Experimental Lung Research

ISSN: 0190-2148 (Print) 1521-0499 (Online) Journal homepage: https://www.tandfonline.com/loi/ielu20

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To cite this article: Nabil H. Al-Humadi, Paul D. Siegel, Daniel M. Lewis, Mark W. Barger, Jane Y. C. Ma, David N. Weissman & Joseph K. H. Ma (2002) THE EFFECT OF DIESEL EXHAUST PARTICLES (DEP) AND CARBON BLACK (CB) ON THIOL CHANGES IN PULMONARY OVALBUMIN ALLERGIC SENSITIZED BROWN NORWAY RATS, Experimental Lung Research, 28:5, 333-349, DOI: 10.1080/01902140290091976

To link to this article: https://doi.org/10.1080/01902140290091976

Published online: 02 Jul 2009.

Experimental Lung Research 28:333–349, 2002

0190-2148/02 $12.00 + .00
DOI: 10.1080/01902140290091976

Taylor & Francis healthsciences

THE EFFECT OF DIESEL EXHAUST PARTICLES (DEP) AND CARBON BLACK (CB) ON THIOL CHANGES IN PULMONARY OVALBUMIN ALLERGIC SENSITIZED BROWN NORWAY RATS

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□ *Brown Norway rats were exposed by intratracheal instillation of saline, carbon black (CB), or diesel exhaust particles (DEP) (5 mg/kg) on day 1, followed by exposure to ovalbumin (OVA, 90 mg/m^3) or saline for 30 minutes on days 1, 8, 15, and 29. Animals were sacrificed on day 30. The DEP, CB, or OVA exposure alone did not result in abnormal levels of inflammatory cells, lactate dehydrogenase (LDH), or total protein in the lavage fluid. In combined OVA-DEP or OVA-CB exposure, however, these markers were significantly increased. The adjuvant effect of CB and DEP on OVA sensitization was evidenced by the marked increases in serum OVA-specific IgG (5.6-fold) and IgE (3.5-4 fold) levels, and the increase in interleukin-4 (IL-4) mRNA levels in lung tissue. The OVA exposure markedly reduced glutathione (GSH) levels in both cell types. In combined DEP-OVA exposure, the level of GSH in lymphocytes was further decreased, indicating a possible interactive effect between DEP and OVA exposures. These results show that both DEP and CB augmented OVA-induced allergic sensitization, and that particle composition of DEP may not be a critical factor for the adjuvant effect. OVA exposure causes significant depletion of intracellular GSH in lymphocytes, which may play a key role in OVA-mediated immune responses.*

Keywords *diesel exhaust particles, immunoglobulin, interleukin-4, ovalbumin, thiols*

The prevelance and severity of asthma has increased worldwide in the last 20 years [1, 2]. The reasons for this increase are not well understood, but are likely to involve the complex interplay of genetic, social-economic,

This work was supported in part by NIH grant HL62630-02.
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behavioral, and environmental factors. Environmental factors include combined exposure to airborne particulates (PM 10, PM 2.5) and a variety of allergens such as house dust mites and pollens. The most prevalent form of asthma is allergic asthma. An allergic immune response to an allergen, is characterized by the production of interleukin (IL)-4, IL-5, and IL-10 by the Th2 subset of CD4+ T lymphocytes [3]. These cytokines trigger immunoglobulin E (IgE) production by B cells and the recruitment of eosinophils [3]. Inflammation orchestrated by Th2 cytokines (IL-4, IL-5, and IL-13) and thickening of all layers of the airway walls are common pathological observations in asthmatics [4].

Recently, a number of studies have shown that short-term exposure to diesel exhaust particles (DEP) may exert a strong effect on the respiratory and immune system, suggesting that DEP and perhaps, other particles as well, may be key environmental factors in augmenting pulmonary allergic reaction. Indeed, the ability of DEP to enhance an immune response to intranasally administered allergen has been demonstrated in both humans and rodents. Diaz-Sanchez and colleagues [5, 6] showed that in vivo nasal challenge with 300 μg of DEP of both healthy and allergic subjects enhances the amount of total IgE (but not of other immunoglobulin isotypes) in human upper airways and increased the number of local IgE-secreting cells. This DEP exposure also increased both Th1- and Th2-type cytokines (including IL-4, IL-6, IL-13, and interferon) in the nasal mucosa. In later studies, the same laboratory [7] showed the combined exposure of ragweed allergen and DEP resulted in marked enhancement in the production of ragweed-specific IgE and mRNA for Th2-type cytokines by cells in the nasal mucosa, when compared to challenge with ragweed allergen or DEP alone in subject with ragweed allergy.

The adjuvant effect of DEP on allergic sensitization has also been demonstrated in mice using ovalbumin (OVA) as the allergen. Takano and colleagues [8] showed that intranasally instilled DEP aggravated OVA-induced airway inflammation, and markedly increased IL-5 levels and the mRNA levels of IL-4, IL-2, and granulocyte-macrophage colony-stimulating factor in lung tissue. DEP exposure also exhibited adjuvant activity for anti-OVA–specific IgG and IgE production. In addition, mice exposed both to DEP (3 or 6 mg/m^3) by inhalation for 6 weeks and to intranasally administered OVA resulted in higher anti-OVA IgE antibody titers than mice exposed to DEP or OVA alone.

