

Diesel exhaust particulate induces airway hyperresponsiveness in a murine model: Essential role of GM-CSF

Ken Ohta, MD, PhD,^a Naomi Yamashita, MD, PhD,^a Makoto Tajima, MD,^a Takashi Miyasaka, MD, PhD,^a Junichi Nakano, MD, PhD,^a Mikio Nakajima, MD,^a Akira Ishii, MD,^b Tadashi Horiuchi, MD, PhD,^c Kenji Mano, MD, PhD,^a and Terumasa Miyamoto, MD, PhD^d Tokyo, Japan

Background: Inhaled pollutants were recently shown to be responsible for an increased incidence of airway allergic diseases, including asthma. A common feature of all forms of asthma is airway hyperresponsiveness.

Objective: Our purpose was to elucidate the effects of diesel exhaust particulate (DEP), one of the most prevalent inhaled pollutants, on airway responsiveness.

Methods: A/J and C57Bl/6 mice were used; the former are genetically predisposed to be hyperresponsive to acetylcholine, whereas the latter are not. DEP was administered intranasally for 2 weeks, after which pulmonary function was analyzed by whole-body plethysmography.

Results: Intranasal administration of DEP increased airway responsiveness to acetylcholine in both A/J and C57Bl/6 mice and induced displacement of ciliated epithelial cells by mucus-secreting Clara cells. The effect was mediated by M₃ muscarinic receptors. Acetylcholine-evoked bronchial constriction was reversed by administration of terbutaline, a β_2 -adrenergic antagonist, which is also characteristic of human asthma.

Intranasal administration of antibody raised against GM-CSF abolished DEP-evoked increases in airway responsiveness and Clara cell hyperplasia. The antibody raised against IL-4 also inhibited DEP-evoked increases in airway responsiveness.

However, it was to a lesser extent compared with antibody against GM-CSF. In addition, DEP stimulated GM-CSF messenger RNA expression in the lung.

Conclusion: DEP induces airway hyperresponsiveness by stimulating GM-CSF synthesis. (*J Allergy Clin Immunol* 1999;104:1024-30.)

Key words: Asthma, airway hyperresponsiveness, GM-CSF, diesel exhaust particulate, acetylcholine, bronchial epithelial cells

A common feature of asthma is hypersensitivity of the airways to a variety of airborne and endogenous stimuli. The characteristic bronchial hyperresponsiveness has been thought to be caused in part by an imbalance between adrenergic and cholinergic innervation,¹ whereas recent evidence suggests that allergic inflammation

Abbreviations used

ACh:	Acetylcholine chloride
BALF:	Bronchoalveolar lavage fluid
Cdyn:	Dynamic compliance
DEP:	Diesel exhaust particulate
mRNA:	Messenger RNA
Raw:	Airway resistance
RT:	Reverse transcription

may also be a critical contributing factor.^{2,3} Air pollutants also appear to modulate airway responsiveness: inhalation of nitrogen dioxide and ozone causes airway hyperresponsiveness in normal subjects and induces attacks in patients with asthma.⁴⁻⁷ More specifically, diesel exhaust particulate (DEP) derived from diesel automobiles and trucks is responsible for a recent increase in airway allergic disease.^{8,9} DEP also exerts an adjuvant effect on IgE production^{10,11} mediated in part by enhanced production of IL-4^{12,13} and in part by direct effects on B cells.¹⁴ DEP exerts an adjuvant effect on the airway hyperresponsiveness induced by allergen immunization.¹⁵ The direct effects of DEP on airway hyperresponsiveness are unknown, although there is evidence that it stimulates bronchial epithelial cells to produce the proinflammatory cytokine GM-CSF.^{16,17}

Bronchial epithelial cells contribute to allergic inflammation by acting as both effector and producer cells, and they synthesize and secrete such cytokines as GM-CSF, IL-6, IL-8, and TNF- α .¹⁸⁻²⁶ Among these, GM-CSF promotes differentiation of eosinophils from promyelocytes, activates eosinophils, and prolongs eosinophil survival in peripheral tissues.²⁷⁻²⁹ Furthermore, augmented GM-CSF production is observed in cells located at sites of asthmatic activity.³⁰⁻³²

The aim of the current study was to clarify the mechanisms underlying airway hyperresponsiveness, and within that context we assessed the extent to which GM-CSF contributes to its development. We found that in our murine model DEP induced airway hyperresponsiveness and caused hyperplasia of mucus-secreting Clara cells, which is also characteristic of human asthma. The effects of DEP were completely inhibited by the presence of a neutralizing anti-GM-CSF antibody, suggesting that DEP augments airway hyperresponsiveness by GM-CSF.

