

## Diesel exhaust particles exert acute effects on airway inflammation and function in murine allergen provocation models

Minqi Hao, MD, PhD,<sup>a</sup> Stephania Comier, PhD,<sup>b</sup> Meiyong Wang, MD,<sup>a</sup>  
James J. Lee, PhD,<sup>b</sup> and Andre Nel, MD, PhD<sup>a</sup> *Los Angeles, Calif, and Scottsdale, Ariz*

**Background:** Epidemiologic studies show that sudden surges in ambient particulate matter (PM) levels can trigger acute asthma exacerbations. Although diesel exhaust particles (DEPs) act as an adjuvant for allergic sensitization, this is a delayed response and does not explain acute PM effects on airway hyperreactivity (AHR).

**Objective:** Our aim was to determine the acute effects of DEPs on AHR using a mouse model.

**Methods:** Three protocols were developed, 2 of which require OVA sensitization, whereas the third was OVA independent. In the mild sensitization protocol BALB/c mice receive intraperitoneal OVA without alum and are then challenged with aerosolized OVA with or without DEPs. In the postchallenge model DEPs are delivered after OVA challenge to animals sensitized by intraperitoneal OVA plus alum. In the third protocol nebulizer DEPs were also delivered to IL-5-overexpressing mice that exhibit constitutive airway inflammation. Animals were subjected to whole-body plethysmography (WBP) and then killed for performance of bronchoalveolar lavage, histology, and serology.

**Results:** DEP delivery concomitant with OVA challenge or after the induction of airway inflammation with this allergen induced increased AHR in models 1 and 2, respectively. Although these animals showed DEP-induced inflammation and mucus production in the intermediary airways, there was no effect on OVA-specific IgE or T<sub>H</sub>2 cytokine production. In the IL-5 transgenic mice it was possible to induce similar effects with DEPs in the absence of an allergen.

**Conclusion:** We demonstrate that DEPs induced AHR independent of their adjuvant effects, suggesting the use of these

models to study the mechanism or mechanisms of acute asthma exacerbation by means of PM. (*J Allergy Clin Immunol* 2003;112:905-14.)

**Key words:** Diesel exhaust particle, airway hyperreactivity, asthma, mouse models

Recent epidemiologic studies have reported an association between short-term increases in ambient particulate matter (PM) levels and acute cardiorespiratory events, including acute asthma flares.<sup>1,2</sup> Although the exact mechanism or mechanisms of these effects are unknown, there is accumulating evidence from controlled-exposure studies that PM induces acute inflammation in the human lung.<sup>3</sup> This is consistent with data showing that diesel exhaust particles (DEPs) act as an adjuvant for allergen sensitization in animals and human subjects.<sup>4,5</sup> These effects are dependant on redox cycling DEP chemicals that exert pro-oxidative and pro-inflammatory effects in the lung.<sup>6</sup>

Although the adjuvant effects of PM could affect asthma prevalence in the long term,<sup>7</sup> this does not explain acute asthma flares within a few hours after the pollution event.<sup>2</sup> This suggests that in addition to their subacute or chronic effects, DEPs exert acute effects on airway hyperreactivity (AHR). Almost all of the animal protocols used to date to show that DEPs affect AHR have been long-term studies in which either the particles are directly delivered to the trachea or nose or the animals are exposed to diesel fumes in an inhalation chamber for extended time periods (6-16 weeks).<sup>8-15</sup> This limits the use of these protocols in studying PM effects on asthma.

We used a classical sensitization approach (intraperitoneal OVA plus aluminum hydroxide) to determine whether DEPs coadministered with OVA exert effects on AHR in BALB/c mice. Because this did not lead to a discernable increase in OVA-induced AHR, we developed alternative approaches to studying short-term DEP effects in mice. Success was achieved with a milder OVA sensitization model<sup>16</sup> in which aerosolized DEPs could induce increased AHR without enhancing IgE production. We also demonstrated that DEPs could induce increased AHR and mucin production if delivery of the nebulizer particles is delayed until after the peak airway inflammatory response. DEPs could also induce AHR in animals with constitutive airway inflammation caused by

From <sup>a</sup>the Division of Clinical Immunology and Allergy, Department of Medicine, UCLA School of Medicine, University of California, Los Angeles, and <sup>b</sup>the Division of Pulmonary Medicine, Mayo Clinic Scottsdale.

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Reprint requests: Andre E. Nel, MD, PhD, Department of Medicine, Division of Clinical Immunology and Allergy, 10833 Le Conte Ave, 52-175 CHS, Los Angeles, CA 90095.

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**Abbreviations used**

AHR: Airway hyperreactivity  
BAL: Bronchoalveolar lavage  
DEP: Diesel exhaust particle  
MBP: major basic protein  
OVA: Ovalbumin  
PenH: Enhanced pause  
PM: Particulate matter  
WBP: Whole-body plethysmography

transgenic IL-5 overexpression, suggesting that DEP exposure is capable of exacerbating ongoing inflammatory responses in the absence of allergen provocation.