The cellular reactions to DEP and/or their particulate and organic components leading to increased Th2 response are not yet clear. Studies in our laboratory have shown that DEP suppresses pulmonary bacteria clearance and bacteria-induced macrophage production of tumor necrosis factor alpha (TNF-α) [9, 10]. This effect was attributed to the organic component of DEP. However, a number of studies have shown that various particles including carbon black (CB), which resemble the carbonaceous core of DEP, can enhance allergic sensitization [11–13]. CB, in particular, has been demonstrated to enhance proliferation of antibody forming cells and both IgE and IgG levels [14, 15]. These studies suggested that DEP may skew the immune response toward the Th2 side, whereas CB stimulates both Th1 and Th2 responses.

The specific hypothesis of this research is that the adjuvant effect of DEP on IgE and pulmonary allergic responses is, at least in part, mediated through its effects on thiol levels. The inflammatory responses associated with particle stimulation involve oxidative stress in the lung and possible alteration of thiol levels that are critical to the cytokine production and cytotoxic activities of lymphocytes. Glutathione (GSH), for example, is known to affect nuclear factor kappa B (NF-κB) activation and regulate lymphocyte immune responses [16–18]. Studies by Shukla and colleagues [19] have shown that inhalation of CB (300 $\mu g/m^3$ air for 6 hours followed by 24 hours' clean air exposure) causes an early increase in intracellular oxidants and the mRNA levels of a number of NF-κB–regulated genes, including TNF-α, IL-6, and interferon. These studies suggest that GSH may play a role in DEP- and/or CB-induced immune responses. To provide more insight into the effects of DEP and their components on the pulmonary immune system, we have studied the adjuvant activities of DEP and CB on OVA sensitization in Brown Norway rats, and the potential role of GSH in OVA- and/or particle-mediated Th2 lymphocyte responses.

MATERIALS AND METHODS

Particulate Sample Preparations

CB particles (Fisher Scientific, Fair Lawn, NJ) were autoclaved at 120°C for 4 hours before use. DEP (standard Reference Material 1650, representing heavy duty engine with a mass median aerodynamic diameter of 0.5 μm) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD). Endotoxin free sterile saline (Baxter Healthcare Corporation, Deerfield, IL) was used to suspend the particles. The suspensions of CB and DEP were sonicated for 1 minute using an ultrasonic processor with a micro tip (Heat System-Ultrasonics, Plainview, NY) prior to intratracheal instillation.

Animal Exposure

Male Brown Norway rats (Charles River, Stoneridge, NY), weighing 200 to 250 g, were used. Animals were housed in an American Association for

Accreditation of Laboratory Animal Care- (AAALAC) approved facility that was maintained at 23°C ± 1°C with 50% relative humidity and a 12-hour light/dark cycle. Food and water were given ad libitum. Methohexital sodium (35 mg/kg body weight, intraperitoneal [IP]; Eli Lilly, Indianapolis, IN) was used to lightly anesthetize the animals prior to their placement in a vertical position for intratracheal instillation. A curved ball-tipped cannula (18 guage) was used to briskly inject the CB and DEP particulate suspension (5 mg/kg body weight) into the trachea. Control animals received the sterile saline vehicle. The instillation volume was 2 mL/kg body weight of rats. A solution of OVA (Sigma, St. Louis, MO) in endotoxin-free saline was aerosolized using a DeVilbiss-646 (DeVilbiss, Somerset, PA) nebulizer for OVA exposure. Constant feeding of the OVA solution to the nebulizer was achieved using a syringe pump (Model no. 901, Harvard Apparatus, Dover, MA). To achieve desired concentrations, filtered air was passed through the nebulizer, and used as a diluent for the aerosolized OVA. The chamber OVA aerosol concentration was determined by collecting samples onto 0.45 μm (Polycarbonate Membrane, Poretics Corporation, Livermore, CA) filters from a chamber side port at a rate of 1 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 exposed to OVA for 30 minutes at day 1 after intratracheal (IT) exposure (on day 0) to DEP, CB, or saline, and then once a week for 30 minutes on days 8, 15 and 29 (Table 1). The OVA chamber concentration was 90 ± 18 mg/m^3. Control animals were exposed to aerosolized endotoxin free saline. All rats were sacrificed at day 30. Booster OVA exposures were given to augment the antibody response and to enhance any particulate induced alteration. Rats were sacrificed 24 hours after the last OVA challenge exposure to maximize potential for identifying alteration in pulmonary inflammatory responses.

TABLE 1 Experimental Design

	Route of exposure		
		Inhalation**	
Group	IT Day 0	Days 1, 8, 15, 29	Day 30
Saline/saline	Saline	Saline	Sacrifice
CB/saline	CB*	Saline	Sacrifice
DEP/saline	DEP*	Saline	Sacrifice
Saline/OVA	Saline	OVA	Sacrifice
CB/OVA	CB	OVA	Sacrifice
DEP/OVA	DEP	OVA	Sacrifice

*5 mg/kg body weight.
**90 ± 18 mg OVA/m^3 air or saline vehicle.