From the ^aDepartment of Medicine, Teikyo University School of Medicine, the ^bDepartments of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo, ^cKanto Chyuo Hospital, and the ^dJapan Clinical Allergy Institute, Tokyo, Japan.

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Reprint requests: Ken Ohta, MD, PhD, Department of Medicine, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan.

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METHODS

At all times during this study we adhered to the guidelines contained within the principles of Laboratory Animal Care formulated by the National Society for Medical Research.

Reagents

DEP was kindly provided by Dr Fujimaki (National Institute for Environmental Studies) after being collected on glass fiber filters placed at the end of a dilution tunnel connected to a 4-cylinder diesel engine (model 4JB1, Isuzu Automobile, Tokyo, Japan).¹² As measured with an Anderson air sampler, the mean diameter of the particles was 0.4 μ m; their carbon content was 26% of the total concentration. Goat antimouse GM-CSF and IL-4 neutralizing antibodies were purchased from R&D systems (Minneapolis, Minn); the 50% neutralization doses were 0.6 to 0.8 μ g/mL and 3 to 5 μ g/mL, respectively. Goat IgG was obtained from Chemicon International (Temecula, Calif), pentobarbital sodium was from Pitman More (Mundelein, Ill), pancuronium bromide was from Organ (Teknika, the Netherlands), acetylcholine chloride was from Wako Chemicals (Tokyo, Japan), and the muscarinic receptor antagonist 4-DAMP was from Hoffman-La Roche (Nutley, NJ).

Sensitization

Pathogen-free male A/J and C57Bl/6 mice (10-12 weeks old) were purchased from Charles River (Sulzfeld, Germany). The mice were intranasally administered 0.25 mg/mL DEP in 40 μ L of saline solution (0.9 mol/L sodium chloride) every other day for 2 weeks. The control mice were administered saline solution alone.

Assessment of airway responsiveness

After anesthesia was induced with pentobarbital, the mice were injected with pancuronium bromide, tracheostomized, and ventilated with a Harvard Instruments ventilator (tidal volume 0.5 mL, respiratory frequency 120/min). The mice were then placed into whole-body plethysmographs (Buxo Electronics, Troy, NY) to measure airway resistance (Raw) and dynamic compliance (Cdyn). Increasing doses of acetylcholine (ACh) were administered by ultranebulization for 3 minutes. In some experiments ACh-induced changes in Raw are expressed as the percent change from the Raw measured before ACh inhalation. Although airway conductance was also sensitive to ACh, it was less so than Raw; consequently, Raw was used as our index of airway constriction.

The effect of neutralizing antibody administration

Anti-GM-CSF (1 mg/mL) or anti-IL-4 (1 mg/mL) neutralizing antibody was administered intranasally in a 50 μ L volume immediately before each administration of DEP. The same volume and concentration of goat IgG was administered to sham control animals.

Measurements of total serum IgE

Serum IgE levels were measured with use of ELISA kits purchased from Yamasa Shoyu (Chiba, Japan). Plates were coated with rat antimurine IgE antibody of defined specificity.³³ Samples (25-fold dilution) were then incubated on the plates, after which horseradish peroxidase-labeled rat antimurine antibody specific for a different epitope was added. The color was developed and measured at 450 nm. The kit was able to detect murine IgE at concentrations as low as 10 ng/mL.

Reverse transcription-PCR for analysis of cytokine messenger RNA

The expression of messenger RNA (mRNA) encoding GM-CSF, IL-4, IL-5, TNF- α , and β -actin were measured by reverse transcrip-

