TOXICOLOGICAL SCIENCES **88(1)**, 202–212 (2005)
doi:10.1093/toxsci/kfi280
Advance Access publication August 17, 2005

Effect of Diesel Exhaust Particles on Allergic Reactions and Airway Responsiveness in Ovalbumin-Sensitized Brown Norway Rats

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Received April 14, 2005; accepted July 12, 2005

We have previously demonstrated that exposure to diesel exhaust particles (DEP) prior to ovalbumin (OVA) sensitization in rats reduced OVA-induced airway inflammation. In the present study, Brown Norway rats were first sensitized to OVA (42.3 ± 5.7 mg/m^3) for 30 min on days 1, 8, and 15, then exposed to filtered air or DEP (22.7 ± 2.5 mg/m^3) for 4 h/day on days 24–28, and challenged with OVA on day 29. Airway responsiveness was examined on day 30, and animals were sacrificed on day 31. Ovalbumin sensitization and challenge resulted in a significant infiltration of neutrophils, lymphocytes, and eosinophils into the lung, elevated presence of CD4$^+$ and CD8$^+$ T lymphocytes in lung draining lymph nodes, and increased production of serum OVA-specific immunoglobulin (Ig)E and IgG. Diesel exhaust particles pre-exposure augmented OVA-induced production of allergen-specific IgE and IgG and pulmonary inflammation characterized by marked increases in T lymphocytes and infiltration of eosinophils after OVA challenge, whereas DEP alone did not have these effects. Although OVA-sensitized rats showed modest response to methacholine challenge, it was the combined DEP and OVA exposure that produced significant airway hyperresponsiveness in this animal model. The effect of DEP pre-exposure on OVA-induced immune responses correlated with an interactive effect of DEP with OVA on increased production of reactive oxygen species (ROS) and nitric oxide (NO) by alveolar macrophages (AM) and alveolar type II (ATII) cells, NO levels in bronchoalveolar lavage fluid, the induction of inducible NO synthase expression in AM and ATII cells, and a depletion of total intracellular glutathione (GSH) in AM and lymphocytes. These results show that DEP pre-exposure exacerbates the allergic responses to the subsequent challenge with OVA in OVA-sensitized rats. This DEP effect may be, at least partially, attributed to the elevated generation of ROS in AM and ATII cells, a depletion of GSH in AM and lymphocytes, and an increase in AM and ATII cell production of NO.

Key Words: **diesel exhaust particles; airway inflammation; airway hyperresponsiveness; glutathione; reactive oxygen species; nitric oxide.**

INTRODUCTION

In recent decades there has been an increase in the prevalence of allergic conditions in many developed countries. Although the cause for this increase is not yet clear, epidemiological evidence suggests that exposure to certain components of air pollution such as ozone, nitrogen oxides, and the fine fractions of airborne particulate matter, which rise with increasing industrialization, plays a major role in this development (Graham, 2004; Koren, 1995). Diesel exhaust particles (DEP), a major airborne particulate in urban and industrialized areas, have been shown to enhance allergen-induced airway inflammation and production of antigen-specific immunoglobulin (Ig)E and/or IgG (Ichinose *et al.*, 1997, 2002; Miyabara *et al.*, 1998; Takano *et al.*, 1997, 1998). Exposure to DEP was also shown to enhance ovalbumin (OVA)-induced airway hyperresponsiveness (AHR) to inhaled acetylcholine in mice when animals were exposed to the allergen during the same time period (Miyabara *et al.*, 1998; Takano *et al.*, 1998). Although these results indicate that DEP exposure augments the immune response of animals to allergen challenge, other studies have shown that the organic component of inhaled DEP and other pollutants may in fact inhibit allergen-induced airway inflammatory responses (Dong *et al.*, 2005; Melgert *et al.*, 2004). In addition, the effect of DEP on allergic asthma has been demonstrated, primarily in various mouse models (Ichinose *et al.*, 1997, 2002; Miyabara *et al.*, 1998; Takano *et al.*, 1997, 1998). Several studies have pointed out that strain differences in the modulation of allergic airway inflammation and antigen-specific IgE and/or IgG response by DEP existed, and apparently contradictory effects of DEP were observed among different mouse models (Ichinose *et al.*, 1997; 2002). Thus, it is possible that DEP exposure may exert varied or complex effects on experimental allergic asthma depending on animal models, the timing of DEP exposure, and the allergen sensitization process. The latter is of particular significance because, in practical settings, subjects may be sensitized or non-sensitized to an allergen, and may encounter

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DEP and allergen exposure at the same or different times. This suggests that different exposure protocols for the timing of DEP and allergen exposures should be pursued to gain insight into the complex effects of DEP on the pulmonary immune system.

We have established an allergic asthma model using Brown Norway (BN) rats sensitized by aerosolized ovalbumin (OVA) administered once a week over a month-long period that produced significant levels of OVA-specific IgE and IgG (Al-Humadi *et al.*, 2002). Exposure of rats to DEP (5 mg/kg) through intratracheal instillation prior to the OVA-sensitization enhanced production of antigen-specific antibodies at the end of allergen sensitization, and this enhancement correlated with an increase in interleukin (IL)-4 mRNA expression in lung tissue. Steerenberg and colleagues (1999, 2003) also reported the use of BN rats in elucidating the adjuvant effect of DEP on timothy grass pollen allergy after intranasal or intratracheal instillation of DEP in combination with the pollen allergen. In a recent study, we further showed that short-term exposure to DEP by inhalation (20 mg/m^3, 4 h/day for 5 days) prior to OVA sensitization enhanced OVA-induced antibody production but attenuated the OVA-induced inflammatory responses that included inflammatory cell infiltration, lactate dehydrogenase (LDH) activity, albumin, and nitric oxide (NO) contents in bronchoalveolar lavage (BAL) fluid, the development of T lymphocytes and their $CD4^+$ and $CD8^+$ subsets in lung lymph nodes, and production of NO, IL-10, and IL-12 by alveolar macrophages (AM) (Dong *et al.*, 2005). The inhibition of OVA-induced airway inflammation by DEP at 30 days post-exposure contradicted the DEP effect reported previously, where DEP were administered to the already-sensitized animals or to those during the sensitization phase (Ichinose *et al.*, 1997, 2002; Miyabara *et al.*, 1998; Takano *et al.*, 1997, 1998). This suggests that there may be acute and delayed responses to DEP exposure that produce different effects on allergic immune/inflammatory reactions.