Bronchoalveolar Lavage (BAL) and Biochemical Assays

Rats were anesthetized with sodium pentobarbital (50 mg/kg; IP; Butler, Columbus, OH) and euthanized by exsanguination of the abdominal aorta 1 day after the last inhalation exposure. The trachea was cannulated and the lungs were lavaged with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS, 45 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), at a volume of 6 mL for the first lavage and 8 mL for the subsequent lavages. The total lavage volume was 80 mL. The BAL fluid was centrifuged at 500 G for 10 minutes at 4°C. The supernatant from the first lavage was saved separately for the determination of protein content and lactate dehydrogenase (LDH) activity using an automated Cobas Fara II analyzer (Roche Diagnostic Systems, Montclair, NJ) with standard diagnostic reagents and manufacturer's procedures. Cell pellets from all lavage fluids of an individual rat were combined and suspended in 1 mL of PBS to determine the total cell and differential cell counts using an electronic cell counter (Coulter Electronics, Hialeah, FL) equipped with a cell sizing unit [20]. Alveolar macrophages (AM) and granulocytes were determined by their unique cell diameters and used in primary cell culture experiments.

Lymph Node Cells Preparation

Lymph nodes (parathymic and tracheal) were collected in 1 mL, pH 7.4, Hepes buffer. The lymph node capsules were broken with a glass bar using a tissue culture screen. Lymph node cells were washed, transferred into a 15-mL tube, and centrifuged at 2000 rpm for 10 minutes. Cell pellets were reconstituted with 1 mL Hepes buffer and lymphocytes were isolated by Histopaque (d = 1.083, Sigma). In brief, the samples were centrifuged for 30 minutes at 2500 rpm, and lymphocytes were collected, washed twice, and resuspended in 1 mL of Hepes buffer for cell counts, cell differentiation, and cell culture experiments. The cell samples thus prepared showed a lymphocyte content of greater than 98%.

Cell Cultures for Cysteine (CYSH) and GSH Synthesis in AM and Lymphocytes

AM isolated from each rat were suspended in Eagle Minimum Essential Medium (EMEM) culture medium (EMEM, Sigma) containing 1 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 10% heat-inactivated fetal bovine serum, and 10 mM Hepes. Aliquots of 1 mL cell suspension, adjusted to 1×10^6 AM, were added to each well of a 12-well tissue culture plate. AM were allowed to adhere to the plastic plater for 2 hours in a humidified incubator (37°C and 5% CO_2) [21]. The nonadherent cells were removed by rinsing the monolayers 3 times with Hepes buffer. The AM-enriched cells were then incubated (37°C and 5% CO_2) in fresh Hepes buffer with or without cystine (24 μg/mL) for 16 hours. After centrifugation at 500 g for 5 minutes, the cell pellet was washed twice and analyzed for CYSH and GSH contents according to the following method. One microliter of a solution containing 80 mM monobromobimane (mbb) was added to 100 μL of the cell suspension. The fluorescence derivitization of the reduced thiols was allowed to proceed at 4°C overnight. The cells were then lysed by sonication for 30 seconds using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA), centrifuged at 10,000 rpm for 10 minutes to remove cell membranes and debris, and filtered through a 0.2-μm filter (Microspin Nylon Filter, PGC, Frederick, MD). Analysis of CYSH and GSH levels in the solution was carried out using a reverse phase high performance liquid chromotography (HPLC) method.

For lymphocytes, aliquots of 2×10^6 cells were incubated in microcentrifuge tubes with or without custine (24 μg/mL) for 16 hours in a humidified incubator (37°C and 5% CO_2). Following incubation, cultures were centrifuged at 500 g for 5 minutes and washed twice with Hepes. The pellets were resuspended in 1 mL Hepes buffer and analyzed for CYSH and GSH following the method described above.

HPLC Analysis

A Shimadzu HPLC system equipped with a RF-551 fluorometric detector and a C18 reverse-phase column (Phenomenex, Luna, 5 μm) was used for the analysis of CYSH and GSH. Seperation of the mbb-thiol derivatives was achieved using a gradient system of acetonitrile in 0.1 M sodium acetate (pH 5.0), delivered at a flow rate of 1 mL/min. The gradient system consisted of a mobile phase of 7% acetonitrile for the first 6 minutes, and stepped increases to 15%, 25%, and 40% after 10, 15, and 18 minutes, respectively. The CYSH and GSH derivatives were detected at an excitation of 380 nm and an emission of 480 nm, with retention times at 8.0 and 10.2 min, respectively.

Serum OVA Specific IgE and IgG Measurements

Blood samples were collected from the vena cava at sacrifice. Three sera dilutions with 5% horse serum albumin (HOSA)/PBS of 1/100, 1/1000, and 1/10,000 were analyzed for OVA-specific IgE. The OVA-specific IgG determinations were obtained on sera dilutions of 1/1000, 1/10,000, 1/50,000, and 1/100,000. 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 [22]. The plates were incubated overnight at 4°C and subsequently incubated with sheep IgG, anti-rat IgE (100 μL, 1/5000 dilution in HOSA/PBS; Cat. No. 64-352, ICN Biomedicals, Costa Mesa, CA), and horseradish peroxidase–bound donkey IgG, anti-sheep IgG (100 μL, 1/10,000 dilution in HOSA/PBS; Cat. No. 67541, ICN Biomedicals) for 2 hours each at room temperature. The plates were washed 3 times following each incubation, treated with tetramethylbenzidine (TMB, Sigma), and read at 630 nm. OVA-specific IgG were determined using Goat IgG, anti-rat IgG (1/1000 dilution in HOSA/PBS; Cat. No. R5005, Sigma) and peroxidase-labeled rabbit IgG anti-goat IgG (1/25,000 dilution in HOSA/PBS; Cat. No. A-3540, Sigma) as detection antibodies following the same protocol described above. The serum from 1 animal exposed to OVA was extensively titered (dilutions of 1/10,000 and 1/50,000 for IgE and IgG respectively was assigned a value of 100) and concentration/response curves were obtained. These curves were then used as references to obtain relative concentrations for the OVA-specific IgE and IgG of different exposure groups.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