tion (RT)-PCR. mRNA of the lung was isolated with use of the Quickprep mRNA purification kit, which is a combination of guanidium thiocyanate with oligodeoxythymidine cellulose chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). Synthesis of complementary DNA and PCR was carried out with use of an RNA PCR kit (Takara Biochemicals, Kyoto, Japan). Single-stranded DNA was synthesized at 25°C for 10 minutes, at 42°C for 15 minutes, at 99°C for 5 minutes, and at 5°C for 5 minutes in a reaction mixture consisting of 1 μ g mRNA obtained from lung tissue in 5 mmol/L magnesium chloride, 1 mmol/L deoxyribonucleoside triphosphate, 0.25 U of reverse transcriptase, 125 μ mol/L oligodeoxythymidine (Takara Biochemicals). The resultant complementary DNA mixture was added by 2.5 mmol/L magnesium chloride, 2.5 U of Taq polymerase, and 0.2 μ mol/L oligonucleotide primer (Takara Biochemicals). The specific primers for the mouse GM-CSF gene were 5'-TGTGG TCTAC AGCCT CTCAG CAC (64-87) and 5'-CAAAG GGGAT ATCAG TCAGA AAGG (407-431) (Clontech Lab, Palo Alto, Calif). The expected size of the amplified product was 368 bp. The primers for IL-4, IL-5, TNF- α , and β -actin were also purchased from Clontech, and the expected lengths of the amplified products were 344, 424, 354, and 540 bp, respectively. The mixture was amplified with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn). Denaturation was performed at 95°C for 1 minute, annealing at 65°C for 1 minute, and primer extension at 72°C for 2 minutes. These steps were repeated for 35 cycles. The amplified products were separated by electrophoresis on 2% agarose gels and were visualized by staining with ethidium bromide.

Histologic examination

After their pulmonary function was evaluated, mice were killed by exsanguination. The lungs were then fixed in 20% formaldehyde and stained with hematoxylin and eosin. During fixation, the lungs were fully inflated at a pressure of 10 cm H₂O.

Statistics

For comparisons of multiple parameters, we used ANOVA. Values of $P < .05$ were considered to be statistically significant.

RESULTS

Airway hyperresponsiveness induced by DEP

As expected, ACh evoked bronchial constriction in A/J mice, which are genetically predisposed to ACh hypersensitivity.^{34,35} The ACh significantly induced bronchoconstriction from 2500 μ g/mL, and 10,000 μ g/mL elicited a 100% increase in Raw (Fig 1, closed circles). ACh-evoked increases of Raw were reversible by the injection of terbutaline, a β_2 -adrenergic agonist, which is also characteristic of human asthma (Fig 1, after BD). Cdyn was less sensitive to ACh than Raw (open triangles); for that reason, Raw was used as the marker of bronchial constriction in the experiments described below.

In contrast to A/J mice, C57Bl/6 mice were normally unaffected by ACh; indeed, concentrations of ACh as high as 10,000 μ g/mL had no effect on bronchial tone in C57Bl/6 mice (Fig 2, closed circles). However, after a 2-week regimen in which mice were repeatedly administered DEP, ACh elicited airway constriction in both A/J and C57Bl/6 mice (Fig 2, open triangles and open circles, respectively). Although A/J mice remained more

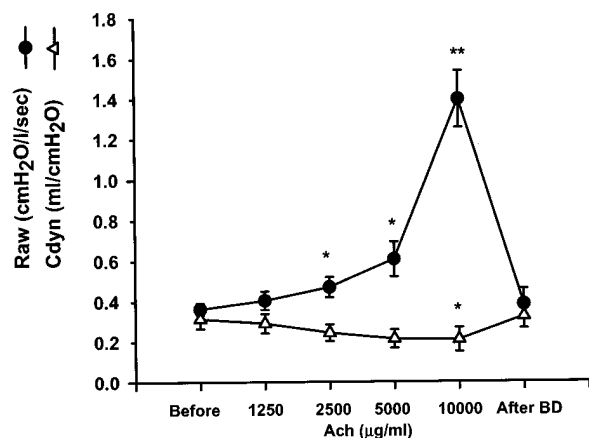


FIG 1. Airway hyperresponsiveness in A/J mice. Indicated concentrations of ACh were inhaled for 3 minutes. Raw (closed circles) and Cdyn (open triangles) were measured with use of whole-body plethysmographs as described in Methods. Arrowhead, Response obtained after injection of bronchial dilator terbutaline sulfate (BD, 0.5 µg). Data shown are means $\pm$ SEM for 5 mice. Asterisk, $P < .05$; two asterisks, $P < .01$.

