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Exposure of Brown Norway Rats to Diesel Exhaust Particles Prior to Ovalbumin (OVA) Sensitization Elicits IgE Adjuvant Activity but Attenuates OVA-Induced Airway Inflammation

Caroline C. Dong,* Xuejun J. Yin,* Jane Y. C. Ma,† Lyndell Millecchia,† Mark W. Barger,† Jenny R. Roberts,† Xing-Dong Zhang,† James M. Antonini,† and Joseph K. H. Ma*,[1]

School of Pharmacy, West Virginia University, Morgantown, West Virginia 26506, and †Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505

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Exposure to diesel exhaust particles (DEP) during the sensitization process has been shown to increase antigen-specific IgE production and aggravate allergic airway inflammation in human and animal models. In this study, we evaluated the effect of short-term DEP exposure on ovalbumin (OVA)-mediated responses using a post-sensitization model. Brown Norway rats were first exposed to filtered air or DEP (20.6 ± 2.7 mg/m^3) for 4 h/day for five consecutive days. One day after the final air or DEP exposure (day 1), rats were sensitized with aerosolized OVA (40.5 ± 6.3 mg/m^3), and then again on days 8 and 15, challenged with OVA on day 29, and sacrificed on days 9 or 30, 24 h after the second OVA exposure or the final OVA challenge, respectively. Control animals received aerosolized saline instead of OVA. DEP were shown to elicit an adjuvant effect on the production of antigen-specific IgE and IgG on day 30. At both time points, no significant airway inflammatory responses and lung injury were found for DEP exposure alone. However, the OVA-induced inflammatory cell infiltration, acellular lactate dehydrogenase activity and albumin content in bronchoalveolar lavage (BAL) fluid, and numbers of T cells and their CD4$^+$ and CD8$^+$ subsets in lung-draining lymph nodes were markedly reduced by DEP on day 30 compared with the air-plus-OVA exposure group. The OVA-induced nitric oxide (NO) in the BAL fluid and production of NO, interleukin (IL)-10, and IL-12 by alveolar macrophages (AM) were also significantly lowered by DEP on day 30 as well as day 9. DEP or OVA alone decreased intracellular glutathione (GSH) in AM and lymphocytes on days 9 and 30. The combined DEP and OVA exposure resulted in further depletion of GSH in both cell types. These results show that short-term DEP exposure prior to sensitization had a delayed effect on enhancement of the sensitization in terms of allergen-specific IgE and IgG production, but caused an attenuation of the allergen-induced airway inflammatory responses.

Key Words: **diesel exhaust particles; adjuvant effect; airway inflammation; glutathione; nitrite oxide; cytokines.**

There has been a dramatic increase in the incidence of asthma in many developed countries during the last decades. Although the causes for this increase are not well known, a considerable amount of epidemiological evidence suggests that certain components of air pollution such as ozone, oxides of nitrogen, and particulate matter as well, as a variety of allergens may play important roles (Graham, 2004; Koren, 1995). Fine particles with widely different composition, such as carbon black (CB), titanium dioxide (TiO_2), polystyrene, polytetrafluoroethylene, residual oil fly ash (ROFA), house dust, and urban ambient particles, have been reported to exhibit adjuvant effects on the production of immunoglobulin (Ig)E in response to concurrently administered allergens (Dybing *et al*., 2004; Granum *et al*., 2001; Hamada *et al*., 2000; Lambert *et al*., 1999; Ormstad *et al*., 1998; van Zijverden and Granum, 2000). An important advance in this area is the discovery that diesel exhaust particles (DEP), a major component of environmental particulate pollutants in most industrialized urban areas, exert an adjuvant effect on IgE or IgG production in response to coadministered common allergens in humans and various animal models (Diaz-Sanchez *et al*., 1994, 1997; Lovik *et al*., 1997; Takano *et al*., 1997). *In vitro* and *in vivo* studies showed that polyaromatic hydrocarbons (PAHs) extracted from DEP increased IgE production by human B cells, and that mucosal stimulation with the combination of DEP and ragweed allergen induced isotype switching to IgE with ragweed allergy (Fujieda *et al*., 1998; Takenaka *et al*., 1995). Elevation of allergen-specific IgE is one of the hallmarks of allergic diseases. IgG, on the other hand, has been reported to be related to late-phase asthmatic reactions and mast cell activation (Durham *et al*., 1984). Although the adjuvant effect of DEP has been demonstrated by a number of investigations, this result was mainly obtained from studies where DEP were administered in combination with an allergen during the sensitization process, or already-sensitized human/animal models were employed. Considering the real-life circumstances where persons may encounter DEP and allergens at the same or different time points, one of the important questions raised,

[1] To whom correspondence should be addressed at School of Pharmacy, West Virginia University, 1 Medical Center Drive, Morgantown, WV 26506–9530. Fax: (304) 293-2576. E-mail: jma@hsc.wvu.edu.

therefore, is whether DEP, when given separately from an allergen, can have the adjuvant effect, i.e., augmentation of specific IgE or IgG response to an allergen encountered before or after DEP exposure.

Another important question regarding the involvement of DEP in allergic asthma is whether DEP may contribute to the chronic airway inflammation that has been recognized as an essential feature of the disease. A number of studies pointed out that fine and ultrafine particles, such as DEP, CB, ROFA, and metal particles, have the potential to trigger inflammatory mechanisms by modulating intracellular calcium concentrations, activation of transcription factors, and production of inflammatory cytokines through an oxidant-mediated mechanism (Brown *et al*., 2004; Dick *et al*., 2003; Sagai *et al*., 1996). These particles have also been shown to aggravate the antigen-induced inflammation when given together with an antigen (Ichinose *et al*., 2002; Lovik *et al*., 1997; Takano *et al*., 1997). DEP are carbon-based particles containing approximately 30% by weight various organic compounds, including PAHs, nitroaromatic compounds, quinines, aldehydes, and heterocyclic compounds, adsorbed onto the carbonaceous core (Draper, 1986; Schuetzle, 1983). Due to the complexity of their chemical properties, DEP may be different from the other particles in their modulating effect on inflammatory response and pulmonary immunity. Indeed, we have previously shown that DEP or their organic extracts, but not the carbonaceous core, CB, or silica, suppress the production of lipopolysacchride (LPS)-stimulated pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin- (IL-) 12, but increase the production of anti-inflammatory IL-10 by rat alveolar macrophages (AM) (Yang *et al*., 1999; Yin *et al*., 2004a,b). Previous results have shown that DEP aggravate ovalbumin (OVA)-induced airway inflammation when given together with the antigen (Ichinose *et al*., 2002; Miyabara *et al*., 1998; Takano *et al*., 1997, 1998). We hypothesized, however, that short-term exposure to DEP prior to sensitization may exert an anti-inflammatory effect which potentially attenuates the allergen-induced airway inflammation.