The RT-PCR experiments were carried out using the method of Noble and colleagues [23]. Total RNA was extracted from lung and lung-associated lymph node (LALN) tissue by guanidine isothiocyanate lysis (Trizol; Life Technologies, Rockville, MD). Tissues were processed immediately after sacrifice. Reverse transcription was performed using 5 μg total RNA, oligo(dT)12–18 primer, and M-MLV reverse transcriptase (Superscript First-Strand cDNA Synthesis Kit, Life Technologies, Rockville, MD).

PCR reactions were performed using cDNA derived from 0.4 μg RNA, primers listed below, Taq polymerase (Sigma-Aldrich, St. Louis, MO), dNTP mix, $10\times$ PCR buffer, $MgCl_2$, and H_2O. The PCR conditions involved denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds. The initial cycle contained a 4-minute denaturation at 94°C and the final cycle contained a 7-minute extension at 72°C. 37 cycles were performed.

Primers used for RT-PCR were as follows: interferon gamma (IFN-γ), 5′-ATCTGGAGGAACTGGCAAAAGGACG-3′ and 5′-CCTTAGGCTAGATTCTGGTGACAGC-3′, which amplify a 288-bp fragment; IL-4, 5′-ACCTTGCTGTCACCCTGTTCTGC-3′ and 5′-GTTGTGAGCGTGGACTCATTCACG-3′, which amplify a 352-bp fragment [23]; TNF-α, 5′-TACTGAACTTCGGGGTGATTGGTCC-3′ and 5′-CAGGCTTGTCCCTTGAAGAGAACC-3′, which amplify a 295-bp fragment; IL-6, 5′-CAAGAGACTTCCAGCCAGTTGC-3′ and 5′-TTGCCGAGTAGACCTCATAGTGACC-3′, which amplify a 614-bp fragment; and glceraldehyde-3-phosphate dehydragenase (G3PDH), 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGA-GGTCCACCAC-3′, which amplify a 983-bp fragment. With the exception of IL-4, all primer sequences were obtained commercially (Clontech, Palo Alto, CA).

PCR products were seperated on 1.5% agarose gels in Tris Borate EDTA (TBE) buffer. Gels were stained with ethidium bromide and digital images captured using a still video system (Eagle Eye II; Stratagene, La Jolla, CA). For gels used to semiquantitate PCR product, samples were loaded in volumes that equalized the size of bands for G3PDH PCR product as determined by visual inspection. Band densities of captured digital images made from these gels were then quantified by densitometry (Eagle Eye II, Stratagene, LaJolla, CA).

Data Analysis

All data were expressed as mean ± standard error (n = 5) of 2 to 4 separate experiments. Each measurement was run in duplicate or triplicate per experiment. Statistical analysis of data was performed using Sigma Stat (version 2.0, Jandel Scientific Software, San Rafael, CA) statistical software for Windows 95, NT, and 3.1. Analysis of variance (ANOVA) was conducted and values of $P < .05$ were considered statistically significant.

RESULTS

Figure 1 shows that rats at 4 weeks post IT exposure to DEP or CB alone exhibited normal levels of total protein and LDH in the BAL fluid as compared to the saline control. Rats exposed to OVA for 4 weeks also exhibited

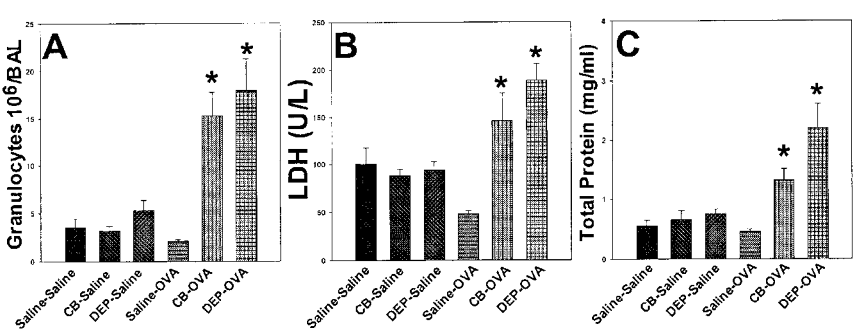

FIGURE 1 The effect of combined exposure to DEP or CB and OVA on the pulmonary inflammatory responses in Brown Norway rats as indicated by elevations of PMN (*A*), lactate dehydrogenase activity (*B*), and total protein content (*C*) in the bronchoalveolar lavage fluid (BAL). n = 5/group. Each point represents average ± SEM. *Significantly different from saline-saline and saline-OVA controls at $P < .05$, ANOVA.

normal levels of total protein and LDH in the lavage fluid. These results show that OVA or the particles alone did not cause persistent pulmonary epithelial damage or cell toxicity. There was also no increase in BAL inflammatory cells in these groups. In the combined exposures with OVA, however, both DEP and CB resulted in significantly elevated levels of inflammatory cells, total protein, and LDH as compared to OVA or particle exposure alone, indicating that both DEP and CB aggravated the airway immune inflammatory responses to OVA.