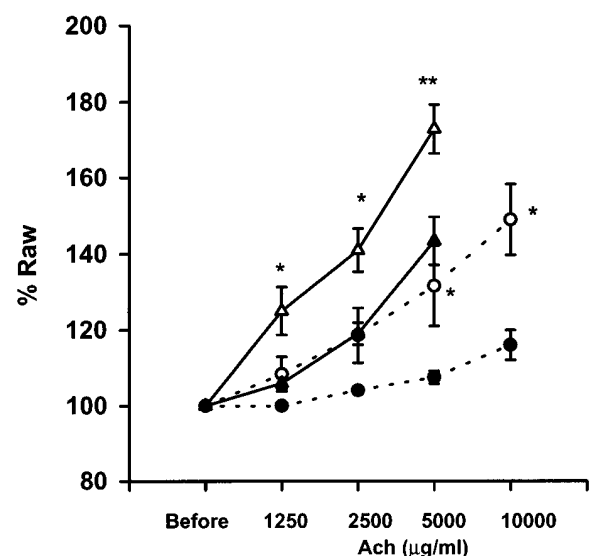


FIG 2. Airway hyperresponsiveness induced by DEP. After 2 weeks of intranasal DEP administration, airway responses to indicated concentrations of ACh were measured. Open triangles, DEP-treated A/J mice; closed triangles, untreated A/J mice; open circles, DEP-C57Bl/6 mice; closed circles, untreated C57Bl/6 mice. ACh (5 mg/mL)-evoked Raw is expressed as percentage of Raw observed before ACh inhalation (%Raw5). Data shown are means $\pm$ SEM of 5 independent experiments. Asterisk, $P < .05$.

sensitive to ACh, even C57Bl/6 mice exhibited increases in Raw when 5000 µg/mL ACh was inhaled. With respect to the duration of DEP-induced airway hyperresponsiveness, Fig 3 shows that the hyperresponsiveness to ACh was significantly elevated in DEP-treated mice for as long as 13 days after the final administration of DEP.

We next examined the extent to which DEP-induced airway hyperresponsiveness was mediated by signaling

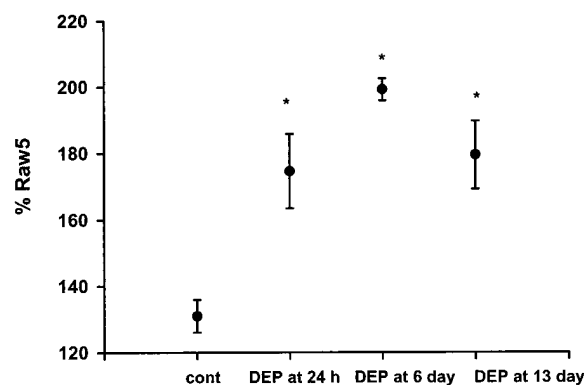


FIG 3. Duration of DEP-evoked airway hyperresponsiveness. Responses to ACh were measured 24 hours and 6 days after 2 weeks of intranasal DEP administration. ACh (5 mg/mL)-evoked Raw is expressed as percentage of Raw observed before ACh inhalation (%Raw5). Data shown are means $\pm$ SEM of 5 mice. Asterisk, $P < .05$.

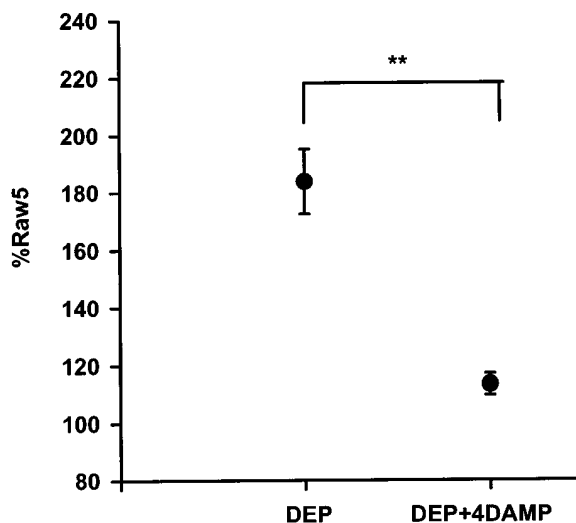


FIG 4. Effects of muscarinic antagonists on DEP-induced increases in airway responsiveness. M₃ muscarinic receptor antagonist 4-DAMP (0.2 µg/kg) was administered intraperitoneally 15 minutes before ACh inhalation. ACh (5 mg/mL)-evoked Raw is expressed as percentage of Raw observed before ACh inhalation (%Raw5). Data shown are means $\pm$ SEM of 7 mice. Two asterisks, $P < .01$.

by the muscarinic receptor.³⁵⁻³⁷ The M₃ muscarinic receptor antagonist 4-DAMP (0.2 µg/kg) significantly attenuated ACh hyperresponsiveness induced by DEP (Fig 4). Thus DEP apparently augments bronchial sensitivity to ACh through M₃ muscarinic receptors, not through nonspecific irritant effects.