**MATERIALS AND METHODS****DEP source and preparation**

Light-duty DEPs collected from a 4-cylinder Isuzu diesel engine under a 10-torque load in a cyclone impactor were obtained from Dr Masaru Sagai (National Institute of Environment Studies, Tsukuba, Ibaraki, Japan). The DEP stock was prepared by resuspending 100 mg of particles in 10 mL of PBS, followed by vortexing and dispersion with a sonic disrupter for 10 minutes on ice.<sup>6</sup>

**Animal sensitization and exposure conditions**

Six- to 7-week-old female BALB/c mice were obtained from Charles River Laboratories (Hollister, Calif). NJ.1726 mice (C57BL/6J background) that exhibit transgenic IL-5 expression in the lung epithelium were generated as previously described.<sup>17</sup> Mice were housed in filter-topped cages under standard laboratory conditions and maintained on autoclaved food and acidified water.

For the mild sensitization protocol, animals received 20 µg of OVA without alum in 500 µL of PBS intraperitoneally on day 1 (Fig 1, A).<sup>16</sup> The negative control group (group 1) received 500 µL of PBS, whereas the positive control group (group 2) received 20 µg of OVA plus 2 mg of alum in 500 µL of PBS intraperitoneally. Each group included 6 animals. All mice were exposed on days 14 to 17 to nebulized saline (group 1), saline for an hour followed by 1% OVA (20 minutes; group 2), 2 mg/m<sup>3</sup> DEPs for an hour followed by 1% OVA (20 minutes; group 3), or saline for an hour followed by 1% OVA (20 minutes; group 4). OVA and DEP nebulization was performed with a Schuco 2000 (Allied Health Care Products, St Louis, Mo), which delivers 0.5- to 4-µm particles at a flow rate of 6 L/min.<sup>6</sup> The animals were subjected to whole-body plethysmography (WBP) on day 18 and were killed on day 19. NJ.1726 mice were exposed to nebulized saline or 200 µg/m<sup>3</sup> DEPs for 1 hour daily for 3 days, used for WBP, and killed for sample collection on day 5 (Fig 4, A).

All animal groups in the postchallenge model, with the exception of the negative control group, received 20 µg of OVA plus 2 mg of alum intraperitoneally on day 1 (Fig 6, A). Thereafter, the animals were challenged with nebulizer saline or 1% OVA daily for 20 minutes on days 15 to 17, after which the animals received nebulizer saline, 1% OVA (20 minutes), or 2 mg/m<sup>3</sup> DEPs for 1 hour daily on days 18 to 21 (Fig 6, A). The animals were used for WBP on day 22 and killed on day 23.

**Determination of airway responsiveness**

Total pulmonary airflow in unrestrained conscious mice was estimated with a WBP device (Buxco Electronics, Troy, NY).<sup>17,18</sup> Mice were challenged for 2 minutes with a series of aerosolized

methacholine administrations. Pressure differences between the chambers containing individual animals and a reference chamber were used to extrapolate minute volume, tidal volume, breathing frequency, and enhanced pause (PenH).<sup>18</sup> Airway reactivity was monitored for 3 minutes after each aerosol challenge. PenH is a function of total pulmonary airflow during the respiratory cycle and is described by the following equation:

$$\text{PenH} = (\text{PEP}/\text{PIP}) \times \text{pause},$$

where PEP is the peak expiratory pressure, PIP is the peak inspiratory pressure, and pause is a component of expiration time. This parameter is dependent on the breathing pattern and correlates with airway resistance as measured by traditional invasive techniques with ventilated mice.<sup>18</sup>

**Animal death and sample collection**

Mice were killed by administering pentobarbital intraperitoneally. Blood was collected by means of cardiac puncture. The trachea was cannulated, and the lungs were gently lavaged 3 times with 1 mL of sterile PBS to obtain bronchoalveolar lavage (BAL) fluid. Lungs were expanded with 10% buffered formalin phosphate before excision and sectioning. The BAL fluid was centrifuged at 1000 rpm for 10 minutes, and total and differential cell counts were performed as previously described.<sup>6,17</sup>

**Determination of OVA-specific IgE and IgG1 antibody titers**

Serum OVA-specific IgE and IgG1 levels were measured by means of ELISA, as previously described.<sup>6</sup>

**Lung histology and immunohistochemistry**

Lung sections were stained with hematoxylin and eosin and periodic acid-Schiff stain.<sup>17</sup> For conducting major basic protein (MBP) immunohistochemistry, tissue sections were deparaffinized, followed by quenching of endogenous peroxidase activity, blocking with 1% normal goat serum, and sequential overlay with rabbit anti-MBP (1:1000) antibody and goat anti-rabbit antibody.<sup>19</sup> Staining was conducted by using Vectastain Elite ABC and DAB kits (Vector Laboratories, Burlingame, Calif).

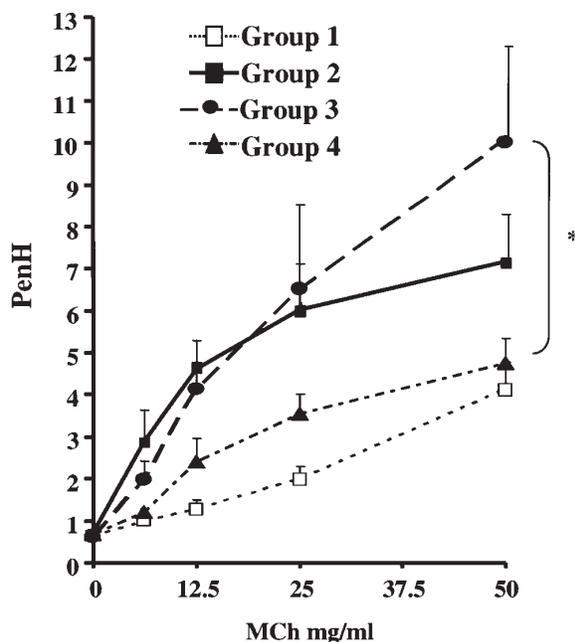
**Statistical analysis**

All data were expressed as means ± SD or means ± SEM. Differences between groups were evaluated by means of ANOVA. If differences between groups were significant ( $P < .05$ ), the Student *t* test was used to distinguish between pairs of groups. A *P* value of less than .05 was considered significant.