The aim of the present study was to examine the effects of DEP inhalation just prior to the last dose of OVA exposure on allergen-mediated immune responses in the BN rat model. We have shown previously that the organic component of DEP induces cellular generation of reactive oxygen species (ROS) that leads to an antioxidative response and a switch from T helper (Th)1 to Th2 immunity (Yin *et al.*, 2004b). This would enhance the allergic responses in antibody production. Nitric oxide produced from lung cells, such as AM and epithelial cells, on the other hand, has been shown to induce eosinophil-mediated airway inflammation (Liu *et al.*, 1997). Both the reactive oxygen and nitrogen intermediates may deplete intracellular glutathione (GSH) and cause a change in the redox state of GSH, which has been shown to regulate cellular production of Th1/Th2 cytokines and T cell development (Murata *et al.*, 2002). To gain more insight into the underlying mechanism(s) through which DEP alter the asthmatic immune responses, the potential involvement of ROS, NO, and GSH depletion in relation to DEP, OVA, and the combined DEP and OVA exposures was investigated.

MATERIALS AND METHODS

Animals and Exposure Schedule

Male Brown Norway rats [BN/CrlBR] weighing 200–225 g were obtained from Charles River Laboratories (Wilmington, MA). The animals were housed in a clean-air and virus-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.

OVA immunization. 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 the desired concentration, filtered air was passed through the nebulizer and used as a diluent for the aerosolized OVA. The concentration of OVA in the 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 using the Coomassie blue dye reagent (Bio-Rad Laboratories, Hercules, CA). Rats were sensitized to OVA at an average chamber concentration of 42.3 ± 5.7 mg/m^3 for 30 min on days 1, 8, and 15, and challenged with OVA on day 29. Non-sensitized animals were exposed to aerosolized endotoxin-free saline following the same exposure schedule.

DEP exposure. A standardized DEP sample (standard reference material 2975), representing heavy-duty diesel engine with a mass median aerodynamic diameter of 0.5 μm, was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). Diesel exhaust particles were suspended in endotoxin-free sterile saline (Baxter Healthcare Corporation, Deerfield, IL), followed by sonication for 2 min in an ultrasonic processor with a micro tip (Heat System-Ultrasonics, Plainview, NY) prior to use. The DEP inhalation exposure system used in this study has been described and characterized elsewhere (Yin *et al.*, 2002, 2004a). Rats were exposed to either filtered air or DEP (22.7 ± 2.5 mg/m^3), 4 h/day for 5 consecutive days, on days 24–28, 24 h prior to the last (challenge) dose of OVA, using a nose-only directed flow exposure unit (CH Technologies, Inc., Westwood, NJ). The DEP concentration in the exposure unit was 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 with 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 above-described inhalation exposure, calculated based on the method of Leong *et al.* (1998), was 402 ± 58 μg/rat.

The exposure groups (5 rats/group) for the present study were: non-sensitized/air-exposed/saline challenged (saline + air); non-sensitized/DEP-exposed/saline challenged (saline + DEP); OVA-sensitized/air-exposed/OVA challenged (OVA + air); and OVA-sensitized/DEP-exposed/OVA challenged (OVA + DEP). The animals were subjected to whole-body plethysmography on day 30, 24 h after the last OVA dose, and they were sacrificed on day 31 for biochemical and cellular measurements. All parameters were measured after saline/OVA challenge (the last dose) with and without DEP exposure.

Measurement of Airway Responsiveness

Airway responsiveness was assessed by inducing airflow obstruction with a methacholine (MCh) aerosol using a noninvasive method (Hamelmann *et al.*, 1997). Minute volume, tidal volume, breathing frequency, and enhanced pause (Penh) were obtained from conscious rats placed in a whole-body plethysmograph (Buxco Electronics Inc., Troy, NY). In this system, rats were unrestrained

and tolerated repetitive measurements. Measurements of MCh responsiveness were obtained by exposing rats for 3 min to aerosolized PBS and incremental doses (6.25–25 mg/ml) of aerosolized MCh (Sigma) in PBS, and monitoring the breathing pattern for 3 min after each MCh challenge. The Penh values measured during each 3-min sequence were averaged and expressed, for each MCh concentration, as a percentage of baseline Penh values observed after PBS exposure.

Bronchoalveolar Lavage (BAL) and Determination of BAL Markers

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. 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 parameters. The lungs were further lavaged with 6 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 PBS and evaluated as described below.

BAL cell differentiation. The BAL cell were numerated 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). Three hundred cells per 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.

Albumin and LDH. The albumin content, which indicates injury to the bronchoalveolar-capillary barrier, and LDH activity, which indicates cytotoxicity, were determined in the first fraction of acellular BAL fluid, using 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). Lactate dehydrogenase 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).

Chemiluminescence (CL). The light generation as CL by resting or stimulated AM as a result of ROS production was determined in a total volume of 0.5 ml HEPES buffer. Resting CL was determined by incubating BAL cells containing 0.5×10^6 AM at 37°C for 10 min in 0.008 % (w/v) luminol (Sigma) followed by the measurement of CL for 15 min. Luminol was used as an amplifier to enhance detection of the light and was first dissolved in a small amount of ethanol before being brought up to its final concentration in HEPES buffer. To determine zymosan-stimulated CL, unopsonized zymosan (2 mg/ml, Sigma) was added immediately prior to the measurement of CL. Measurement of CL was performed with an automated Berthold Autolumat LB 953 luminometer (Wallace, Inc., Gaithersburg, MD) for 15 min, and the integral of counts versus time was calculated. Zymosan-stimulated CL was calculated as the total counts of stimulated cells minus the total counts of the corresponding resting cells. The zymosan-stimulated CL was attributed to AM only, as rat neutrophils do not respond to unopsonized zymosan in this system.

NO production. The production of NO by AM was determined as follows. 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 (FBS). Aliquots of 1 ml cell suspension containing 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 non-adherent BAL cells were removed by rinsing the monolayer three times with culture medium. The remaining AM-enriched cells were incubated in 1 ml medium for 24 h at 37°C and 5% CO_2. The level of nitrite produced from NO in the AM-conditioned media was measured colorimetrically with the Greiss reaction using sodium nitrite as a standard (Green *et al.*, 1982). The levels of NO in the first fraction of acellular BAL fluids were also determined using the Greiss assay.