The objectives of this study were to develop a rat model, in which animals were exposed to DEP before sensitization, and use this model to examine the effects of short-term DEP exposure on allergen-induced IgE and IgG production and allergic airway inflammation. To investigate the underlying mechanism of these effects, we measured alterations in intracellular glutathione (GSH) levels, production of nitric oxide (NO) and cytokines by AM and lymphocytes from lung-draining lymph nodes (LDLN), expression of inducible NO synthase (iNOS) in lung tissue, and alterations of $CD4^+$ and $CD8^+$ T-cell subsets in LDLN. The results show that DEP exposure prior to sensitization had a delayed effect on enhancement of allergen-specific IgE and IgG production, but caused an attenuation of the allergen-induced airway inflammatory responses.

MATERIALS AND METHODS

Experimental design. Rats were divided into four groups, namely air + saline, DEP + saline, air + OVA, and DEP + OVA (Fig. 1). The animals were exposed to filtered air or DEP by inhalation for 4 h/day for 5 days. One day after the final DEP exposure (day 1), rats were sensitized by aerosolized saline or OVA for 30 min, then once a week for 30 min on days 8, 15, and then challenged with aerosolized saline or OVA on day 29. At 9 or 30 days after DEP exposure (24 h after the second OVA exposure and the final OVA challenge), bronchoalveolar lavage (BAL) was performed, and lactate dehydrogenase (LDH) activity and albumin content in acellular BAL fluid were determined as a measure of lung injury/cytotoxicity. The recovered BAL cells were differentiated and counted as an indicator of pulmonary inflammation, and serum concentrations of OVA-specific IgE and IgG were used as indicators of allergic immune response. Meanwhile, levels of intracellular GSH, lymphocyte phenotype, and production of NO and/or cytokines by AM and lymphocytes from LDLN were determined.

Animal exposure. Male Brown-Norway (BN) rats [BN/CrlBR] weighing 200–225 g were obtained from Charles River Laboratories (Wilmington, MA). The animals 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 the inhalation exposure, the animals were conditioned to the exposure unit. Rats were placed in the tubes of the exposure unit for increasing time period from 1–4 h/day for 4 successive days.

A standardized DEP sample (standard reference material 2975) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). The rats were exposed to either filtered air or DEP (20.6 ± 2.7 mg/m^3) for 4 h/day for five consecutive days using a nose-only directed flow exposure unit (CH Technologies, Inc., Westwood, NJ) which has been previously described and characterized (Yin *et al*., 2002, 2004a). Briefly, 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) which allows continuous measurement of the particle concentration in the exposure unit in real time. The estimated mean lung deposition of DEP for the inhalation exposure (4 h × 5 days), according to the calculating method of Leong *et al*. (1998), was estimated to be 396 ± 53.3 μg/rat.

A solution of OVA (Grade V, Sigma Chemical Co., St. Louis, MO) in endotoxin-free saline (1%) was aerosolized using a DeVilbiss-646 nebulizer (DeVilbiss, Somerset, PA). To achieve desired concentration, filtered air was passed through the nebulizer and used as a diluent for the aerosolized OVA. The concentration of OVA in chamber was determined by collecting samples onto 0.4-μm filters (Polycarbonate Membrane, Poretics Corporation, Livermore, CA) from a chamber side port at a rate of 1 l/min. Filters were washed with 10 ml of endotoxin-free saline and analyzed for protein content using the Coomassie blue dye reagent (Bio-Rad Laboratories, Hercules, CA). Rats were exposed to OVA for 30 min at 1 day after the final DEP inhalation exposure (day 1), and then once a week for 30 min on days 8 and 15, and challenged with aerosolized saline or OVA at the same concentration for 30 min on day 29. The average chamber concentration of OVA was 40.5 ± 6.3 mg/m^3. Control animals were exposed to aerosolized endotoxin-free saline.

BAL. Rats were deeply anesthetized with an overdose of sodium pentobarbital (200 mg/kg, ip; Butler, Columbus, OH) and euthanized by exsanguination *via* the vena cava on days 9 or 30, 24 h after the second OVA exposure and the final OVA challenge, respectively. After clamping off the right apical lobe, the remaining lung lobes were first lavaged with 6 ml Ca^{2+}/Mg^{2+}-free phosphate-buffered solution (PBS, 145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4). The first BAL fluid sample was centrifuged at 500 × *g* for 10 min at 4°C, and the resultant cell-free supernatant (~4 ml/rat) was analyzed for various biochemical

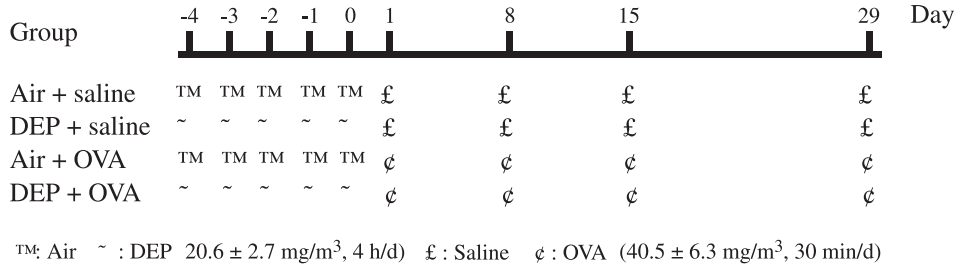

FIG. 1. Experimental design for animal exposure. BN rats (five rats/group) were exposed to DEP (20.6 ± 2.7 mg/m^3) for 4 h/d for 5 days. One day after the final DEP exposure, rats were sensitized by OVA (40.5 ± 6.3 mg/m^3) for 30 min, then once a week for 30 min on days 8 and 15, and challenged with OVA on day 29. Control animals received filtered air and saline instead of DEP and OVA under the same procedure.

parameters. The lungs were further lavaged with 8-ml aliquots of PBS until 80 ml of BAL fluid was collected. These samples were also centrifuged for 10 min at 500 × *g*, and the cell-free BAL fluid discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 ml PBS and evaluated as described below.

Biochemical parameters of injury. Albumin content, a measure to quantify increased permeability of the bronchoalveolar-capillary barrier, and LDH activity, an indicator of general cytotoxicity, were determined in the first fraction of acellular BAL fluid. 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.). LDH activity was determined by measuring the oxidation of lactate to pyruvate coupled with the formation of reduced form of nicotinamide adenine dinucleotide at 340 nm using the Roche Diagnostic reagents and procedures (Roche Diagnostic Systems, Indianapolis, IN).