**RESULTS****Use of a mild sensitization model to demonstrate DEP effects on AHR**

Delivery of aerosolized DEPs concomitant with OVA challenge in animals sensitized with intraperitoneal OVA plus alum did not affect OVA-specific IgE production, BAL eosinophilia, or methacholine-induced AHR (data not shown). This suggests that in this model OVA alone induced a near-maximal response that does not leave room for a superimposed DEP effect. This hypothesis was tested by using a mild sensitization protocol that involves submaximal responses after allergen provocation.<sup>16</sup> The mild protocol was accomplished by eliminating the T<sub>H</sub>2 adjuvant, alum, from the intraperitoneal sensitization protocol





**FIG 2.** DEP exposure leads to an increase in methacholine-induced AHR in the mild sensitization protocol. PenH was measured in a Buxco box, as previously described.<sup>17</sup> \* $P < .05$ , group 3 versus group 4.

**TABLE I.** BAL cytokines in the mild sensitization model

Cytokines	Group 1	Group 2	Group 3	Group 4
IL-13	9.09 ± 0.69	52.33 ± 6.97*	8.81 ± 0.86	13.95 ± 3.56
GM-CSF	4.77 ± 0.39	4.99 ± 0.27	5.49 ± 0.52	5.17 ± 0.56
IL-4	12.85 ± 1.42	14.37 ± 3.09	17.47 ± 0.90	14.90 ± 0.89
IL-5	16.88 ± 0.93	18.82 ± 1.27	26.27 ± 4.83	16.74 ± 4.12

Values are shown as means ± SD in picograms per milliliter.

\* $P < .05$  compared with group 1.

Histologic assessment of lung tissue samples from each group revealed that peribronchial and perivascular inflammatory cell infiltrates were less prominent in the mild compared with the classical sensitization model (Fig 3, A, panels a-d). Although DEPs did not affect small airways, aerosolized particles did induce foci of inflammation associated with large and intermediary airways (Fig 3, A, panels c-e). These changes were accompanied by increased MBP deposition, as well as an influx of MBP-positive eosinophils in the mucosa (Fig 3, B). DEP treatment also induced increased mucin staining in large and intermediary airways in sensitized (panel c) compared with saline-treated (panel d) animals (Fig 3, C).

Taken together, these data indicate that DEPs induce AHR and airway inflammation without affecting the magnitude of the anti-OVA response. This raises the question as to whether DEPs can induce AHR independent of an allergen.

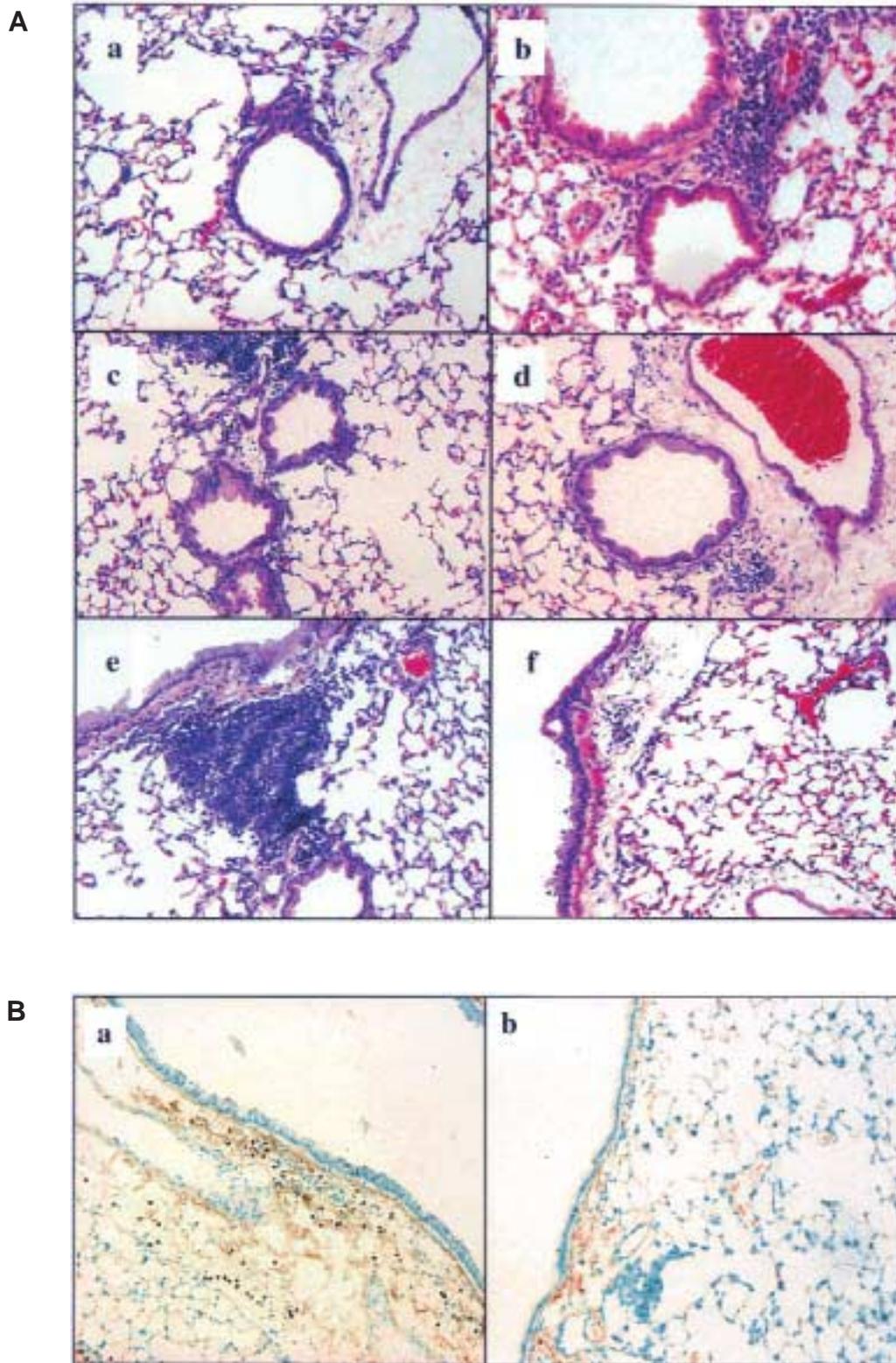
## IL-5 transgenic mice show that DEPs can affect AHR independent of an allergen