Western blot analysis. The recovered AM-enriched cells were washed with PBS and then suspended in 100 μl of a lysis buffer (50 mM Tris-HCl, 1% NP-40, 2 mM EDTA, 100 mM NaCl, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride; pH 7.5) and left on ice for 10 min. Cytoplasmic extracts were separated from the nuclei by centrifugation at 14,000 rpm for 10 min at 4°C and determined for protein content using a BCA Protein Assay Kit (Pierce, Rockford, IL). An equal amount of protein (30 μg/well) for each sample was boiled for 5 min, loaded, and run for electrophoresis in a 4–20% Tris-Glycine gel (Invitrogen, Carlsbad, CA) at 125 V. The gel was transferred electrophoretically (Bio-Rad Laboratories, Hercules, CA) to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), and the blots were blocked with 5% milk in TBST buffer (20 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20; pH 7.5) for 1 h at room temperature. Membranes were then probed with a polyclonal rabbit antibody against inducible NO synthase (iNOS) and a horseradish peroxidase–conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed using commercially developed enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). For quantification, bands in photographs were scanned by a densitometer linked to a computer system (Personal Densitometer SI, Amersham Biosciences, Piscataway, NJ).

Analysis of Alveolar Type II (ATII) Cells

Isolation of ATII cells. Type II cells were isolated using a standard protocol (Dobbs *et al.*, 1986). Briefly, after removal of AM and blood cells, elastase solution (MP Biomedicals, Inc., Irvine, CA) was instilled via the trachea to dissociate the cells from lung tissue. The lung tissue was then minced in the presence of DNase I (Sigma) and FBS, and the suspension was sequentially filtered through nylon mesh. The cell suspension was plated on bacteriological plastic dishes coated with rat IgG (Sigma). After 1 h at 37°C, the nonadherent ATII cells were removed from the plate to which AM and other immune cells were adherent. Cells obtained by this method contained ~90% ATII cells and >90% excluded trypan blue.

Western blot analysis. Expression of iNOS in ATII cells was determined by Western blot analysis using the same procedures for AM as described above, using cytoplasmic protein (30 μg/well) extracted from the freshly isolated ATII cells.

Flow cytometric analysis. Freshly isolated ATII cells (5×10^5 cells) were washed with a washing buffer (PBS with 2% FBS and 0.02% NaN_3, pH 7.4), and re-suspended in DMEM/F12 medium (Invitrogen, Carlsbad, CA) containing 10 μM of 4,5-diaminofluorescein diacetate (DAF, Sigma) or 5 μM dihydroethidium (DHE, Molecular Probes, Eugene, OR) at 37°C for 30 min. After washing, the flow cytometric data were immediately 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).

Analysis of Lymphocytes from Lung-Draining Lymph Nodes (LDLN)

Isolation of lymphocytes. Lymphocytes were isolated from LDLN as described previously (Yin *et al.*, 2003). Briefly, LDLN were excised from each rat after BAL, teased apart, 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, and washed twice with PBS. Lymphocytes were isolated by Histopaque (density 1.083; Sigma) gradient centrifugation. Samples were centrifuged for 30 min at 2500 rpm, and lymphocytes were collected, washed, re-suspended in 1 ml of PBS, and counted using a standard hemocytometer. The cell samples thus prepared showed predominance of lymphocytes and cell viability of greater than 98% as determined by the trypan blue exclusion technique.

Flow cytometric analysis. The effects of OVA and/or DEP exposures on frequencies of T cell subsets in LDLN, *i.e.*, their expressions of CD3, CD4, and CD8 cell surface markers, were examined with a flow cytometric method described elsewhere (Yin *et al.*, 2003). Lymphocytes (10^6 cells) were stained with the addition of FITC-labeled conjugated antibodies against these cell surface markers (BD Pharmingen, San Diego, CA). The flow cytometric data were collected with a Becton-Dickinson FACScan using FACScan Research Software (Becton-Dickinson Immunocytometry System), and analyzed using the PC-LYSYS software (Becton-Dickinson).

Determination of Intracellular GSH

Alveolar macrophages 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 during exsanguination from vena cava of rats at sacrifice. 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) which, according to manufacturer provided information, is specific for IgE class and does not cross-react with the other Ig classes, including IgG. The plates were then incubated with 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 3 times after each incubation, treated with tetramethylbenzidine (Sigma), and read at 630 nm. Ovalbumin-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 concentrations 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, rehydrated, and stained for iNOS expression (Porter *et al.*, 2002). Briefly, microwave antigen retrieval with citrate buffer (pH 6.0) of rehydrated tissue 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, San Francisco, CA) as the chromogen. Tissues were counterstained with Mayer's hematoxylin, dehydrated and covered with a coverslip. Non-stained sections where the primary antibody was omitted were obtained as negative controls, and sections from rats that had been intratracheally instilled with lipopolysacchride (LPS, Sigma, 10 mg/kg) 24 h prior to sacrifice were stained for positive controls.

Statistical Analysis

Results are expressed as means ± standard error (SE). The significance of the interaction among different treatment groups for different parameters at each time point was assessed by 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 $p < 0.05$.

RESULTS

Effect of DEP on OVA-Induced Allergic Responses

Airway reactivity. The effect of DEP exposure on OVA-induced AHR was evaluated from Penh values in rats from various treatment groups after MCh challenge (Fig. 1). The baseline Penh values were not significantly affected by OVA or DEP. However, airway responsiveness to MCh was significantly increased in rats from the combined OVA and DEP exposure group, but not in those from other exposure groups. Thus, DEP exposure had a synergistic effect with OVA on inducing AHR in rats.

OVA-Specific IgE and IgG production. In all the samples collected from non-sensitized rats, both OVA-specific IgE and OVA-specific IgG concentrations were under the detection

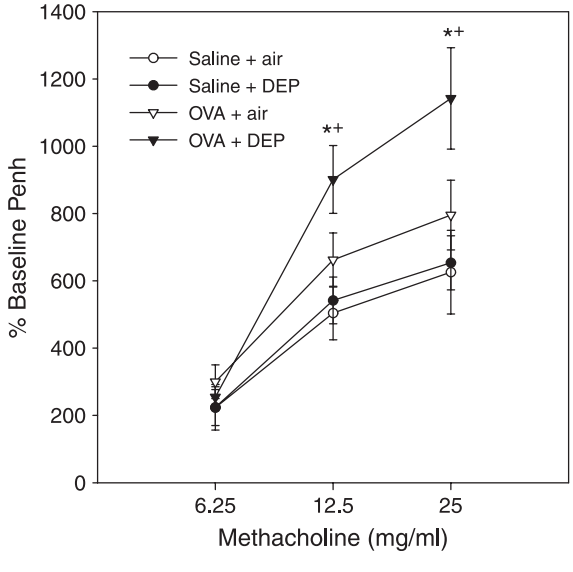

FIG. 1. Airway hyperresponsiveness in rats 24 h after ovalbumin (OVA) challenge. Responsiveness was assessed by measuring enhanced pause (Penh) values of rats in response to inhaled methacholine (Mch) aerosolized from MCh solutions inphosphate-buffered solution (PBS) at various concentrations (6.25–25 mg/ml). Values are expressed as the means ± SE ($n = 5$) of the percentage of baseline Penh values after PBS exposure. *Significantly different from saline + air group, $p < 0.05$; $^+$Significantly different from OVA + air group, $p < 0.05$.