Cellular evaluation. Total BAL cell numbers were determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). Cell suspensions (5 × 10^4 cells) were centrifuged for 5 min at 800 rpm and pelleted onto a slide using a Cytospin centrifuge (Shandon Life Sciences International, Cheshire, England). Cells (300/rat) were identified and differentiated after labeling with Leukostat stain (Fisher Scientific, Pittsburgh, PA). The absolute numbers of cells differentiated were calculated by multiplying the total number of cells by the percentage of the total within each cell type.

Nitrite production. The BAL cells were suspended in Eagle's minimum essential medium (MEM, Biowhittaker, Walkersville, MD) supplemented with 1 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% heat-inactivated fetal bovine serum. Aliquots of 1 ml cell suspensions, adjusted to contain 2 × 10^6 AM, 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 sterile PBS. The remaining AM-enriched cells were then incubated in 1 ml medium for 24 h at 37°C and 5% CO_2. The NO oxidation product nitrite in the AM-conditioned media and acellular BAL fluid was measured colorimetrically with the Greiss reaction (Green *et al.*, 1982). Nitrite levels were determined by comparing values to sodium nitrite standards.

Lymphocyte isolation and T-cell subset phenotypes. All LDLN were excised from each rat after the BAL procedure, teased apart with forceps, and homogenized with a glass pestle in a screen cup (Sigma). Single-cell suspensions were obtained by passing the cell clumps through a 22-gauge needle attached to a 10-ml syringe. The cells were washed twice with PBS, and lymphocytes were isolated by Histopaque (density 1.083; Sigma) gradient centrifugation. In brief, the samples were centrifuged for 30 min at 2500 rpm, and lymphocytes were collected, washed, and resuspended in 1 ml of 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%.

To determine the effects of OVA and DEP exposures on phenotypes of T cell subsets in LDLN, expression of CD3, CD4, and CD8 cell surface markers on isolated lymphocytes was examined with flow cytometry as previously described (Yin *et al*., 2003). Briefly, lymphocytes (10^6 cells) were stained with the addition of FITC-labeled conjugated antibodies against these cell surface markers (BD Pharmingen, San Diego, CA), respectively. The flow cytometric data were collected with a Becton-Dickinson FACScan using FACScan Research Software (Becton-Dickinson Immunocytometry System, San Jose, CA) and analyzed using the PC-LYSYS software (Becton-Dickinson).

Cytokine production. The BAL cells were suspended in Eagle's minimum essential medium (MEM, Biowhittaker, Walkersville, MD) supplemented with 1 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% heat-inactivated fetal bovine serum. Aliquots of 1 ml cell suspensions, adjusted to contain 2 × 10^6 AM, were added to each well of a 24-well tissue culture plate and 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 (2 × 10^6) isolated from LDLN were then incubated in 1 ml medium at 37°C and 5% CO_2 for 24 h. The AM- or lymphocyte-conditioned media were collected, centrifuged (1200 × *g* for 4 min), and aliquots of the supernatants were stored at −70°C until assayed.

The production of IL-10 in AM-conditioned media under various exposure conditions was quantified by the enzyme-linked immunosorbent assay (ELISA) using the OptEIA ELISA Sets (BD PharMingen) as previously described (Yin *et al*., 2004a). The levels of IL-12 in the AM-conditioned media and IL-4 and interferon (IFN)-γ in the lymphocyte-conditioned media and serum were quantified by ELISA using commercial ELISA kits (BioSource International, Inc., Camarillo, CA). The range of detection was: 7.8–500 pg/ml for IL-4 and IL-12, 15.6–1000 pg/ml for IL-10, and 31.3–2000 pg/ml for IFN-γ.

Determination of intracellular GSH. AM or lymphocytes (2 × 10^5 cells) were plated in 96-well microplates, washed twice with PBS, and lysed with 240 μl of a cold lysing buffer (0.1% triton X-100 in 0.1 M sodium phosphate buffer, 5 mM EDTA, pH 7.5). The lysates were acidified with 0.1 N HCl (12 μl), and protein precipitated with 50% sulfosalicyclic acid (12 μl) followed by centrifugation at 4°C. Samples of the supernatants were assayed for total GSH according to the method of Buchmuller-Rouiller *et al*. (1995). Briefly, 50 μl of cell supernatants or GSH standards were distributed to each well of a 96-well microplate, followed by 50 μl of 2.4 mM 5,5′-dithio-bis(2-nitrobenzoic acid). After the mixture was incubated at room temperature for 10 min, 50 μl each of NADPH (0.667 mg/ml) and glutathione reductase (40 μg/ml) were added. The results of the GSH-specific reaction were monitored by OD readings at 405 nm every minute for 8 min with a Spectramax 250 plate spectrophotometer using Softmax Pro 2.6 software (Molecular Devices Corp., Sunnyvale, CA). One of the OD readings obtained with the most satisfied standard curve was selected as the final result.

Determination of OVA-specific IgE and IgG. Blood samples were collected from the vena cava on days 9 and 30. The sera dilutions with 5% horse serum albumin (HOSA)/PBS of 1/50 were analyzed for OVA-specific IgE

and IgG. Diluted sera (100 µl) were added to a 96-well plate (ICN Biomedicals, Horsham, PA) that had been previously coated with 200 µl of 1% OVA carbonate coating buffer and blocked with a 5% HOSA/coating buffer according to the method of Voller and Bidwell (1986). The plates were incubated overnight at 4°C and subsequently incubated with sheep anti-rat IgE (100 µl, 1/2500 dilution in HOSA/PBS, ICN Biomedicals, Costa Mesa, CA), and horseradish peroxidase-bound donkey anti-sheep IgG (100 µl, 1/5000 dilution in HOSA/PBS, ICN Biomedicals) for 2 h each at room temperature. The plates were washed three times following each incubation, treated with tetramethylbenzidine (Sigma), and read at 630 nm. OVA-specific IgG was determined using goat anti-rat IgG (1/500 dilution in HOSA/PBS, Sigma) and peroxidase-labeled rabbit anti-goat IgG (1/12,500 dilution in HOSA/PBS, Sigma) as detection antibodies following the same protocol described above. The serum from one animal exposed to OVA was assigned a value of 100 and used as a reference to obtain relative concentration for the OVA-specific IgE and IgG in serum samples from each group.