We have previously shown that transgenic mice with constitutive IL-5 expression in the lung leads to accumulation of peribronchial eosinophils, as well as an expansion of bronchus-associated lymphoid tissue, goblet cell hyperplasia, epithelial hypertrophy, and focal collagen deposition.<sup>17</sup> This provides us with a model to study DEP effects in the absence of allergen challenge. Exposure of these animals to aerosolized DEP daily for 3 days (Fig 4, A) resulted in increased AHR compared with that of animals receiving saline only (Fig 4, B). This was not accompanied by changes in the BAL cell count, which is constitutively increased in this model (not shown). No change in BAL cytokine levels occurred during DEP treatment (not shown). However, as for the mild sensitization model, IL-5 transgenic mice responded to DEP administration with increased airway inflammation and mucin production in large and intermediary airways (Fig 5, A and B). Because these data show that DEPs affect AHR independent of an allergen, this raises the question of whether DEPs can exert similar effects after the dissipation of the allergen-dependant effect.

## Delayed DEP delivery induces AHR and mucin production in the classical sensitization model

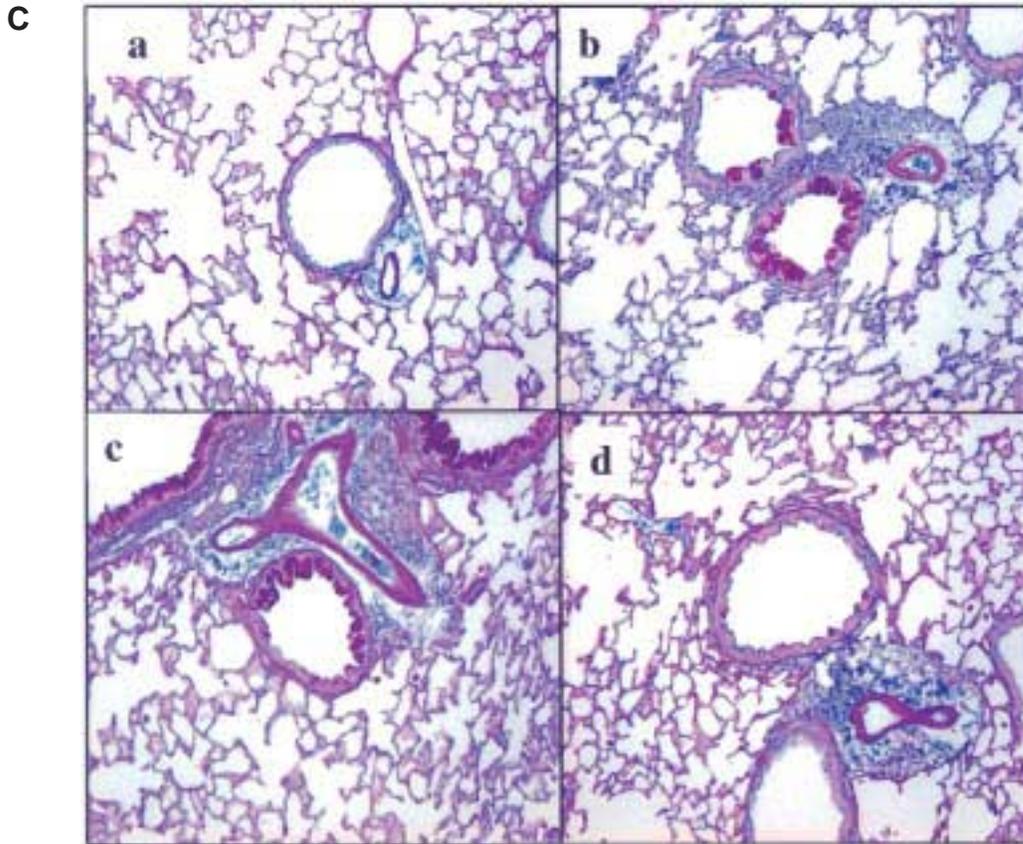
In a modification of the classical OVA sensitization and challenge model, mice were sensitized to intraperitoneal OVA and alum, challenged with aerosolized OVA on days 15 to 17, and then treated with aerosolized saline or DEPs for 4 days (Fig 6, A, groups 2 and 5, respectively). PenH was measured on day 22, and sample collection occurred on day 23 (Fig 6, A). The negative controls included animals receiving intraperitoneal PBS followed by aerosolized saline on days 15 to 21 (group 1) or sensitized animals receiving saline on days 15 to 17 and DEPs on days 18 to 21 (group 4). The positive control group was sensitized animals receiving OVA on days 18 to 21 (group 3). Statistically significant ( $P < .05$ ) methacholine-induced AHR was observed when comparing group 3 with group 1, whereas the AHR in group 2 declined to near-basal values (Fig 6, B). There was no response in animals receiving DEPs without prior OVA challenge (group 4), although animals receiving OVA challenge on days 15 to 17, followed by DEPs on days 18 to 21 (group 5), showed a statistically significant increase in AHR compared with that seen in groups 2 and 4 (Fig 6, B).