IgE production is unaffected by DEP

DEP has been reported to augment IgE synthesis,^{10,11,13,14} raising the possibility that DEP-induced IgE synthesis might be responsible for the increased airway sensitivity to ACh. However, administration of DEP

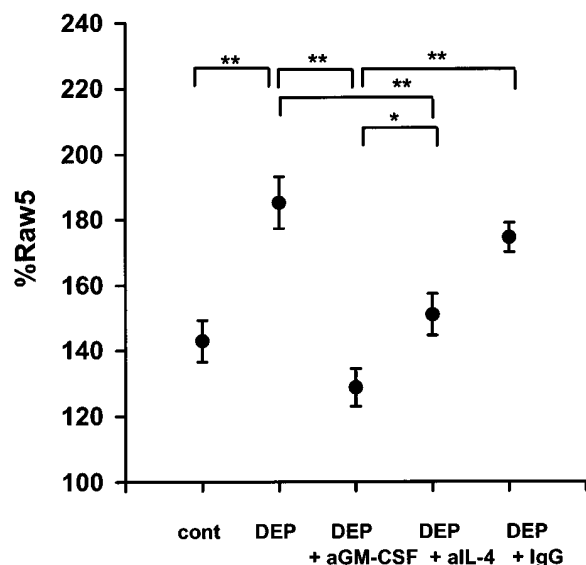


FIG 5. Effects of anti-GM-CSF antibody on DEP-evoked increases in airway responsiveness to ACh. Anti-GM-CSF or anti-IL-4 neutralizing antibody or goat IgG (sham control) was administered intranasally for 2 weeks immediately before each DEP treatment. Data are expressed as percentages of Raw measured before intranasal administration of 5 mg/mL ACh (%Raw5). Data shown are means $\pm$ SEM of 8 mice. Asterisk, $P < .05$; two asterisks, $P < .01$.

alone did not induce IgE synthesis (DEP treated mice 12.2 ± 6.8 ng/mL [$n = 4$] vs untreated mice 8.9 ± 3.8 ng/mL [$n = 5$], $P > .05$), indicating that DEP-induced airway hyperresponsiveness was not mediated by IgE.

Role of GM-CSF in airway hyperresponsiveness

The involvement of GM-CSF in the development of airway hyperresponsiveness was assessed with use of an anti-GM-CSF neutralizing antibody. Fig 5 shows the percent increase of Raw elicited by inhalation of 5 mg/mL ACh by DEP-treated mice. Pretreatment with anti-GM-CSF neutralizing antibody (5 μ g/mouse, every other day for 2 weeks) completely blocked the augmentation in airway responsiveness induced by DEP (DEP 185% $\pm$ 8% vs DEP + anti GM-CSF 142% $\pm$ 6.4%, $P < .01$). On the other hand, pretreatment with goat IgG did not attenuate DEP capacity to sensitize the airways. Although anti-IL-4 neutralizing antibody also inhibited airway hyperresponsiveness, the effect was less than that of anti-GM-CSF neutralizing antibody ($P < .05$, Fig 5).

Analysis of BALF revealed that DEP administration increased the cell numbers present (Fig 6). Although most of the cells in the BALF of both untreated and DEP-treated mice were macrophages, macrophages of DEP-treated mice were activated and contained carbon black particles. Anti-GM-CSF neutralizing antibody, but not control IgG, significantly reduced the number of cells present in the BALF.

Histologic examination confirmed the involvement of GM-CSF in our murine model. In untreated mice the