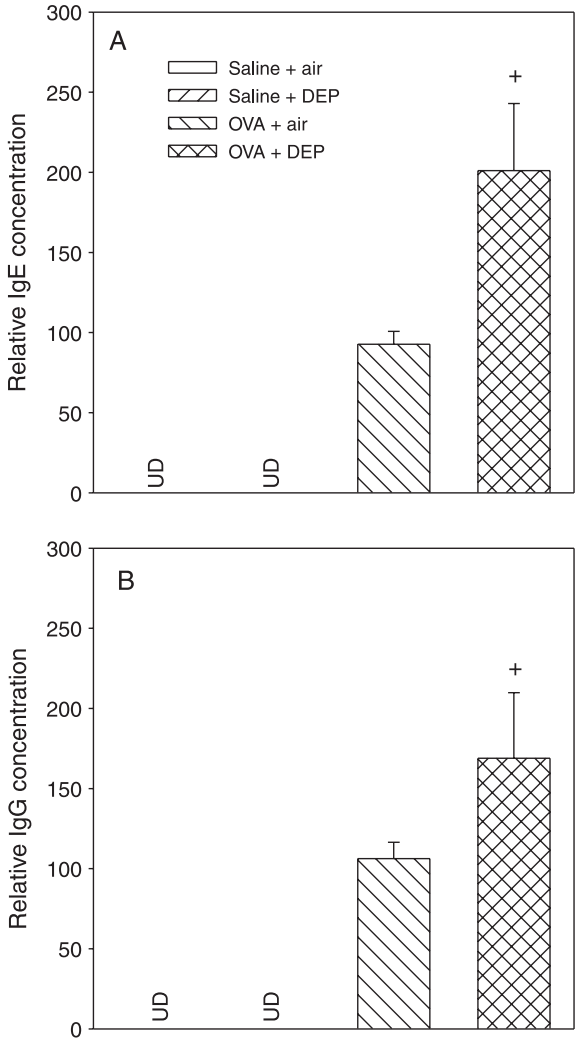

FIG. 2. Relative concentrations of OVA-specific IgE (A) and IgG (B) in serum collected from rats. Values are expressed as the means ± SE ($n = 5$). $^{+}$Significantly different from OVA + air group, $p < 0.05$. UD: under detection limit.

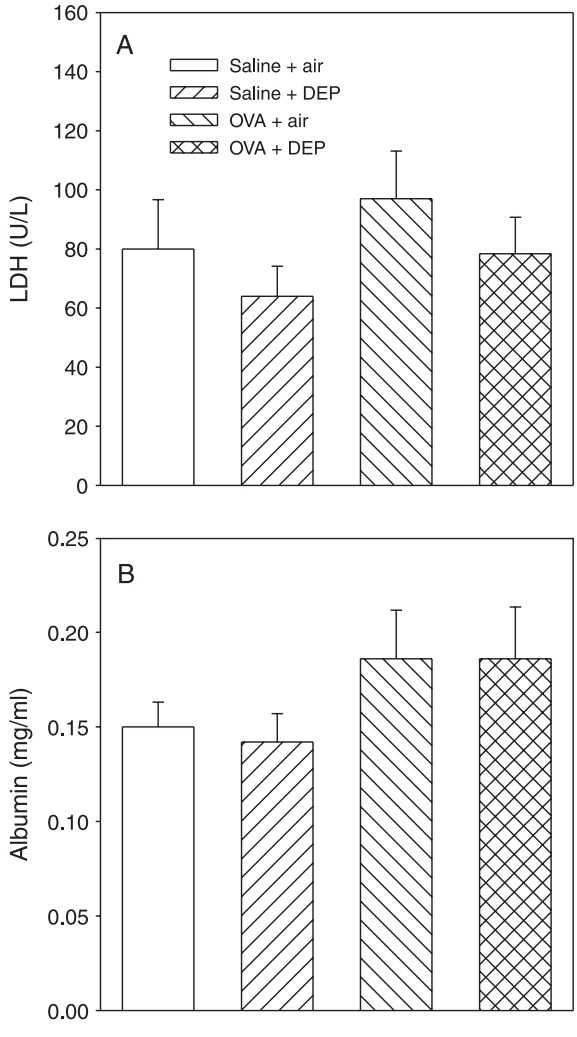

FIG. 3. LDH activity (A) and albumin content (B) in the first fraction of BAL fluid collected from rats. Values are expressed as the means ± SE ($n = 5$). No significant difference was found among any treatments for the two measurements.

limits. Ovalbumin sensitization resulted in a significant production of antigen-specific IgE. The levels of OVA-specific IgE in rats receiving combined OVA and DEP exposure were 2 times higher than that in rats sensitized to OVA but exposed to air (Fig. 2A). Ovalbumin sensitization also resulted in the production of OVA-specific IgG, whose level was likewise increased, 1.7 fold, by DEP pre-exposure (Fig. 2B). Diesel exhaust particle exposure alone had no effect on IgG production. These results indicate that DEP pre-exposure augments the immune responses of rats to OVA in the production of allergen-specific IgE and IgG.

Effect of DEP on OVA-Induced Cell Differentiation

BAL fluid and cells. The LDH activity and albumin content in the first fraction of acellular BAL fluid (Fig. 3) and the numbers of AM, eosinophils, neutrophils, and lymphocytes recovered from BAL fluid (Fig. 4) were determined as measurements of lung injury and airway inflammation. Neither DEP, OVA, nor the combined OVA and DEP exposure induced elevated levels of LDH activity and albumin content, indicating that the exposure protocols did not cause significant lung injury that may complicate the experimental results. Diesel exhaust particle exposure alone induced a moderate but significant increase in the number of neutrophils recovered from BAL fluid. Ovalbumin exposure induced a greater infiltration of neutrophils than DEP, and it also resulted in infiltration of eosinophils and lymphocytes. The OVA-induced eosinophil count was markedly increased by DEP pre-exposure, even though DEP exposure alone had no effect on this cell type. In comparison, the OVA-mediated infiltration of neutrophils and lymphocytes was not augmented by DEP in the combined OVA-DEP exposure. These results show that DEP, under the current