Examination of the BAL fluid showed the expected increase in total and differential cell counts in the positive control group (group 3, Table II). The increase in BAL cell counts in groups 2 and 5 was smaller but without a discernible DEP effect (Table II). Similarly, there was a smaller increase in the OVA-specific IgE and IgG1 antibody levels (Table III) and the BAL fluid IL-13 levels (Table IV) in groups 2 and 5 compared with that in group 3. DEPs did not exert any effects on  $T_H2$  cytokine



Environmental and occupational disorders

**FIG 3.** Histology to show airway inflammation and MBP and mucin staining in the mild sensitization protocol. **A,** Hematoxylin and eosin staining (*a*, group 1; *b*, group 2; *c* and *e*, group 3; *d* and *f*, group 4). **B,** MBP staining (*a*, group 3; *b*, group 4). (Figure continued on next page.)



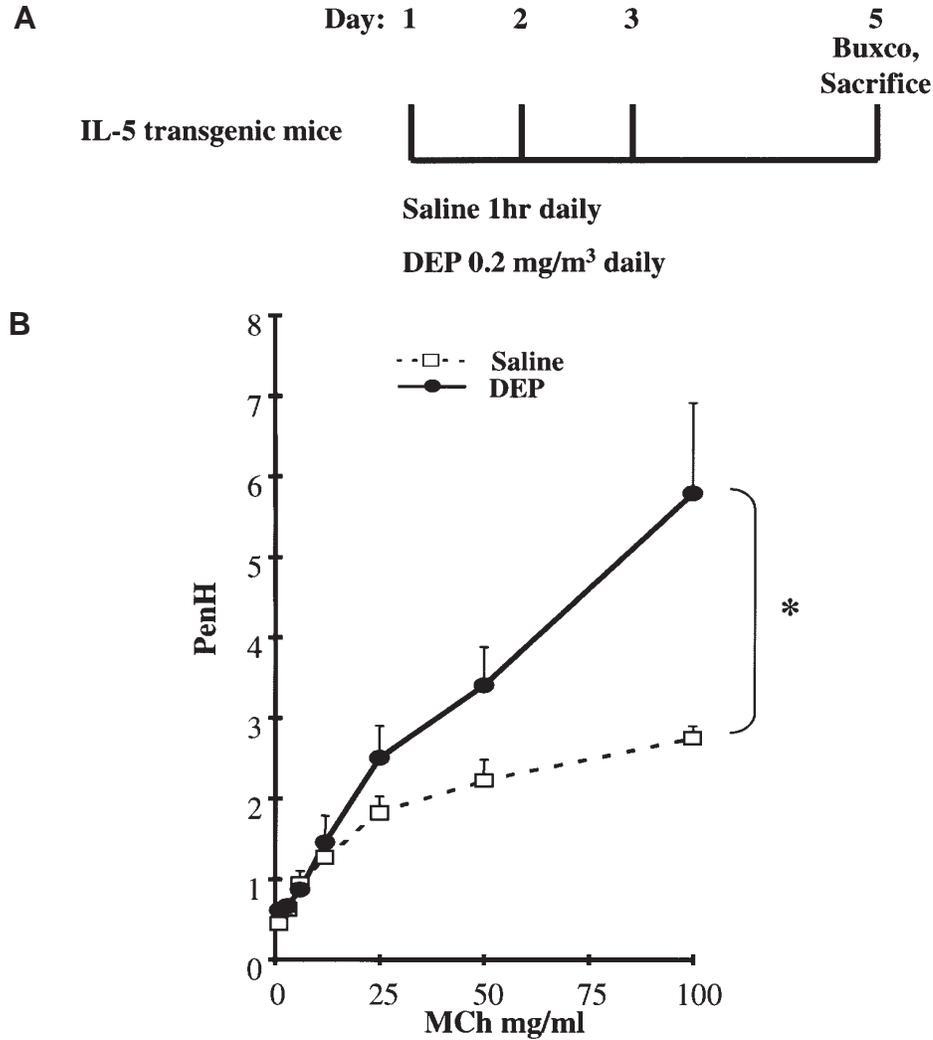
**FIG 3.** (continued) **C**, Periodic acid-Schiff staining (*a*, group 1; *b*, group 2; *c*, group 3; *d*, group 4; original magnification 100 $\times$ ).