Immunohistochemistry. Before BAL, the right apical lobe was clamped off to prevent entry of lavage fluid. Following BAL and excision of LDLN, the clamp was removed, and all lobes were inflated intratracheally with 10% formalin. The right apical lobe was processed within 24 h and embedded in paraffin. Sections were cut at 5 µm, deparaffinized in xylene and rehydrated, and stained for iNOS expression as previously described (Porter *et al.*, 2002). Briefly, after rehydration, microwave antigen retrieval with citrate buffer (pH 6.0) was performed, followed by peroxidase blocking with a 1:1 mixture of 3% H_2O_2 and methanol. Slides were incubated overnight at 4°C with iNOS monoclonal antibody (N32020, Transduction Laboratories, Lexington, KY, 1:50 dilution). Localization was achieved using a streptavidin-biotin-peroxidase system for use on rat specimens (K0609, Dako, Carpinteria, CA), with diaminobenzidene (Zymed Laboratories, South San Francisco, CA) as the chromogen. Tissues were counterstained with Mayer's hematoxylin, dehydrated, and covered with a coverslip. Negative controls were sections in which the primary antibody was omitted; these had no staining, except for occasional slightly positive mast cells. Positive controls for iNOS were lung sections from BN rats that had been intratracheally instilled with LPS (Sigma, 10 mg/kg) 24 h prior to sacrifice.

Statistical analysis. Results are expressed as means ± standard error (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 Different Test. For all analyses, the criterion of significance was set at $p < 0.05$.

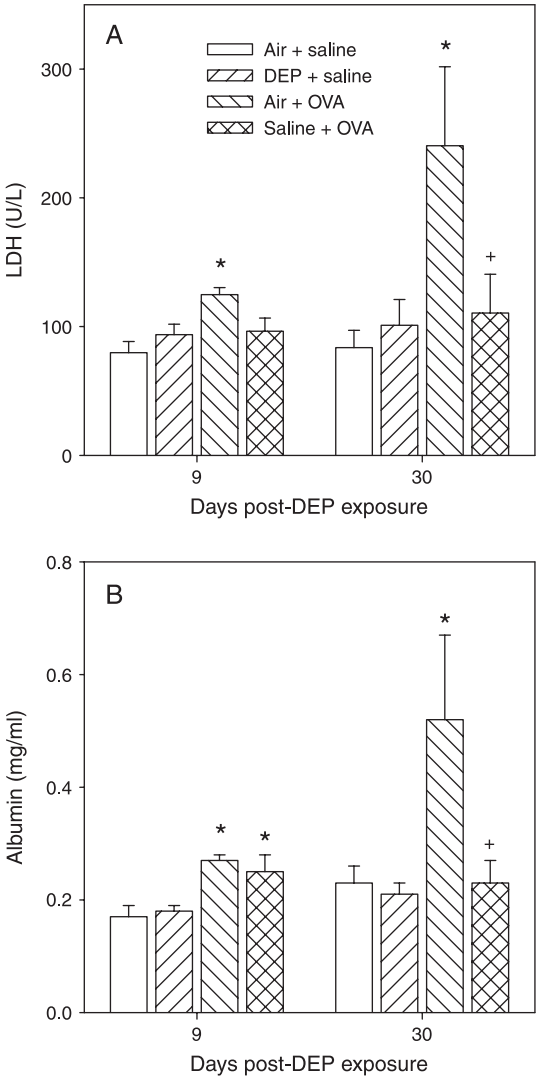

FIG. 2. Yield of (A) LDH activity and (B) albumin content in bronchoalveolar lavage (BAL) fluid from rats ($n = 5$). *Significantly different from air + saline group, $p < 0.05$; $^{+}$Significantly different from air + OVA group, $p < 0.05$.

RESULTS

Lung Inflammation/Injury

The LDH activity and albumin content in the first acellular BAL fluids of rats from various exposure groups were determined as markers of lung injury and cytotoxicity (Fig. 2), whereas the numbers of recovered AM, neutrophils, lymphocytes, and eosinophils in BAL fluids were counted as an index of airway inflammation (Table 1). These data show that both the BAL proteins and inflammatory cell counts for DEP exposure alone, when measured at 9 and 30 days after exposure, were not different from those of the air-exposed control, suggesting that DEP exposure did not cause lung injury at 9 or 30 days post-exposure. Exposure of rats to OVA (administered weekly at a dose of 40.5 ± 6.3 mg/m^3) caused significant increases in neutrophils, lymphocytes, eosinophils, albumin, and LDH activity in the rat lung after two exposures (measured at day 9). These increases were further substantiated in rats receiving five OVA exposures (measured at day 30), with a clear increase in total BAL cells when compared to that of the air-exposed control. Although DEP exposure alone did not show significant effect on lung inflammation at the two time points examined, it had a strong effect on OVA-induced inflammatory responses. As shown in Table 1 and Figure 2, the combined DEP and OVA exposure resulted in a significant decrease in OVA-induced inflammatory cell infiltration at day 30 and LDH and albumin at days 9 and 30 of DEP exposure. It should be noted, however, the number of total cells recovered from rats exposed to air or DEP alone was slightly increased on day 30 compared to their corresponding groups on day 9 (Table 1). This increase, according to the calculating method

TABLE 1
Total Numbers and Differentials of BAL Cells ($\times 10^6$)

Treatment	Total cells	AM	Neutrophils	Lymphocytes	Eosinophils
			Day 9 post-DEP exposure		
Air + saline	3.36 ± 0.88	3.26 ± 0.85	0.01 ± 0.01	0.03 ± 0.02	0.05 ± 0.01
DEP + saline	3.53 ± 0.84	3.31 ± 0.51	0.02 ± 0.01	0.05 ± 0.01	0.11 ± 0.05
Air + OVA	3.55 ± 0.54	3.05 ± 0.20	0.05 ± 0.01[a]	0.07 ± 0.02[a]	0.19 ± 0.02[a]
DEP + OVA	3.16 ± 0.54	2.78 ± 0.47	0.08 ± 0.03[a]	0.09 ± 0.03[a]	0.21 ± 0.04[a]
			Day 30 post-DEP exposure		
Air + saline	5.82 ± 1.10	4.52 ± 0.50	0.09 ± 0.02	0.05 ± 0.01	0.15 ± 0.08
DEP + saline	5.00 ± 1.22	4.68 ± 1.15	0.09 ± 0.03	0.04 ± 0.01	0.19 ± 0.09
Air + OVA	12.37 ± 4.07[a]	5.59 ± 1.48	3.22 ± 0.84[a]	0.62 ± 0.29[a]	3.24 ± 1.65[a]
DEP + OVA	5.56 ± 0.68[b]	3.21 ± 0.56[b]	1.40 ± 0.25[a,b]	0.17 ± 0.02[a,b]	0.77 ± 0.33[a,b]

Note. Values are means ± SE ($n = 5$).
[a]Significantly different from air + saline group; $p < 0.05$.
[b]Significantly different from air + OVA group; $p < 0.05$.

used in this study, would result in a slight increase in each individual cell type differentiated on day 30, although no direct comparison between data of day 9 and day 30 was attempted. The reason for this difference is not clear, but may be related to the gain in body weight by animals on day 30.