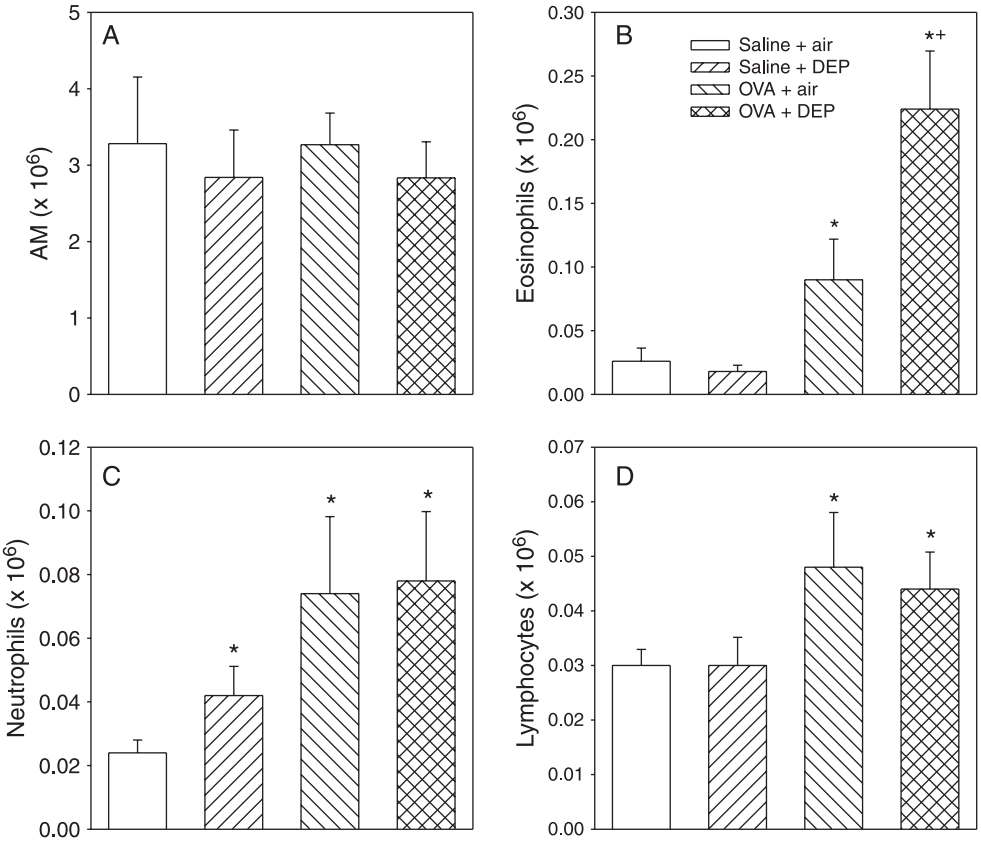

FIG. 4. Differentials of BAL cells recovered from rats. Values are expressed as the means ± SE ($n = 5$). *Significantly different from saline + air group, $p < 0.05$; $^{+}$Significantly different from OVA + air group, $p < 0.05$.

dose and exposure protocol, did not cause substantial inflammation to the lung or augment OVA-induced neutrophil and lymphocyte infiltration, but strongly enhanced the infiltration of eosinophils that mediates airway inflammation and AHR.

LDLN. The numbers of total lymphocytes, T cells, and their $CD4^+$ and $CD8^+$ subsets in LDLN from rats sensitized and challenged by OVA were significantly higher than those of the air-exposed, non-sensitized rats (Fig. 5). Although DEP exposure alone moderately increased these lymphocyte counts, the combined DEP and OVA exposure resulted in a substantial increase in the numbers of $CD4^+$ and $CD8^+$ T cells compared to cell counts measured for OVA exposure alone.

Effect of DEP on OVA-Induced Oxidant Generation and GSH Depletion

ROS and NO production. Alveolar macrophages from various exposure groups were assessed for ROS production by measurement of CL in response to zymosan stimulation (Fig. 6A). Exposure to DEP or OVA alone had no effect on CL production by AM. However, a substantial elevation in CL was observed for the combined OVA and DEP exposure group. The production of NO, a highly reactive nitrogen intermediate, was measured in the acellular BAL fluid (Fig. 6B) and in AM-conditioned media (Fig. 6C). Both OVA and DEP exposures resulted in increased presence of NO in the acellular BAL fluid and in AM-conditioned media, and these levels were further increased in samples from the combined OVA and DEP exposure.

The effect of DEP pre-exposure on OVA-induced generation of reactive oxygen and nitrogen species was also studied in ATII cells isolated from the rats. Figure 7 shows the intracellular presence of NO (Fig. 7A) and superoxide (Fig. 7B) determined by flow cytometry after the cells were stained with DAF-DA and DHE, the relatively specific probes for intracellular generation of NO and superoxide, respectively. The ATII cells from OVA-exposed rats exhibited a higher percentage of cells that produce NO and superoxide than those of the air-exposed, non-sensitized rats. Although DEP exposure alone increased the percentage of ATII cells that produce superoxide but not NO, the combined DEP and OVA exposure resulted in significant increase in the percentage of the cells that produce NO and superoxide over the control (Fig. 7).

iNOS expression. Immunohistochemical analysis for iNOS expression in lung tissues showed no staining of AM in any exposure group. However, the airway epithelium was found