levels (Table IV). Moreover, tissue histology revealed less inflammation in the small airways of groups 2 and 5 compared with group 3 (not shown). Similar to the mild sensitization model (Fig 3), DEP treatment was associated with increased airway inflammation and mucin production in the larger and intermediary airways in group 5 compared with that seen in group 2 (not shown).

## DISCUSSION

A number of animal and human studies have demonstrated that DEPs act as an adjuvant during allergen exposure,<sup>4,8-15</sup> raising the possibility that PM might be involved in the increased prevalence of atopic asthma.<sup>4</sup> However, although there is an epidemiologic link between asthma prevalence and exposure to polluted urban environments or vehicular emissions, there are no definitive data to establish that DEPs play a role in the global asthma epidemic.<sup>20,21</sup> In addition to these subacute and chronic effects, PM exposure induces acute asthma exacerbations, suggesting that PM can effect AHR independent of its effects on allergic sensitization. This notion is compatible with the data presented here. We demonstrate that although allergic airway inflammation is necessary to elicit a DEP-induced response in models 1 and 2, the particles can act independent of the allergen to induce AHR in IL-

5-overexpressing mice. Our study agrees with previous mouse models in which long-term DEP exposures were used to demonstrate particle effects in the presence or absence of an allergen.<sup>8-15</sup> A drawback of these long-term studies is the reliance on intratracheal or intranasal administration or the necessity to use an exposure chamber to deliver diesel exhaust fumes over a 12-hour period daily for 3 to 16 weeks.<sup>8-15</sup> Although many of these studies show a DEP-induced increase in BAL eosinophilia, OVA-specific IgE production, and increased IL-5 and GM-CSF production, our experimental models did not show this adjuvant effect.<sup>8-15</sup> Variation of the dose, frequency, and time of DEP administration did not result in an animal model in which DEPs increase both the level of OVA sensitization, as well as AHR. Although it is possible to enhance OVA-specific IgE production in animals receiving nebulizer DEPs plus OVA,<sup>6</sup> these animals do not have BAL eosinophilia or increased AHR (not shown). Therefore the shortcomings of the inhalation sensitization model is that DEPs do not achieve the level of sensitization and inflammation that is required to induce AHR. Apparently, it is possible to enhance allergic inflammation and achieve this threshold during chronic DEP exposure.<sup>8-15</sup> These chronic exposure protocols require a diesel engine and an exposure chamber, which is costly and not widely available to investigators in this field.



**FIG 4.** The effects of nebulizer DEPs on IL-5 transgenic mice: **A**, protocol outline; **B**, DEP exposure leads to an increase in methacholine-induced AHR in IL-5 transgenic mice. \* $P < .05$ , DEPs versus saline at 100 mg/mL methacholine.