Alveolar Macrophage Function

NO, an important marker of allergic inflammatory responses, in the acellular BAL fluid (Fig. 3A) and in AM-conditioned media (Fig. 3B) from various exposure groups was determined. The OVA exposure resulted in an increase in NO levels in the acellular BAL fluid and AM-conditioned media. This increase, however, was significantly attenuated in rats already exposed to DEP. DEP exposure alone did not affect NO production in samples obtained at day 9, although the NO levels in the BAL fluid at day 30 showed a moderate decrease compared to that of the air-exposed controls.

Figure 4 shows the production of IL-10 and IL-12 by AM. DEP exposure had no significant effect on the production of IL-10 (Fig. 4A) or IL-12 (Fig. 4B) by AM recovered from rats 9 and 30 days after exposure. In contrast, OVA sensitization markedly elevated both IL-10 and IL-12 secretion by AM at both time points. The production of these cytokines by AM from rats exposed to both DEP and OVA was significantly lower than cells from rats exposed to OVA alone.

Lymphocyte Population and Cytokine Production

Figure 5 shows the numbers of lymphocytes, T cells, and their $CD4^+$ and $CD8^+$ subsets recovered from LDLN for each exposure group on day 9 (Fig. 5A) and day 30 (Fig. 5B). DEP exposure was found to increase the numbers of total lymphocytes, T cells and their $CD4^+$ and $CD8^+$ subsets in the LDLN compared to the air-exposed control. Likewise, OVA exposure significantly increased these cell counts on day 9 as well as on day 30. In the combined DEP and OVA exposure, however, there was a significant reduction in total lymphocytes, T cells, and $CD4^+$ and $CD8^+$ subsets in the rat lung on day 30 when compared to data for the OVA-only exposure groups.

Levels of IL-4 and IFN-γ in lymphocyte-conditioned media were below the detection limit of the ELISA kits used. The cytokines were also measured in serum from the rats. Except OVA alone group showing marginally detectable IL-4 and IFN-γ, other rats did not show detectable amount of the cytokines in serum (data not shown). The effects of DEP and OVA exposure on these cytokines, therefore, could not be determined.

Intracellular GSH Levels in AM and Lymphocytes

The effect of DEP and OVA exposures on intracellular GSH levels was studied in AM (Fig. 6A) and lymphocytes (Fig. 6B) from various exposure groups. DEP exposure alone slightly decreased GSH levels in AM, but markedly reduced GSH concentration in lymphocytes on day 9 and day 30. Exposure to OVA significantly decreased intracellular GSH in both cell types. Under the combined DEP and OVA exposure, AM and lymphocytes exhibited a greater depletion of intracellular GSH at day 9 and at day 30 compared to either DEP or OVA exposure alone.

OVA-Specific IgE and IgG Levels in Serum

In all samples collected on day 9, both serum IgE and IgG levels were under the detection limits. On day 30, neither air- nor DEP-exposed rats exhibited measurable IgE levels in the serum (Fig. 7A). The OVA exposure, however, resulted in elevated IgE level, and this antibody production was further enhanced in rats preexposed to DEP (Fig. 7A). The IgE level for the combined DEP and OVA exposure was 2.2-times higher than that of the OVA exposure alone. Similarly, OVA sensitization also increased OVA-specific IgG production,

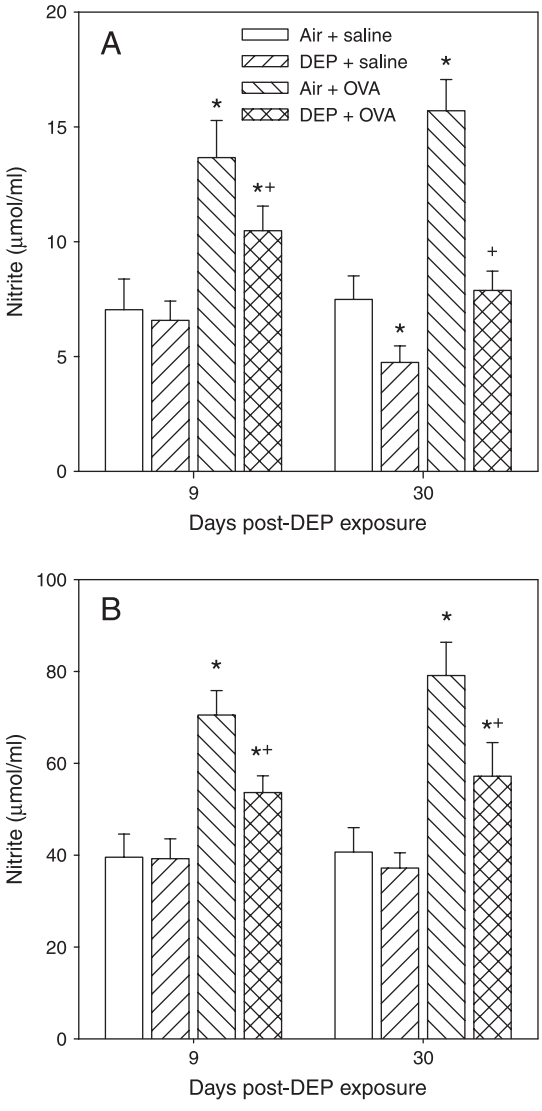

FIG. 3. Concentrations of NO in (A) BAL fluid and (B) AM-conditioned media ($n = 5$). *Significantly different from air + saline group, $p < 0.05$; $^{+}$Significantly different from air + OVA group, $p < 0.05$.

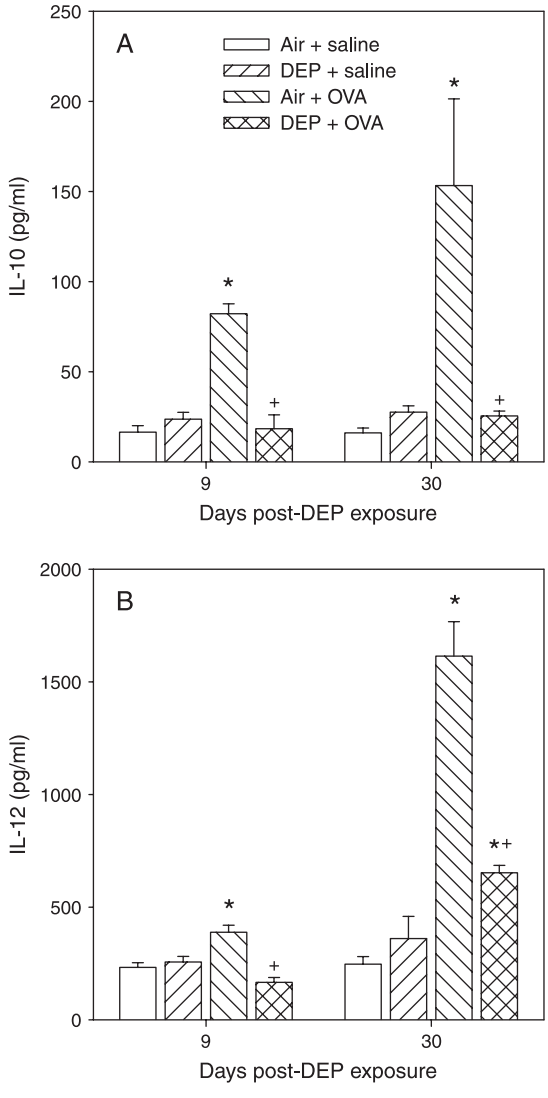

FIG. 4. Production of (A) IL-10 and (B) IL-12 by AM. AM were incubated at 37°C and 5% CO_2 for 24 h. Concentrations of the cytokines in the culture media were quantified ($n = 5$). *Significantly different from air + saline group, $p < 0.05$; $^{+}$Significantly different from air + OVA group, $p < 0.05$.