Figure 2 shows the effect of DEP or CB on OVA-induced serum-specific antibody levels. An antigen-specific IgE and IgG antibody responses in rats exposed to OVA alone were found, and a marked enhancement in antibody production in rats exposed to either particles (DEP or CB) or OVA. The increase in anti-OVA IgE production due to particle interplay was 4- and 3.5-fold that of the OVA control for the CB- and DEP-exposed groups, respectively. The increase in serum anti-OVA IgG production by CB or DEP was 5- 6-fold higher than OVA exposure alone. These results show that both DEP and CB exhibited adjuvant effect on OVA sensitization, and that particle composition, in this case the organic content in DEP, was not critical in inducing the adjuvant effect.

Figure 3 shows the effect of CB or DEP on IFN-γ and IL-4 (*A* and *B*) mRNA expressions in lung tissues of nonsensitized and OVA-sensitized rats. In nonsensitized animals, both DEP and CB appeared to enhance

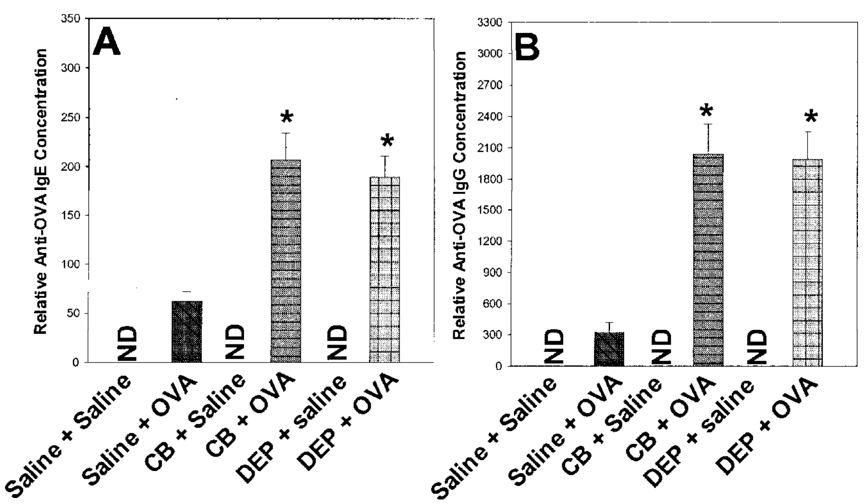

FIGURE 2 The relative serum anti-OVA IgE (*A*) and anti-OVA IgG (*B*) concentration responses to saline, DEP, or CB exposure in nonsensitized (exposed to saline) and OVA-sensitized rats. n = 5/group. Each point represents average ± SEM. ND, "not detected." The reported values are relative to a pooled reference standard as noted in Materials and Methods. *Significantly different from saline-OVA control at $P < .05$, ANOVA.

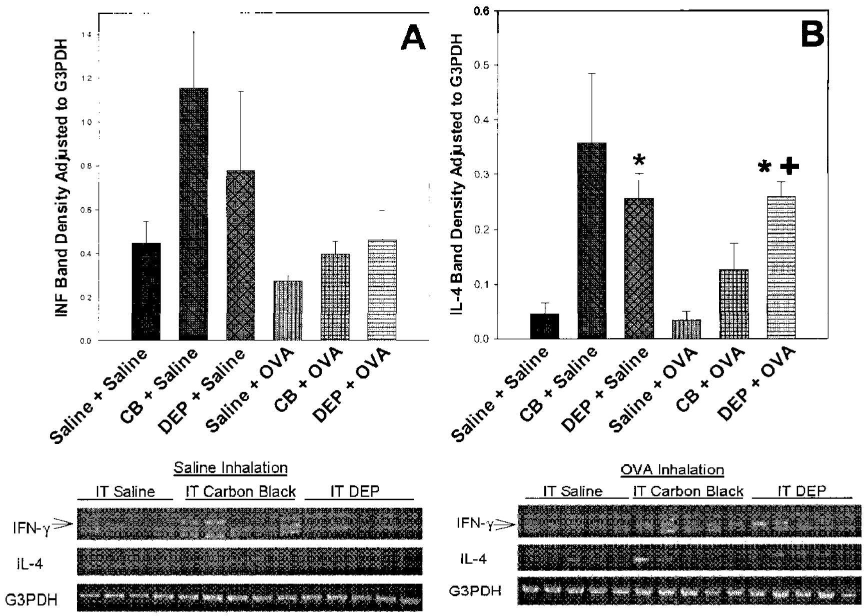

FIGURE 3 Effect of DEP or CB with or without OVA exposure on mRNA expressions for IFN-γ (*A*) and IL-4 (*B*) in lung tissue. n = 5/group. Each point represents average ± SEM. Note: Agaros gel pictures have been included to show the variation between the individual animals. *Significantly different from IL-4 value for saline-saline control at $P < .05$, ANOVA. +Significantly different from IL-4 value for saline-OVA at $P < .05$, ANOVA.

IFN-γ mRNA content as compared to saline controls, but this enhancement was not statistically significant. In contrast, there was a significant increase in IL-4 mRNA by DEP exposure. OVA-sensitization alone did not alter IFN-γ and IL-4 mRNA levels in the lung tissue. In the combined exposures, whereas the IFN-γ mRNA levels remained unchanged in all groups, both CB- and DEP-exposed rats showed elevated levels of IL-4 mRNA when compared to rats exposed to OVA alone. The enhancement of IL-4 mRNA by DEP was significant and was consistent with the fact that DEP alone induced an increase in IL-4 mRNA in lung tissue. There was no significant difference in TNF-α mRNA in lung tissue of all exposure groups (data not shown).