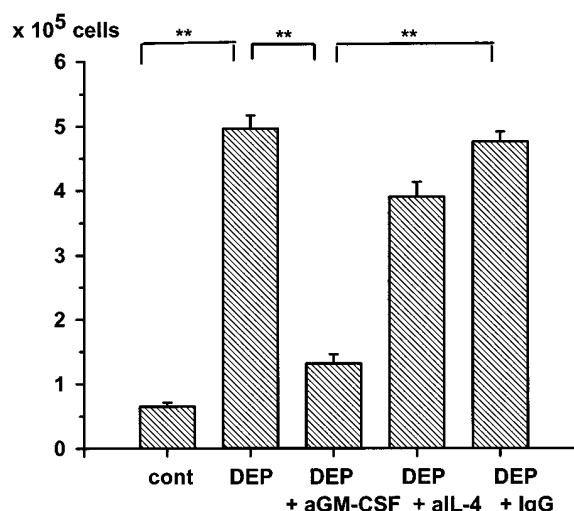


FIG 6. Analysis of bronchoalveolar lavage fluid (BALF). Lungs were subjected to lavage through intubation tubes with 5 mL of saline solution (0.9 mol/L sodium chloride), and the lavage fluid was collected. Cells present in BALF were pelleted, resuspended in 1 mL of saline solution, and placed on glass slides, where they were counted and fixed with Cytospin. The slides were then stained with Diff Quik, and cell differentiation was assessed microscopically. Data shown are means $\pm$ SEM of 8 mice. Two asterisks, $P < .01$.

cells lining the airway consisted of ciliated epithelial cells (Fig 7, A), whereas in DEP-treated mice the epithelial cells had been displaced by mucus-secreting Clara cells (Fig 7, B). Administration of anti-GM-CSF antibody, but not goat IgG, before DEP abolished this effect, and normal epithelial linings were maintained (Fig 7, C and D).

DEP-induced expression of cytokine mRNA

GM-CSF is produced by T cells, macrophages, and bronchial epithelial cells.^{17,31,32,38,39} We tested whether DEP stimulated GM-CSF production in lung tissue. Total mRNA was extracted from lung tissue, after which GM-CSF, IL-4, IL-5, and TNF- α mRNAs were examined by controlled RT-PCR. As shown in Fig 8, the expression of GM-CSF mRNA was up-regulated by the treatment with DEP (Fig 8, lane 4). IL-4 and IL-5 mRNA of total lung tissue was not detected in DEP-treated mice (Fig 8, lanes 6 and 8). In contrast, another inflammatory cytokine, TNF- α , was also up-regulated by the treatment of DEP (Fig 8, lane 10).

DISCUSSION

Airway hyperresponsiveness is one of the most prominent characteristics of asthma.² Its cause has yet to be established, although the rising incidence of bronchial asthma is, in part, attributable to air pollution⁴⁻⁷: inhalation of nitrogen dioxide or ozone induces airway hyperresponsiveness in normal subjects and causes attacks in those with asthma.⁴⁻⁷ In the current study we showed that

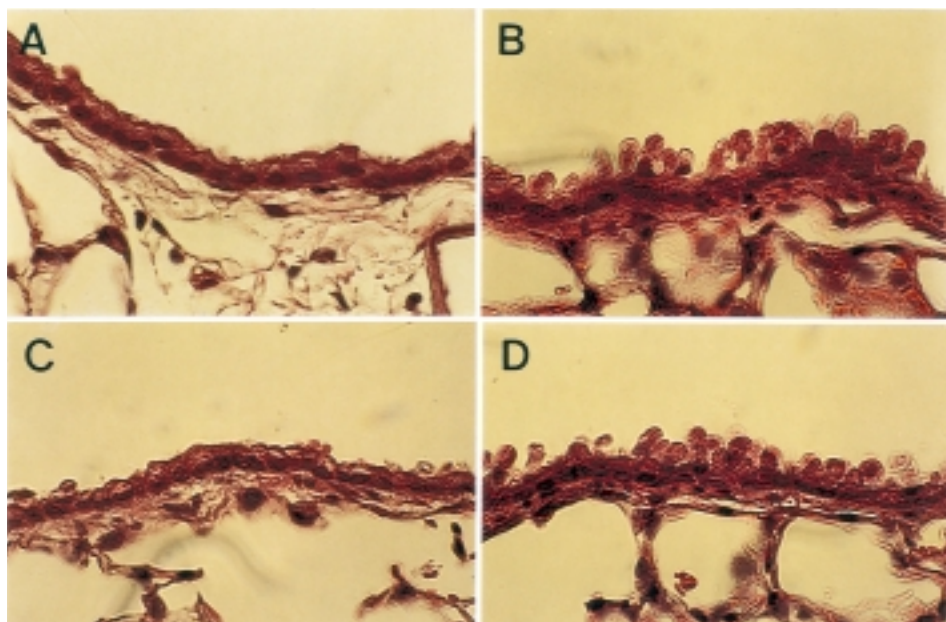


FIG 7. Effect of anti-GM-CSF neutralizing antibody on the histologic characteristics of lung tissue. Mice were treated with anti-GM-CSF antibody and DEP as described in the legend to Fig 4. Tissue specimens from (A) untreated control mouse, (B) DEP-treated mouse, (C) mouse treated with both anti-GM-CSF and DEP, and (D) mouse treated with both goat IgG and DEP.

