of DE and DEP on allergy and asthma. Several recent reviews include Sydbom et al. (2001), Parnia and Frew (2001), and Polosa et al. (2002). DEP has been repeatedly shown to act as an adjuvant, increasing specific IgE titers with antigen sensitization, as well as enhancing the allergic pulmonary inflammatory responses. Studies in our laboratories are consistent with the ability of DEP to enhance both allergic sensitization and responses (Al-Humadi et al., 2002). Brown Norway rats were given DEP or carbon black (CB) by IT injection (5 mg/kg) and then sensitized by aerosol exposure to ovalbumin (OVA; 90 mg/m^3, 30 min/wk, 3 wk). An OVA aerosol challenge was given 2 wk following the last OVA sensitization exposure. Both DEP and CB, equally, increased circulating anti-OVA IgE and anti-OVA IgG by greater than 3- and 6-fold, respectively. Allergen challenge-induced pulmonary inflammation, as noted by BAL granulocytes, LDH, and protein, was also markedly elevated in both CB- and DEP-exposed, OVA-sensitized rats. IL-4 mRNA was also significantly elevated in the lung tissue of DEP-treated, OVA-sensitized versus saline-treated, OVA-sensitized rats. While many of the organic constituents of DEP have been shown to have adjuvant properties, the results of the study just described are consistent with the report that particles, including DEP, regardless of composition and route of administration, exhibit adjuvant activity (Lovik et al., 1997; Granum et al., 2001a, 2001b).

The mechanism by which DEP increases these humoral responses is not known. The particulate effect may be important in augmentation of humoral responses, but the organic constituents may also play a role. Suppression of alveolar macrophage NO production by DEP possibly may lead to enhanced dendritic cell antigen presentation. Yoshino et al. (2002) reported that DEP and DEP extracts were able to block the induction of oral tolerance. Repeated aerosol exposure to OVA has been reported to produce immunological tolerance (Sedgwick & Holt, 1983; McMenamin et al., 1992). Statistically significant reduction of circulating levels of OVA-specific IgE from peak levels attained with exposure to 68 mg/m^3 was noted at a very high OVA aerosol exposure levels of 156 mg/m^3 (30 min of exposure on d 0, 7, 14, and 28; serum taken on d 28; unpublished data). DEP augmentation of humoral responses

may thus be by a combination of increased production of humoral factors and inhibition of immunological tolerance.

In summary, DEPs have the ability to alter immune function and defenses mediated by both particulate effects and components of the organic fraction. The particulate nature of DEP can potentially contribute to acute and allergic inflammatory responses and adjuvantlike activity on the humoral immune system. Alveolar macrophage suppressive activity is mediated by the more polar constituents of the organic fraction of DEP. This suppression of alveolar macrophage activation, that is, production of macrophage cytokines, reactive oxygen species, and NO, is most likely a major factor in potential increased susceptibility to pulmonary infection.

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