The effects of particle and OVA exposures on cellular concentrations of CYSH and GSH were studied in AM and lymphocyte primary cultures incubated for 16 hours in thiol-free medium. Figure 4 shows the intracellular CYSH (*A*) and GSH (*B*) levels in AM from various exposure groups. DEP or CB exposure alone did not alter the thiol levels at 4 weeks post exposure. The OVA exposure increased slightly the CYSH concentration, but markedly decreased the intracellular GSH concentration. In the combined exposure,

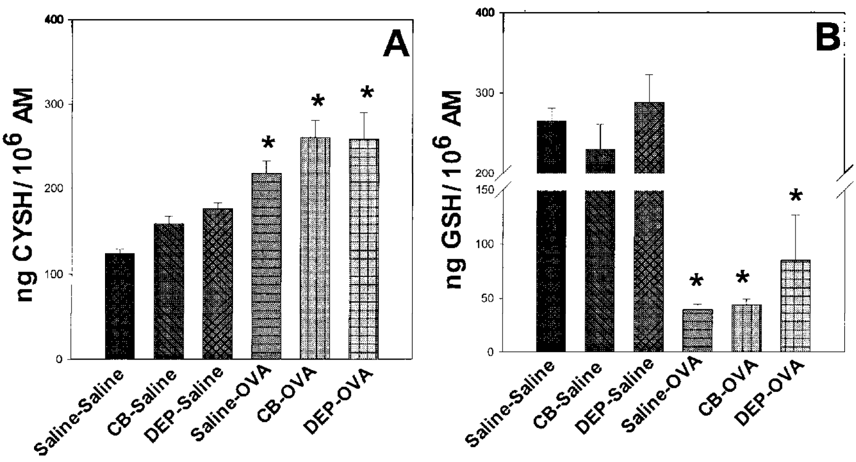

FIGURE 4 Measurement of intracellular cysteine (*A*) and GSH (*B*) levels in AM harvested from rats exposed to saline, CB, DEP, OVA, or both particles (CB or DEP), and OVA, after incubation for 16 hours. n = 5/group. Each point represents average ± SEM. *Significantly different from saline-saline control at $P < .05$, ANOVA.

AM from all groups exhibited elevated concentration of CYSH but lowered GSH when compared to the saline control. Figure 5 shows that DEP or CB exposure alone had no significant effect on either CYSH or GSH levels in lymphocytes. Exposure to OVA, on the other hand, resulted in a substantial

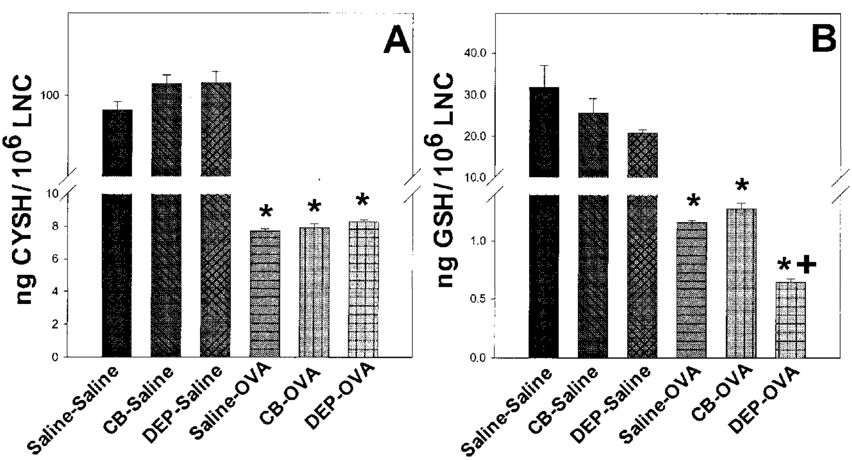

FIGURE 5 Measurement of intracellular cysteine (*A*) and GSH (*B*) levels in parathymic and tracheal lymph node cells (contain >98% lymphocytes) harvested from rats exposed to saline, CB, DEP, OVA, or both particles (CB or DEP), and OVA, after incubation for 16 hours. n = 5/group. Each point represents average ± SEM. *Significantly different from saline-saline control at $P < .05$, ANOVA. + Significantly different from saline-OVA at $P < .05$, ANOVA.

decrease in both CYSH and GSH concentrations. Figure 5*B* also shows that DEP may augment the OVA effect in lowering the intracellular GSH in lymphocytes.

The abilities of AM and lymphocytes from various exposure groups to utilize cystine in CYSH and GSH synthesis are shown in Figures 6 and 7, respectively. Alveolar macrophages had a strong capacity for the uptake and conversion of cystine to CYSH (Figure 6*A*). This ability, however, was significantly suppressed by OVA exposure. Both DEP and CB exposures, on the other hand, enhanced CYSH production by AM, even in cells from OVA-sensitized rats. The addition of cystine to AM culture did not result in a significant increase in GSH synthesis in all exposure groups (Figure 6*B*). GSH was not assessed in the extracellular medium and it is possible that the AM transported much of the GSH out of the cell. Figure 6*B* further shows that OVA decreased GSH concentrations in AM. Figure 7*A* shows that lymphocytes have the capacity to take up and convert cystine to CYSH. This capacity was not significantly altered by DEP or CB exposure, but was strongly inhibited by OVA. The addition of cystine to lymphocyte culture resulted in a slight increase in GSH synthesis in all exposure groups. As shown in Figures 4 and 5, OVA exposure resulted in lowered cellular levels of GSH in AM and lymphocytes. Figures 6 and 7 further show that even under elevated concentrations of CYSH (through cystine utilization), the GSH levels in AM and lymphocytes remained suppressed by OVA sensitization and challenge.