**TABLE II.** BAL cell counts in the post-OVA challenge model

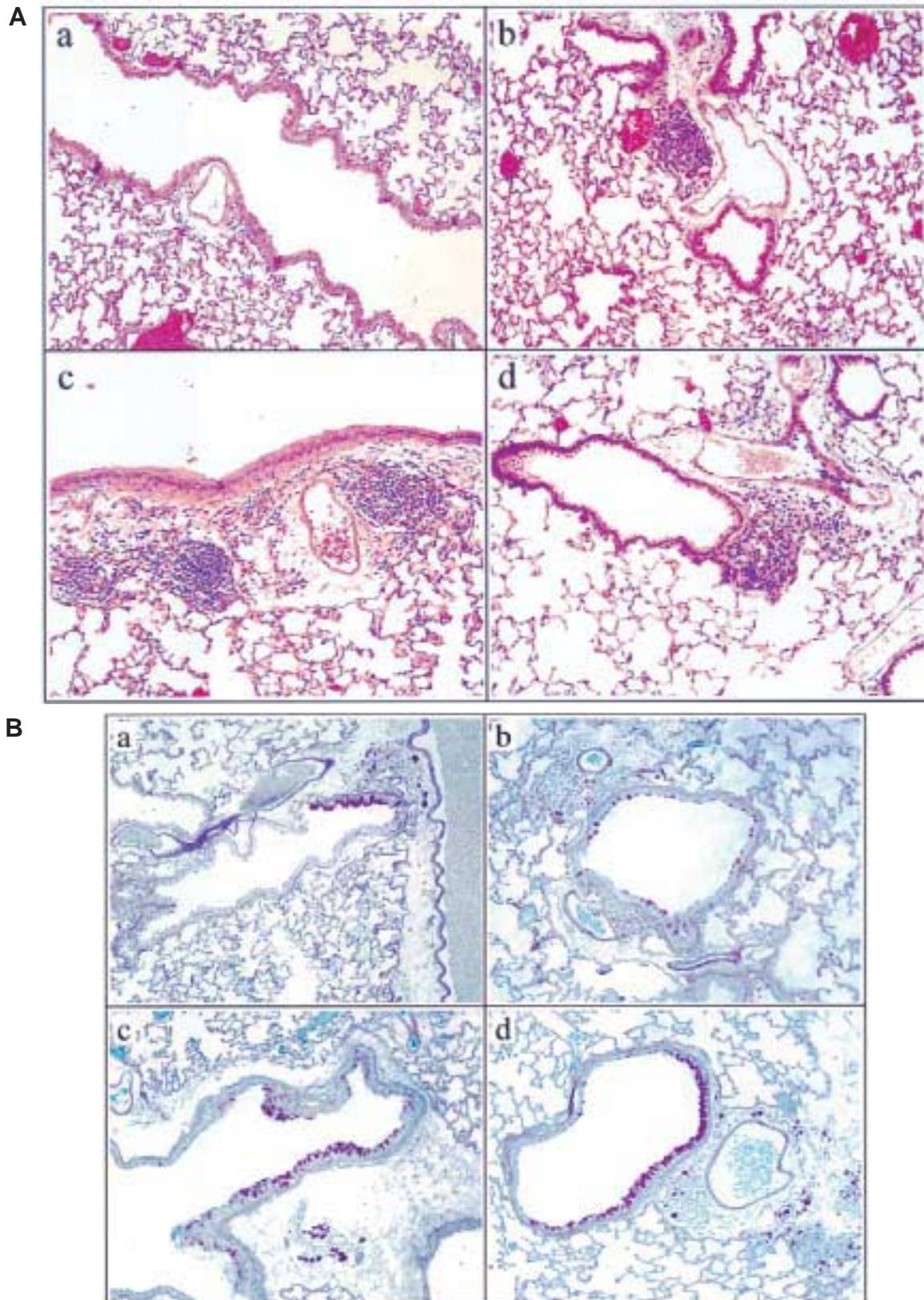
	Group 1	Group 2	Group 3	Group 4	Group 5
Total cell count	0.58 ± 0.10	8.74 ± 1.21	14.51 ± 3.91*	0.70 ± 0.14	7.2 ± 1.89
Eosinophils	0.00	3.52 ± 0.62	7.94 ± 2.66*	0.00	3.83 ± 1.45
Neutrophils	0.00	0.29 ± 0.11	0.26 ± 0.09	0.00	0.08 ± 0.06
Lymphocytes	0.00	1.33 ± 0.33	0.83 ± 0.20	0.004 ± 0.002	0.62 ± 0.16
Monocytes	0.58 ± 0.10	3.60 ± 1.01	5.47 ± 1.15	0.69 ± 0.14	2.66 ± 0.38

Values are shown as means ± SD in cells × 10<sup>5</sup> per milliliter.

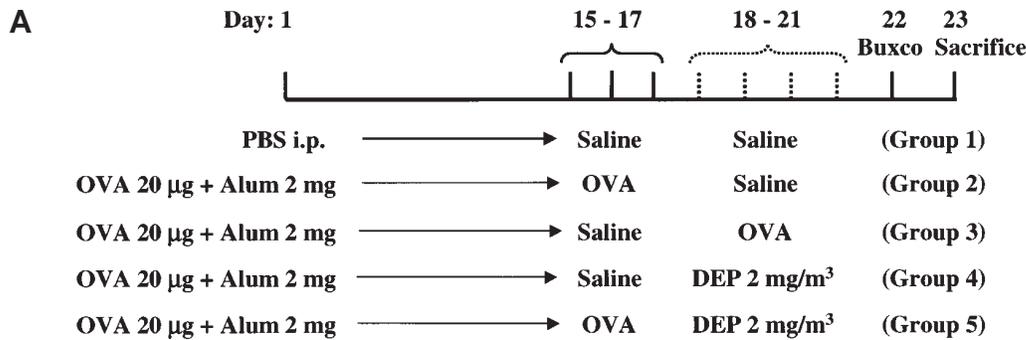
\* $P < .05$  compared with groups 1 and 4.

We demonstrate that DEP-induced AHR could be achieved in 3 different ways. The first model is a mild OVA sensitization protocol, in which DEP delivery concomitant with OVA challenge induces increased airway obstruction, as well as increased inflammatory changes and mucin production, in large and intermediary airways (Figs 1-3). A possible explanation for the effect of nebu-

lizer DEPs on large and intermediary airways is the deposition of the relatively large-sized nebulizer droplets in these airways. Animals in this protocol did not exhibit an increase in OVA-specific IgE or T<sub>H</sub>2 cytokine levels. The second protocol uses DEP administration to sensitized animals after they have been exposed to OVA. The delayed introduction of the nebulizer particles is neces-