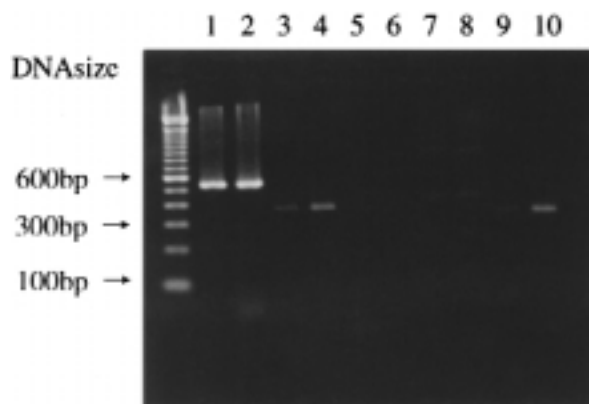


FIG 8. PCR analysis of expression of mRNAs encoding GM-CSF, IL-4, IL-5, and TNF- α . For control (lanes 1, 3, 5, 7, and 9) and DEP-treated (lanes 2, 4, 6, 8, and 10) mice, respectively, lanes 1 and 2 contain mRNAs encoding β -actin; lanes 3 and 4, GM-CSF; lanes 5 and 6, IL-4; lanes 7 and 8, IL-5; and lanes 9 and 10, TNF- α . DNA size standards are shown in the first lane on the left.

DEP augmented airway responsiveness to ACh in a murine asthma model that also exhibited bronchial epithelial damage with Clara cell hyperplasia. DEP affected both A/J and B57Bl/6 mice although the airways of C57Bl/6 mice do not normally exhibit the hyperresponsiveness seen in A/J mice. In addition, we found that DEP-induced hyperresponsiveness is mediated at least in part by GM-CSF, a cytokine affecting several airway-resident cell types.

DEP is a small particulate that at high concentrations accelerates formation of superoxide radical and induces

pulmonary edema; at lower concentrations it appears to increase IgE biosynthesis, which is related to bronchial asthma.^{11,12} These findings suggest that in urban environments, where its levels are comparatively high, DEP may contribute in some parts to the increased numbers of patients with allergic airway reactions. In this report we demonstrated that DEP directly affects airway responsiveness in mice: it exacerbated the hypersensitivity to ACh seen in A/J mice and induced sensitivity to a lesser extent in C57Bl/6 mice.

Although IgE-mediated activation of mast cells is an important trigger, DEP-induced airway hyperresponsiveness cannot be attributed to IgE-mediated inflammation. Instead, DEP may act directly on cells lining airways and thus induce inflammation through the production of cytokines. Our findings indicate that GM-CSF is a key cytokine causing bronchial inflammation and increased bronchial responsiveness when airways are exposed to air pollutants. GM-CSF is required for the maturation and survival of eosinophils as well as for augmentation of eosinophil chemotaxis, degranulation, and cytokine production.²⁷⁻²⁹ GM-CSF also affects basophils, enhancing their survival and augmenting histamine release and chemotaxis,⁴⁰⁻⁴² and augments macrophage and neutrophil function.⁴³ In addition, GM-CSF gene transfer in the lung was recently shown to cause not only accumulation of inflammatory cells but also irreversible fibrotic reaction.⁴⁴

Bronchial epithelial cells and T cells produce GM-CSF in response to a variety of stimuli.^{19,20} We hypothesize that GM-CSF synthesized by bronchial epithelial cells exposed to DEP stimulates TNF- α and IL-1, etc,

production by macrophages. The TNF- α in turn accelerates GM-CSF production by both epithelial cells and T cells. GM-CSF likely augments allergic inflammation by affects on basophils, eosinophils, neutrophils, and macrophages, resulting in bronchial epithelial cell damage and Clara cell hyperplasia. Although GM-CSF is not the sole cytokine involved in asthma, it apparently plays a central role in the cascade inducing the airway hyper-responsiveness. In that context, investigation of new approaches to the treatment of asthma that involve controlling the effects of GM-CSF would seem appropriate.

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