and in combination with DEP exposure, resulted in an even greater production of IgG (Fig. 7B). The IgG level for the combined DEP and OVA exposure was about two-times higher than that of the OVA exposure alone. These results indicate that DEP have an adjuvant effect on the production of both IgG and IgE.

Effects of DEP and OVA on Lung iNOS Expression

Immunohistochemical analysis of iNOS in lung tissues showed that AM from various exposure groups did not stain for iNOS. At day 9 after DEP exposure, one of the five rats in the combined DEP and OVA exposure showed slightly positive iNOS staining in the airway epithelium. On day 30, two of the five rats from the combined exposure and one of five rats from the OVA exposure alone showed positive airway staining. In comparison, both AM and airway epithelia from LPS-treated animals were highly positive in iNOS staining (data not shown).

DISCUSSION

Studies have shown that many environmental pollutants exacerbate asthma by having an adjuvant effect on the allergic responses. DEP, in particular, were shown to aggravate antigen-induced airway inflammation, serum IgE production, infiltration of eosinophils and lymphocytes, and an increase in goblet cells in bronchial epithelium in mice concurrently exposed to both OVA and DEP (Ichinose *et al*., 2002; Takano *et al*., 1997,

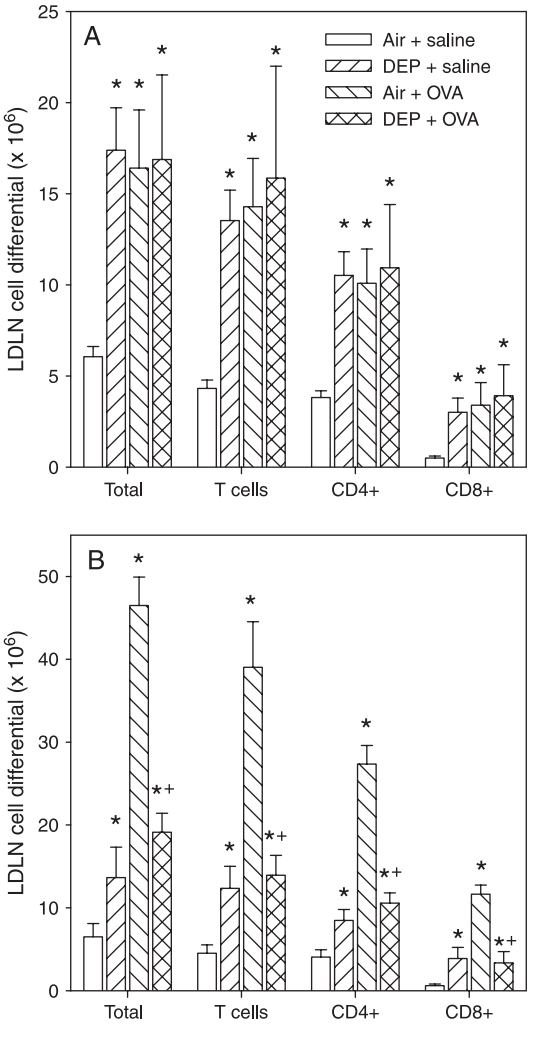

FIG. 5. Lymphocyte differentiation in lung-draining lymph nodes (LDLN) of rats at (A) day 9 and (B) day 30 post-DEP exposure ($n = 5$). *Significantly different from air + saline group, $p < 0.05$; $^{+}$Significantly different from air + OVA group, $p < 0.05$.

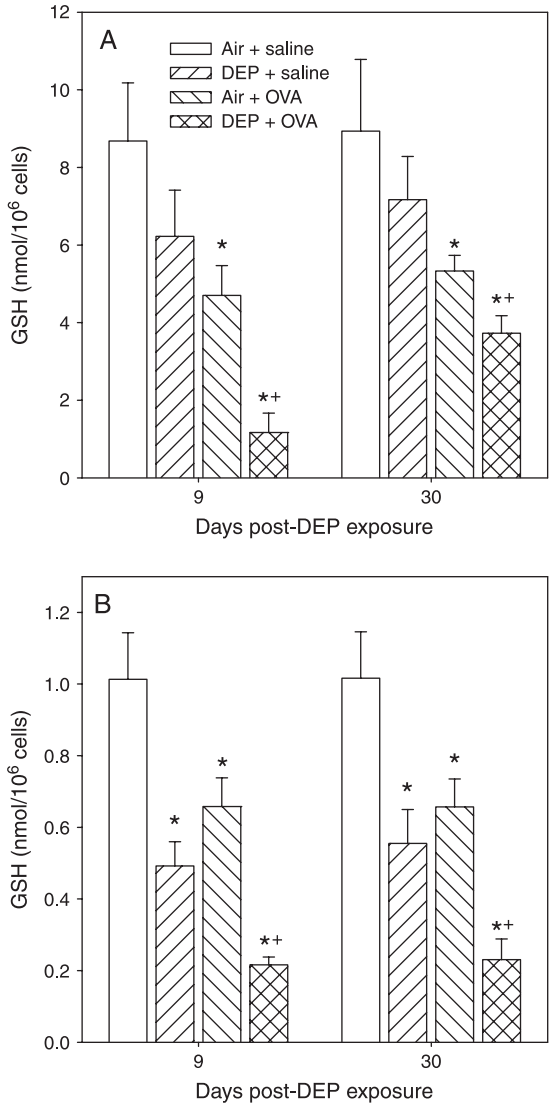

FIG. 6. Concentrations of intracellular GSH in (A) AM and (B) lymphocytes isolated from lung-draining lymph nodes of rats at day 9 and day 30 post-DEP exposure ($n = 5$). *Significantly different from air + saline group, $p < 0.05$; $^{+}$Significantly different from air + OVA group, $p < 0.05$.