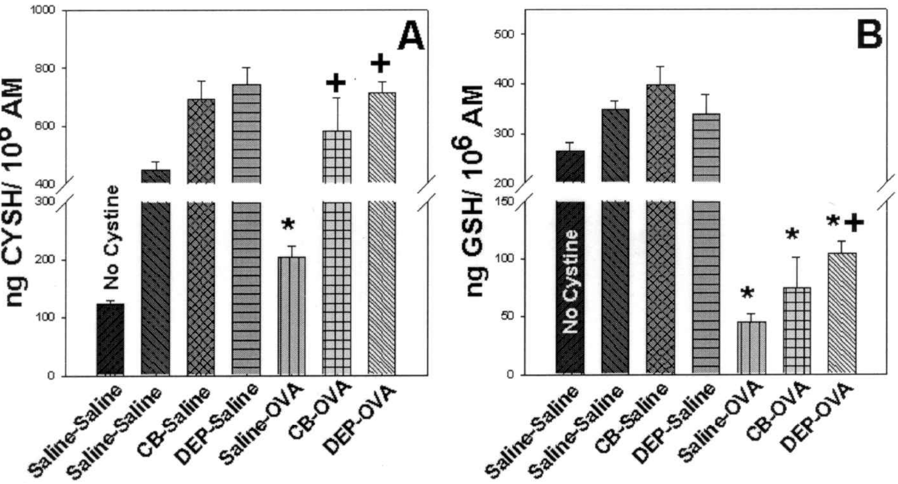

FIGURE 6 Measurement of intracellular cysteine (*A*) and GSH (*B*) levels in AM harvested from rats exposed to saline, CB, DEP, OVA, or both particles (CB or DEP), and OVA, after incubation with 24 μg/mL cystine for 16 hours. n = 5/group. Each point represents average ± SEM. *Significantly different from saline-OVA at $P < .05$, ANOVA.

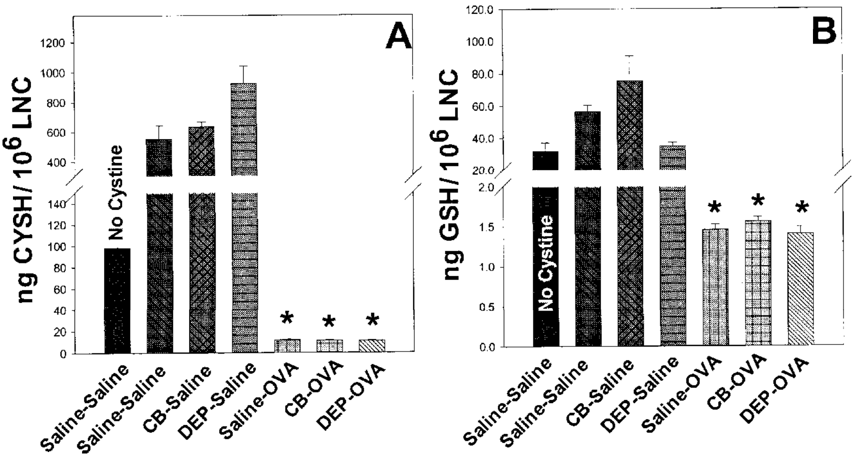

FIGURE 7 Measurement of intracellular cysteine (*A*) and GSH (*B*) levels in parathymic and tracheal lymph node cells (contain >98% lymphocytes) harvested from rats exposed to saline, CB, DEP, OVA, or both particles (CB or DEP), and OVA, after incubation with 24 μg/mL cystine for 16 hours. n = 5/group. Each point represents average ± SEM. *Significantly different from saline-saline with cystine control at $P < .05$, ANOVA.

DISCUSSION

Exacerbation of respiratory allergy has long been associated with particulate air pollution exposure. DEP, which consist of carbonaceous particles and aromatic and aliphatic hydrocarbons adsorbed onto the particle surface, is a major air-borne pollutant. Both the particulate and the organic components may play a role in their biological effects. Studies have already indicated that DEP induce acute pulmonary inflammatory responses, but suppress AM responses to lipopolysaccharide and bacterial stimulation in the production of nitric oxide, IL-1, and TNF-α, thus increasing the susceptibility of the lung to respiratory infection [9, 10]. This effect was attributed to the organic component of DEP. The adjuvant effect of DEP through intranasal administration on allergic sensitization has been reported in humans and in mice [24–27]. This effect is characterized by an increase in antigen-specific IgE production [5], and the secretion of Th2-type cytokines in the nasal mucosa [7]. Recent studies further indicate that carbonaceous particles such as CB also stimulate Th2 responses and exhibit adjuvant activity to allergic sensitization [15]. Indeed, through the present study, we have demonstrated that both CB and DEP aggravated OVA-induced pulmonary allergic inflammatory responses (Figure 1), enhanced anti-OVA IgE and anti-OVA IgG production (Figure 2), and caused an increase in IL-4 mRNA expression in lung tissue (Figure 3). These results suggest that particle composition of diesel exhaust does not play a major role in the adjuvant effect.