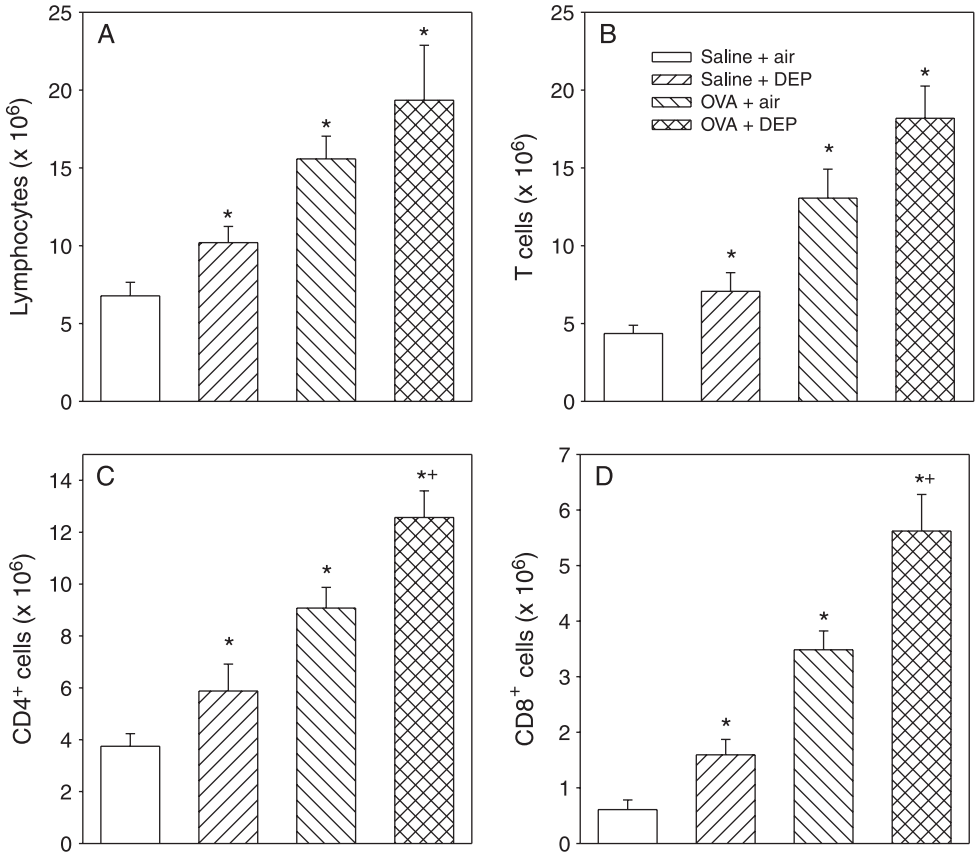

FIG. 5. Numbers and differentials of lymphocytes isolated from lung-draining lymph nodes (LDLN) of rats. Values are expressed as the means ± SE ($n = 5$). *Significantly different from air + saline group, $p < 0.05$; $^{+}$Significantly different from OVA + air group, $p < 0.05$.

positive in all five rats from the combined DEP and OVA exposure, in three out of five rats from DEP or OVA exposure alone, and in two out of five rats from the air-exposed, non-sensitized control (data not shown). The OVA exposure was also found to increase iNOS expression in AM and ATII cells isolated from the rats, as detected by Western blot analysis (Fig. 8). Although DEP exposure alone did not induce iNOS in AM and ATII cells, the combined DEP and OVA exposure resulted in a significantly higher iNOS expression in these cells than those from any other groups.

GSH levels in AM and lymphocytes. The levels of total GSH in AM and lymphocyte were slightly lowered by DEP or OVA exposure, but the decreases were not statistically significant (Fig. 9). Under combined DEP and OVA exposure, however, the intracellular GSH levels were significantly reduced in both cell types compared to all other exposure groups.

DISCUSSION

Experimental models of asthma, as in BN rats, require a sensitization period for the animal to develop the allergic response. Diesel exhaust particle exposure, on the other hand, is known to induce acute inflammatory responses through particle stimulation but anti-inflammatory responses through the organic component of DEP (Ma and Ma, 2002, Yin *et al.*, 2004b). Hence, the effects of DEP on allergic responses may vary depending on the sensitization process and the timing of DEP exposure. The present study was designed to characterize the effect and mechanism of DEP exposure on altering OVA-induced airway inflammation and antigen-specific IgE and IgG production in rats already sensitized to the allergen. The dose for DEP exposure (22.7 ± 2.5 mg/m^3) appears to be high in comparison to the reported environmental and occupational concentrations, but in fact it results in a lung deposition (402 ± 58 μg/rat) that is relevant to both non-occupational and occupational exposure settings as discussed previously (Yin *et al.*, 2002, 2004a). Exposure of non-sensitized rats to DEP resulted in only a moderate increase in neutrophils in the lung, and there was a clear absence of eosinophilic inflammation, antibody responses, or AHR. However, in OVA-sensitized rats, pre-exposure to DEP markedly enhanced OVA-induced AHR, eosinophil infiltration, and serum OVA-specific IgE and IgG production. Although alterations in the lung cytokine profile were unknown, the results show that DEP pre-exposure

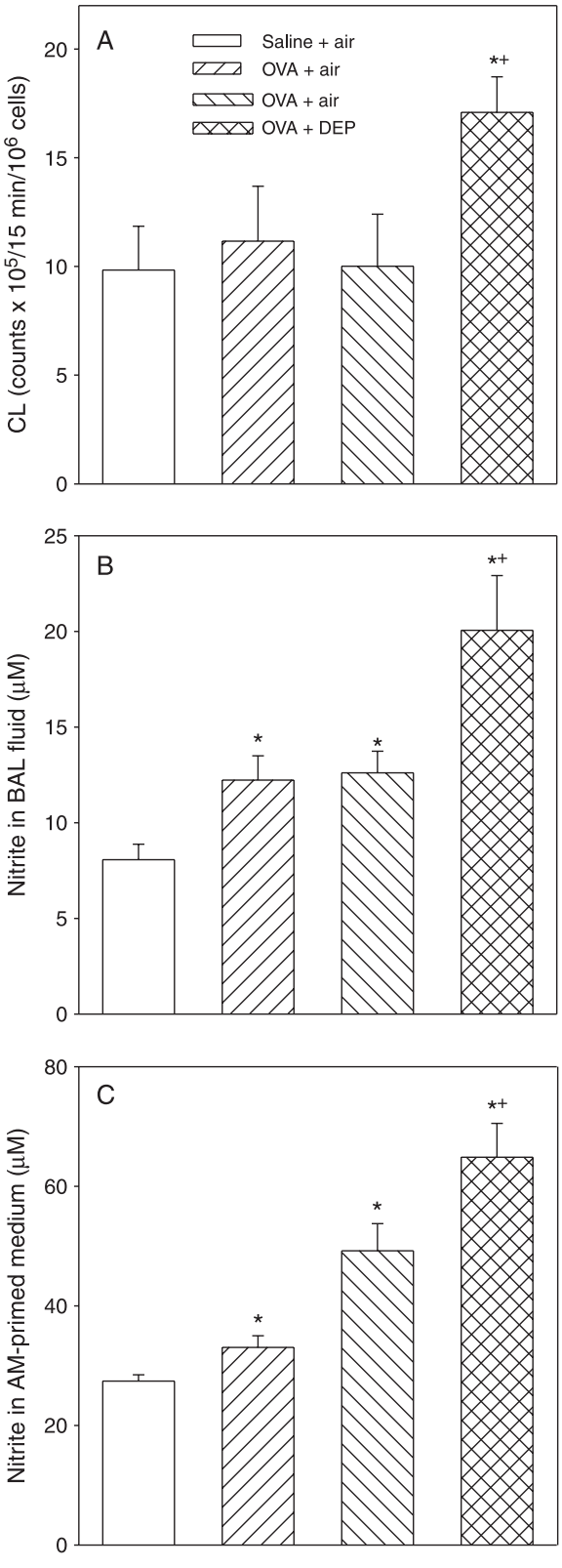

FIG. 6. Zymosan-stimulated CL by AM (A) and concentration of nitrite in BAL fluid (B) and AM-conditioned media (C). Values are expressed as the means ± SE ($n = 5$). *Significantly different from saline + air group, $p < 0.05$; $^{+}$Significantly different from air + OVA group, $p < 0.05$.