1998). In practical settings, subjects may be sensitized or nonsensitized to an allergen and may encounter DEP and allergen exposure at different time points, thus making the timing of DEP exposure an important factor for assessment of allergic responses. The present study was designed to assess whether inhaled DEP, when exposed prior to sensitization, enhance the allergen-induced IgE or IgG responses and airway inflammation. The results showed that, when rats were exposed first to DEP for five consecutive days and then to aerosolized OVA once a week for 4 weeks, the inhaled DEP enhanced the sensitization of rats to OVA in antigen-specific antibody production. This result, together with that from previous studies, suggests that frequent exposure to DEP may alter allergy-related responses in both previously sensitized and nonsensitized individuals, thus contributing to the manifestations and increased prevalence of asthma (Diaz-Sanchez *et al*., 1994, 1997; Lovik *et al*., 1997; Takano *et al*., 1997).

DEP are carbon-based particles containing various organic compounds adsorbed onto the carbonaceous core. The organic component-mediated reactive oxygen species (ROS) generation plays an important role in cell-mediated immune responses (Ma and Ma, 2002; Whitekus *et al*., 2002; Yin *et al*., 2004b). The imbalance between cellular antioxidants and ROS levels can lead to oxidative stress and a depletion of intracellular thiol levels. Increasing clinical, epidemiological, and experimental evidence indicates that excess production of ROS and defective endogenous antioxidant defense mechanisms are associated with asthma (Henricks and Nijkamp, 2001). Glutathione-S-transferases (GSTs), for example, have been shown to be key regulators of the adjuvant effects of DEP on allergic responses. Compared with patients with a functional genotype, individuals

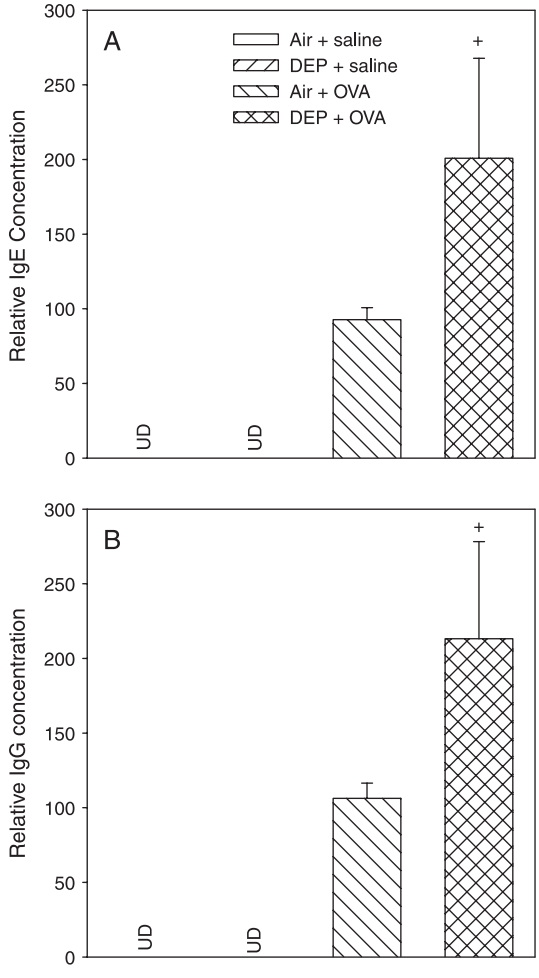

FIG. 7. Relative concentrations of OVA-specific (A) IgE and (B) IgG in serum collected at day 30 post-DEP exposure ($n = 5$). $^{+}$Significantly different from air + OVA group, $p < 0.05$. UD: Under detection limit.

with GSTM1 null or the GSTP1 I105 wild type genotypes showed enhanced nasal allergic responses in the presence of DEP, including increased production of antigen-specific IgE (Gilliland *et al*., 2004). As an important immune modulator, intracellular GSH has a regulatory role in antigen presenting cells, including AM, dendritic cells, and B cells, which is critical for the development of T cell-mediated immunity and the predominance of cellular production of Th1 or Th2 cytokines (Ma and Ma, 2002; Murata *et al*., 2002; Peterson *et al*., 1998). Studies have also shown that macrophages with high GSH content preferentially converted naïve $CD4^{+}CD44^{-}$ Th0 to Th1 cells, whereas GSH deficient macrophages acted oppositely, i.e., converted the naïve Th0 to Th2 cells (Murata *et al*., 2002). This macrophage-mediated Th1/Th2 balance is of significance, since OVA exposure resulted in a dose-dependent depletion of GSH in AM that correlated to increased production of antigen-specific IgE and IgG in rats (Al-Humadi *et al*., 2002). Our studies showed that DEP exposure alone markedly reduced the levels of GSH in lymphocytes and, to a lesser extend, in AM at 9 and 30 days post DEP exposure, and that the combined DEP and OVA exposure resulted in a synergistic depletion of GSH in both cell types. However, the OVA-induced $CD4^{+}$ T cells and AM secretion of IL-10 and IL-12 were significantly decreased by DEP, while two other cytokines (IL-4 and IFN-γ) were not successfully detected. The fact that DEP aggravate intracellular GSH depletion in AM and lymphocytes suggests that the organic component of DEP, which induces intracellular ROS (Yin *et al*., 2004b), may play a role in the elevation of antigen-specific IgE and IgG. Although a clear switch of T-cell immunity toward Th2 response in terms of cytokine profile cannot be concluded from this study, elevated levels of OVA-specific IgE and IgG in the DEP plus OVA group strongly suggest the occurrence of this switch *in vivo*.

The present study also showed that DEP exposure prior to OVA sensitization inhibited allergen-induced airway inflammation measured at 4 weeks post exposure. In agreement with this finding, we have previously shown that preexposure to DEP significantly decreased the number of inflammatory cells (AM and neutrophiles) in the lung and T cells and their $CD4^{+}$ and $CD8^{+}$ subsets in LDLN recovered from *Listeria monocytogenes*-infected BN rats (Yin *et al*., 2004a). Inhibition of OVA-induced airway inflammation by DEP as shown by the BAL cell differentials in this study, albeit not an unexpected effect, contrasted with that reported previously (Miyabara *et al*., 1998; Sagai *et al*., 1996; Takano *et al*., 1998). This discrepancy suggests that DEP may have multiple effects on allergic responses, depending on the status of the lung inflammation induced by both allergens and particles. On one hand, DEP exhibit time-dependent effects on allergen-induced airway inflammatory responses by increasing allergic inflammation in already-sensitized individuals or during the sensitization process, but attenuating these responses induced by delayed sensitization in previously nonsensitized individuals. On the other hand, local inflammation induced by DEP (or other particles), which is significant or not, depending on the doses and timing for exposure, is another important factor for DEP to affect allergen-related inflammation. In concurrent exposure studies, the aggravation of allergen-induced inflammatory responses by DEP, such as those reported by Takano *et al.* (1997, 1998), is expected, since the acute pulmonary responses to DEP exposure alone also involve inflammatory cell infiltration, airway inflammation, and altered lymphocyte function (Miyabara *et al*., 1998; Sagai *et al*., 1996; Takano *et al*., 1998). In the current study, no significant acute inflammatory response or lung injury was expected, while we did not examine the acute pulmonary response of rats to DEP exposure. According to the same exposure dose and protocol, DEP alone did not induce significant acute inflammatory responses and lung injury in BN rats as shown by the previous results (Yin *et al*., 2004a).