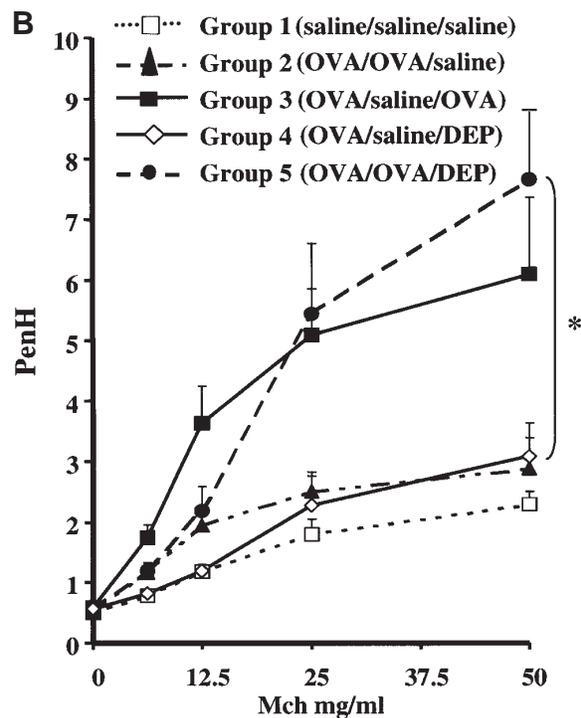


**FIG 5.** Histology to show the effects of nebulizer DEPs on lung inflammation. **A**, Hematoxylin and eosin staining (*a* and *b*, saline exposure; *c* and *d*, DEP exposure). **B**, Periodic acid–Schiff staining (same panels as Fig 5, **A**).



sary to demonstrate a DEP effect that is otherwise obscured by the rigorous inflammatory response to OVA in the classical model. These data are reminiscent of the acute asthma exacerbations that follow a sudden surge in PM levels.<sup>2,3</sup> The realization that a lesser degree of airway inflammation is optimal for elucidating DEP effects led to the introduction of the third model, which uses constitutive eosinophilic inflammation in IL-5 transgenic mice to elicit AHR and pro-inflammatory effects (Figs 4 and 5).

Our exposure methods should be useful to dissect the pathways and chemical components by which DEPs induce acute asthma exacerbations. In addition to enhancing already existing airway inflammation, DEPs might also affect AHR by damaging bronchial epithelial cells.<sup>22</sup> In this regard we have previously shown that DEPs induce apoptosis in bronchial epithelial cells in an oxidative stress-dependent fashion.<sup>22</sup> Shedding of epithelial cells might lead to increased airway reactivity.<sup>23</sup> Tunnel staining of the lung sections from the postchallenge model did not, however, reveal an increase in the apoptotic cells in the bronchial epithelial lining of DEP-treated animals (not shown). Other possible mechanisms of action include effects on irritant receptors, smooth muscle function, cytokine production, and autonomic reflexes. An example of an irritant receptor is the capsaicin receptor, which might be activated by noxious and tissue-damaging stimuli, giving rise to the release of neuropeptides from sensory nerve fibers.<sup>24</sup> Because respiratory epithelial cells are the first to encounter and respond to airborne irritants, it might be relevant that PM has been shown to initiate inflammatory cytokine release by the activation of capsaicin receptors in a human bronchial epithelial cell line.<sup>24</sup> In light of the DEP-induced AHR in IL-5 transgenic animals, it is also possible that DEP chemicals might affect eosinophil degranulation and mediator release. Although aerosolized DEP inhalation per se did not stimulate IL-5 production, it is possible that DEP chemicals might stimulate the release of chemokines that are involved in eosinophil chemotaxis. In this regard our own studies have demonstrated induction of RANTES, macrophage inflammatory protein 1 $\alpha$ , and monocyte chemoattractant protein 3 production in the human nasal mucosa during challenge with DEPs.<sup>25</sup> Our future studies will address the mechanism of DEP-induced AHR in more detail.



**FIG 6.** Delayed DEP delivery induced AHR in the classical sensitization model. **A**, Protocol outline. **B**, Methacholine challenge and WBP. DEP effects in the postchallenge model have been confirmed in numerous repeat experiments. \* $P < .05$ , group 5 versus group 2 at 25 and 50 mg/mL methacholine.

In summary, we have shown that DEPs can induce acute AHR in mice in the absence or presence of an allergen, and we will use this approach to study the PM components and biologic pathways by which PM induce acute asthma exacerbations.

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**TABLE III.** Serum OVA-specific Ig levels in the post-OVA challenge model

	Group 1	Group 2	Group 3	Group 4	Group 5
IgE (U/mL)	0.00	1044 ± 238	2190 ± 419*	349 ± 86	877 ± 194
IgG1 (ng/mL)	0.00	269 ± 61	1246 ± 368*	55 ± 10	319 ± 72

Values are shown as means ± SD.

\**P* < .05 compared with other groups.

**TABLE IV.** Cytokines in the BAL fluid in the post-OVA challenge model

Cytokines	Group 1	Group 2	Group 3	Group 4	Group 5
IL-13	6.84 ± 0.28	24.22 ± 7.83	155.02 ± 40.03*	8.80 ± 1.12	20.03 ± 3.28
GM-CSF	4.36 ± 0.19	4.89 ± 0.14	5.14 ± 0.47	4.50 ± 0.29	4.32 ± 0.11
IL-4	13.35 ± 0.81	18.60 ± 3.99	23.13 ± 2.77	14.40 ± 2.78	21.47 ± 1.88
IL-5	9.46 ± 2.37	18.12 ± 1.02	27.24 ± 6.26	9.60 ± 1.62	11.62 ± 3.38

Values are shown as means ± SD in picograms per milliliter.

\**P* < .05 compared with other groups.

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