CB was found to exhibit similar effects to those of DEP on IFN-γ and IL-4 mRNA tissue levels. The mRNA level of IFN-γ was not significantly affected by CB, DEP, OVA, or combined particle-OVA exposure. Thus, it is inconclusive whether DEP or CB affects the production of IFN-γ or other Th1 cytokines in the lung. It should also be mentioned that at 4 weeks post particle exposure, animals were well recovered from the acute inflammatory stimulation by these particles; however, the tissue cytokine message levels were examined 24 hours after OVA challenge of sensitized rats. In contrast, DEP and CB exposure, even at 4 weeks post exposure, resulted in elevated levels of IL-4 in lung tissues from either nonsensitized or OVA-sensitized rats (Figure 3), although the data on CB did not reach statistical significance in the current experiments. Nevertheless, these results indicate that DEP or CB may augment OVA-induced antibody production through enhanced Th2 responses (i.e., IL-4 production). These results are consistent with those of other studies indicating that DEP enhances IL-5 production and IL-4 mRNA level in OVA-sensitized mice [8].

The cellular mechanism(s) involved in altered immune response and cytokine production remains unclear. The fact that rats exposed to DEP or CB showed a significant increase in lung damage and inflammation, when further challenged with OVA suggests potential alteration in cells, such as AM and lymphocytes, involved in the immune response. For this reason we have studied the role of CYSH and GSH in pulmonary allergic responses associated with particle, OVA, and the combined particle-OVA exposures. Thiols are important molecules for various lymphocyte functions. Depletion of intracellular GSH inhibits the lectin-induced activation response of T lymphocytes [28]. Studies by Gmunder and Droge [29] showed that depletion of intracellular GSH decreases the proportion of CD8+ cells (i.e., it increases the CD4+/CD8+ ratio), and inhibits the generation of large blast-like CD8+ cells and cytotoxic T lymphocyte activity. CYSH has a strong influence on the intracellular GSH and DNA synthesis of cytotoxic T-cell clones [30]. Thiols in fact may play a regulatory role analogous to the hormone-like lymphokines and cytokines in mediating immunologically relevant functions of lymphocytes. Our previous work demonstrated that DEP produced a time-dependent increase in GSH and CYSH levels in AM and lymph node lymphocytes that peaked at 4 and 72 hours post exposure, respectively. Thiol reductase activity was also induced by DEP [31]. The lack of alteration in the thiol levels 4 weeks post exposure (when incubated for 16 hours in thiol-free media) is not surprising. The cultured AM from the Brown Norway rats had only slightly higher levels of CYSH than GSH (on a molar basis), whereas CYSH from cultured pulmonary-associated lymph node lymphocytes was approximately 7 times that of GSH. Both cell types displayed a strong capacity for the uptake and reduction of the disulfide, cystine. The OVA exposure to sensitized rats had a marked effect in decreasing thiol levels in AM and lymph node lymphocytes, and their ability to take up and reduce cystine. Interestingly, AM, but not lymph node lymphocytes, from DEP- and CB-exposed rats had significantly greater levels of cystine uptake and reduction over saline controls. DEP and CB exposure also significantly restored the capacity of AM from OVA sensitized/challenged rats to take up and reduce cystine (Figure 6*A*).

The interplay between particle and OVA exposure on lymphocyte GSH levels from the current study is not clear, probably due to the strong OVA effect. Lymphocytes from animals exposed to both DEP and OVA were not able to maintain their intracellular GSH levels (vs. OVA-only exposure) when incubated for 16 hours in thiol-free media (Figure 5*B*). This enhanced loss of GSH was not seen when the lymphocytes were cultured with cystine (Figure 6*B*). These results suggest that depletion of intracellular GSH and CYSH may have a role in, or be a consequence of, allergic pulmonary responses. This is consistent with the results of our previous work in which in vitro DEP exposure of pulmonary-associated lymph node cells resulted in a depletion of intracellular GSH, even at increased CYSH concentrations and glutathione reductase activity [31]. The role of particulates and antigens in the (altered) thiol regulation in these immunologically relevant cells is not known. Thiols have been suggested to be important in signal transduction pathways involving NF-κB activation [16, 17] which is associated with a number of cytokine genes including interferon, TNF-α, IL-4, IL-6, and transforming growth factor-α [19]. Modulation of thiol regulation by particulates may thus affect allergic outcomes.

In summary, the present study shows that DEP- and CB-exposed rats exhibit an aggravated allergic response to OVA exposure with markedly enhanced pulmonary inflammation, IL-4 mRNA levels in lung tissue, and serum OVA-specific IgG and IgE levels similar to the effect reported in mice. The results also confirm the finding of other reports that the carbonaceous particle alone induce adjuvant effect on allergic asthma; however, the organic component of DEP may also contribute in some aspects aggravating the disease. The intracellular CYSH and GSH levels in lymphocytes were severely depleted in OVA-sensitized/challenged rats, with or without prior exposure to DEP or CB. It cannot be determined from the present study design if the thiol disregulation was a result of the allergic response or contributed sensitization and/or response to the allergen.

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