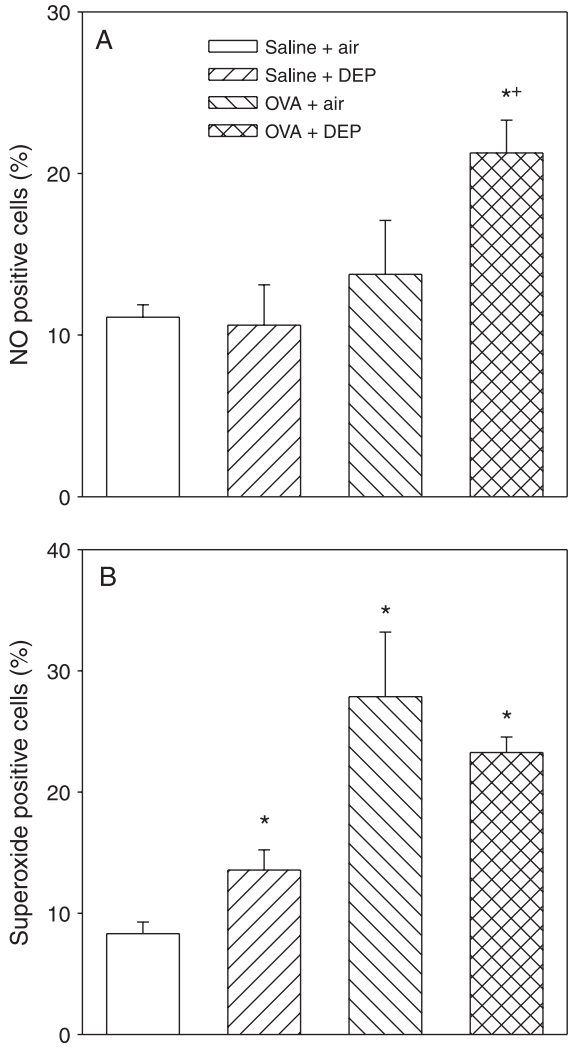

FIG. 7. Flow cytometric analysis of intracellular generation of NO (A) and superoxide (B) by ATII cells. Values are expressed as the means ± SE ($n = 5$). *Significantly different from saline + air group, $p < 0.05$; $^{+}$Significantly different from OVA + air group, $p < 0.05$.

strongly enhances the OVA-induced Th2 responses in antibody production as well as eosinophil-mediated airway inflammation. To our knowledge, this is the first demonstration of a DEP-enhanced allergic AHR in rats. These results are consistent with, and complement those obtained using various mouse models where DEP exposure was co-administered with antigen-challenge (Ichinose *et al.*, 1997, 2002; Miyabara *et al.*, 1998; Takano *et al.*, 1997, 1998). It is interesting to point out that in a previous study, we demonstrated that DEP exposure prior to the sensitization enhanced the sensitization in antibody production but inhibited airway inflammation induced by OVA challenge using the same rat model (Dong *et al.*, 2005). Thus, the action of DEP exposure on allergic asthma is such that it affects both the sensitization and the asthmatic response of animals to the allergen.

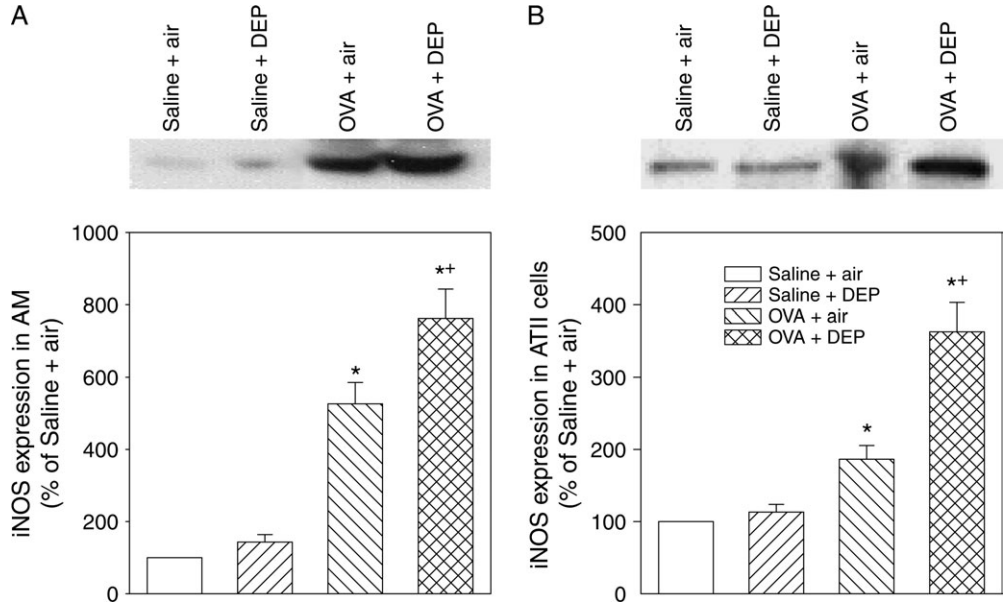

FIG. 8. Western blot analysis of iNOS expression in AM (A) and ATII cells (B). The top panels show representive results of the Western blot analysis and the bottom panels show percentage increase in band density which represents the means ± SE ($n = 5$). *Significantly different from saline + air group, $p < 0.05$; $^{+}$Significantly different from OVA + air group, $p < 0.05$.