The mechanism through which inhaled DEP inhibit the allergen-induced airway inflammation is complex but may

involve a number of factors including NO, ROS, and their effects on cellular cytokine production. Studies in rats and mice have shown that NO production from lung cells (epithelial cells, macrophages, and Th1 cells) may down-regulate Th1 cell-derived IFN-γ production and concomitantly up-regulate local expression of Th2 cell-derived IL-4 and IL-5 (Barnes and Liew, 1995), thus promoting the development of pulmonary eosinophilia and airway hyperreactivity (Feder *et al*., 1997; Liu *et al*., 1997). Although immunohistochemical analysis of lung tissues failed to detect alterations in iNOS expression in AM, the current study shows that inhaled DEP significantly inhibit OVA-induced NO production in AM-conditioned media and BAL fluid, and this corresponds to an attenuation of OVA-induced eosinophilia. It has been known that DPE, through the organic compounds, induce cellular expression of antioxidative enzymes that contribute to the anti-inflammatory responses. One such enzyme is heme oxygenase-1 (HO-1). We have shown previously that DEP up-regulated expression of HO-1 in AM through a mechanism that involves the organic component–induced cytochrome P450 1A1 and ROS generation (Rengasamy *et al*., 2003; Yin *et al*., 2004b). Studies have shown that HO-1 may act through carbon monoxide, a major catalytic byproduct of HO-1-mediated heme degradation, which, interestingly, has also been shown to reduce allergen-induced airway hyperresponsiveness and eosinophilia (Chapman *et al*., 2001). Additionally, studies in animal models of allergic asthma showed that CD8-deficent mice or those with depleted $CD4^+$ T cells developed significantly lowered airway hyperresponsiveness and eosinophilic inflammation, indicating an important role of these T-cell subsets in airway eosinophilia (Gavett *et al*., 1994; Komai *et al*., 2003; Miyahara *et al*., 2004). The inhibition of OVA-induced $CD4^+$ and $CD8^+$ T lymphocytes by DEP exposure, as demonstrated in our study, thus may also contribute to its attenuation of allergic airway inflammation.

AM play a pivotal role in controlling and directing immune responses by secreting a variety of mediators including both pro-inflammatory and anti-inflammatory cytokines. We and others have shown previously that *in vivo* or *in vitro* exposure to DEP suppressed AM phagocytotic capacity and their secretion of pro-inflammatory cytokines, such as IL-1β, IL-12, and TNF-α, in response to bacterial infection or LPS stimulation (Amakawa *et al*., 2003; Saito *et al*., 2002; Yin *et al*., 2002). These studies indicated that DEP exposure dampened AM function and responses to external stimuli and that DEP may play a role in reducing inflammation. Indeed, DEP, at low dose, were shown to exert an anti-inflammatory effect on bacteria-induced lung inflammation in rats (Yin *et al*., 2004a). Consistent with these results, the current study showed that DEP significantly suppressed AM secretion of NO, IL-10, and IL-12 in response to OVA stimulation. In view of the decreased lung inflammation in the combined DEP- and OVA-exposed rats, the observed reduction in OVA-induced IL-10 by DEP seems puzzling, since lines of evidence have led to the hypothesis that IL-10 may act as one of the anti-inflammatory cytokines that regulate ongoing inflammatory responses (de Vries, 1995). In fact, the regulatory role of endogenous IL-10 in asthma remains unclear. Previous studies have reported that IL-10 production in response to LPS was impaired in the monocyte/macrophage cell lineage of asthmatics (Borish *et al.*, 1996; John *et al.*, 1998), whereas other studies have reported an increase of IL-10-producing T cells and macrophages in asthmatic airways and a further increase of those cells after an allergen challenge (Colavita *et al.*, 2000; Magnan *et al.*, 1998; Robinson *et al.*, 1996). Furthermore, experimental asthma studies using IL-10 knockout mice have provided controversial results in which increased or decreased airway inflammatory responses to allergen challenge were found (Justice *et al.*, 2001; Makela *et al.*, 2000; Yang *et al.*, 2000). Interestingly, the IL-10 knockout mice showed increased or no changeable Th2 responses including allergen-specific IgE production (Justice *et al.*, 2001; Yang *et al.*, 2000). These results, together with that obtained from the current study, show that the role of IL-10 in regulating allergen-related airway inflammatory responses may be very complex and need to be further studied. On the other hand, one would speculate on a decreased IgE production in the DEP-plus-OVA exposure group because AM antigen presenting function may be also dampened. Since AM are poor antigen-presenting cells compared to dendritic cells (Nicod *et al*., 2000), inhibition of AM function by DEP may not significantly affect the antigen presenting in the lung and subsequent antibody production. Inhibition of AM function by DEP may be, at least in part, responsible for the decreased production of both Th1 and Th2 cytokines as examined in the DEP plus OVA exposed rats.

In summary, this study showed that short-term DEP exposure prior to a 4-week OVA sensitization process enhanced the production of allergen-specific IgE and IgG but attenuated the antigen-induced airway inflammation in BN rats. These results are different from those obtained from experimental protocols involving long-term DEP exposure or allergic responses to acute DEP exposure (Ichinose *et al*., 2002; Sagai *et al*., 1996; Takano *et al*., 1997, 1998), but allow one to examine the delayed effect of inhaled DEP on the immune system. The synergistic depletion of GSH in AM and lymphocytes by DEP and OVA may be responsible for the adjuvant effect of DEP on OVA-specific antibody production. The fact that inhaled DEP elicited an inhibitory effect on the OVA-induced inflammatory cell infiltration, T lymphocyte development, and the production of NO and pro-inflammatory cytokines by AM suggests that the organic component of DEP may play an important role in the delayed effect of DEP on the allergic responses. Considering the well-known detrimental effects of long-term DEP exposure on increased asthma prevalence, it is important to show whether the observed reduction in allergic airway inflammation by DEP is a temporary change. The accurate time-dependent nature of interactions of exposure to DEP and allergens in producing allergic inflammatory responses is intriguing and warrants for further study.

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