The mechanisms through which DEP alter the allergic reactions remain unclear in part because of the complex, time-dependent effects of DEP on allergen-induced immune responses. One of the cellular actions of DEP is the induction of intracellular ROS through the organic component-mediated activation of cytochrome P450 1A1 and other metabolic enzymes (Ma and Ma, 2002; Whitekus *et al.*, 2002; Yin *et al.*, 2004b). The imbalance of cellular antioxidative responses to ROS generation induced by DEP leads to oxidative stress and a reduction of total intracellular GSH in AM and lymphocytes (Al-Humadi *et al.*, 2002; Dong *et al.*, 2005). The present study shows that ROS generation in AM and ATII cells is an important feature of the combined DEP and OVA exposure. As shown by CL measurements, a marked increase in oxidant activity, which corresponds to a lowered total GSH concentration in AM and lymphocytes, is associated with the combined DEP and OVA exposure, which is greater than either DEP or OVA exposure alone. This suggests that DEP pre-exposure augments ROS production by cells from OVA-sensitized rats. GSH, in addition to its role in protecting cells from oxidative injury, is critical to macrophages and dendritic cells that act as antigen-presenting cells for the development of T-cell–mediated immune responses. Depletion of GSH in these cells has been shown to skew the development of T cells from Th1 to Th2 type (Murata *et al.*, 2002; Peterson *et al.*, 1998), a manifestation of increased allergic asthmatic responses. That DEP augment OVA-specific antibody production is consistent with the fact that DEP facilitate depletion of GSH in AM and lymphocytes.

Nitric oxide has been considered an important marker in allergen-induced inflammatory responses. This reactive nitrogen intermediate can directly react with and deplete intracellular GSH (Folkes and Wardman, 2004) and may play a role in the development of eosinophilia and AHR in mouse and rat allergic models (Feder *et al.*, 1997; Liu *et al.*, 1997). It has been shown that NO derived from iNOS in epithelial cells promotes asthmatic inflammation by downregulating Th1 cells that secrete interferon-γ and concomitantly upregulate Th2 cells that secrete IL-4 and IL-5 (Barnes and Liew, 1995). On the other hand, the constitutive NO-synthase (cNOS)–derived NO has been shown to exert bronchoprotective effects in asthma including airway smooth muscle relaxation and inhibition of smooth muscle proliferation (Ricciardolo *et al.*, 2001, 2003). In fact, NO derived from cNOS and from iNOS may play different roles in the airways. The former seems to protect airways from excessive bronchoconstriction while the latter has a modulatory role in inflammatory disorders of the airways such as asthma. The effect of DEP pre-exposure, as shown in the present study, is to increase iNOS expression in AM and ATII cells of OVA-sensitized and challenged rats, suggesting that DEP interact directly with these lung cells. This interaction results in an acute response of increased production of NO that is known to mediate OVA-induced eosinophilic inflammation and AHR.

Our study shows an apparent linkage between the ROS and NO generation with increased responses of T lymphocytes. Both DEP and OVA exposure enhanced the numbers of T cells and their $CD4^{+}$ and $CD8^{+}$ subsets recovered from the LDLN in the BN rat model. But it was the combined DEP and OVA exposure that yielded a substantial increase in T cell responses, which correlate with increased production of ROS and NO and decreased level of GSH. Clinical investigations have observed that $CD4^{+}$ T lymphocytes and their secretion of Th2 cytokines

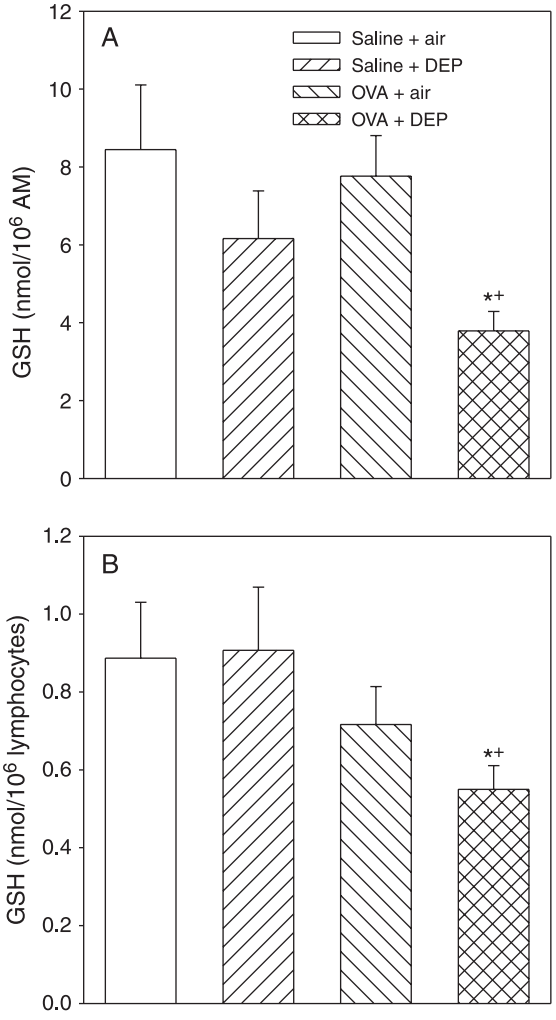

FIG. 9. Concentration of intracellular GSH in AM (A) and lymphocytes (B) isolated from lung-draining lymph nodes of rats. Values are expressed as the means ± SE ($n = 5$). *Significantly different from saline + air group, $p < 0.05$; $^{+}$Significantly different from OVA + air group, $p < 0.05$.

played a central role in initiating and sustaining asthmatic responses in the asthmatic airway (Robinson, 2000). Studies in animal models further showed that depletion of $CD4^+$ T lymphocytes by administration of anti-CD4 antibody inhibited allergen-induced airway eosinophilia and AHR (Gavett *et al.*, 1994; Komai *et al.*, 2003). $CD8^+$ T lymphocytes also play a role in allergic responses. Miyahara *et al.* (2004a,b) showed that CD8-deficient mice had a significantly lower AHR and eosinophilia in response to OVA sensitization and challenge comparing to the wild-type, but the allergic response was fully restored by adoptive transfer of antigen-primed effector $CD8^+$ T cells.

In summary, this study demonstrated that short-term DEP exposure enhances OVA-induced airway inflammation and antigen-specific IgE and IgG production, and that it increases airway responsiveness in allergen-sensitized rats. The adjuvant effect was characterized by an increase in the responses of $CD4^+$ and $CD8^+$ T lymphocytes in LDLN, both of which are known to play a major role in allergic asthma, that was accompanied by an increase in ROS and NO production and iNOS expression in AM and ATII cells and a decrease in GSH levels in AM and lymphocytes.

ACKNOWLEDGMENTS

The authors thank Patsy Willard and Dean Newcomer of the National Institute for Occupational Safety and Health for assisting in immunohistochemical analysis. We also thank Dr. Paul D. Siegel for assisting in ovalbumin exposure. This research was supported in part by a grant from the National Heart, Lung, and Blood Institute, National Institutes of Health (RO1 HL 62630–03).

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