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Diesel Exhaust Inhalation Enhances Airway Hyperresponsiveness in Mice

Abstract

Background: Repeated intratracheal instillation of diesel exhaust particles and ovalbumin-induced airway hyperresponsiveness and airway inflammation in mice. However, the effects of daily inhalation of diesel exhaust may differ from the effects of direct instillation. **Methods:** Therefore, mice were exposed to diesel exhaust by inhalation 12 h per day for 3 months. Before the diesel exhaust exposure, ovalbumin was injected intraperitoneally as a sensitization. After 3 weeks of diesel exhaust exposure, these mice were challenged with ovalbumin every 3 week thereafter. **Results:** Diesel exhaust exposure with antigen challenge induced airway hyperresponsiveness and airway inflammation which was characterized by increased numbers of eosinophils and mast cells in lung tissue. The recruitment of inflammatory cells was accompanied by an increment in goblet cells on bronchial epithelium. Diesel exhaust exposure alone also enhanced airway hyperresponsiveness, but did not induce eosinophilic infiltration and/or an increment in goblet cells. **Conclusion:** Diesel exhaust inhalation enhanced airway hyperresponsiveness and airway inflammation caused by ovalbumin sensitization in mice.

Key Words

Diesel exhaust
Airway hyperresponsiveness
Eosinophils
Goblet cells
Ovalbumin

Introduction

Asthma is a chronic disease of the airways which is clinically characterized by airway obstruction, airway hyperresponsiveness, and airway inflammation. The airway inflammation of asthma is unique in that the airway wall is infiltrated by T lymphocytes bearing the T helper type 2 phenotype, eosinophils and mast cells [1–3]. Each of these cells is thought to contribute to the pathophysiologic changes that characterize asthma.

Recently, we demonstrated that the repeated intratracheal instillation of diesel exhaust particles (DEP)-induced airway hyperresponsiveness, chronic airway inflammation with infiltration of eosinophils and neutrophils, and hypersecretion of mucus in mice [4]. Furthermore, we showed that DEP enhanced airway inflammation and hyperresponsiveness caused by ovalbumin (OVA) sensitization. It was

accompanied by OVA-specific IgG1 production and interleukin-5 expression [5]. These experiments suggest that DEP may cause allergic asthma in humans with allergens, which are increasing in modern buildings with airtightness [6]. Furthermore, it has been shown that the prevalence of allergic rhinitis among school children is significantly higher in districts polluted by automobile exhaust than in non-polluted districts in Japan [7].

However, the effects of intratracheal and intranasal instillation of DEP to mice may not be the same as the effects of diesel exhaust (DE) inhalation. It is necessary to evaluate the effects of DE inhalation on airway hyperresponsiveness and inflammation. Although there are a lot of experiments about the effects of intratracheal and intranasal instillation of DEP suspension on allergic rhinitis, to our knowledge, in none of the experimental trials in mice the effects of DE inhalation on airway hyperresponsiveness have been shown.

To elucidate the involvement of DE in allergic airway inflammation and airway hyperresponsiveness, mice were exposed to DE and sensitized with OVA to examine airway hyperresponsiveness, airway inflammation, hyperplasia of goblet cells, and the production of allergen-specific IgE and IgG1.

Materials and Methods

Materials

Acetylcholine (ACh), diethyl ether, thimerosal, and Tween 20 were purchased from Nacalai Tesque (Kyoto, Japan). Anti-mouse IgE rat horseradish peroxidase-conjugated streptavidin, bovine serum albumin (BSA), ethylenediamine tetraacetic acid, 4-methyl-umbelliferyl- β -galactoside and OVA (grade V) were from Sigma (St. Louis, Mo., USA). Biotinylated rabbit anti-mouse IgG1 and β -D-galactosidase-conjugated streptavidin were from Zymed Laboratories (San Francisco, Calif., USA). Phosphate-buffered saline (PBS; pH 7.4) was from Nissui (Tokyo, Japan). Diff-Quik stain solution was from International Reagents (Kobe, Japan). Anti-mouse IgE rat monoclonal antibody was from Yamasa Shoyu (Chiba, Japan). Schiff's reagent was from Merck (Darmstadt, Germany). All other chemicals were of the highest grade available.

Animals

Male C3H/HeN mice (4 weeks old) were obtained from Japan Clea (Tokyo, Japan). They were fed a commercial stock diet (Japan Clea) and water ad libitum. Six-week-old mice were transferred into a chamber that was maintained at 24–26°C with 55–75% humidity and 14 h/10 h light/dark cycle. The study adhered to the National Institute of Health guidelines for the use of experimental animals.

Generation of DE

A 4JB-1-type, light-duty (2,740 ml), four-cylinder diesel engine (Isuzu Automobile, Tokyo, Japan) was connected to an EDYC dynamometer (Meidensha, Tokyo, Japan) to regulate driving conditions. The engine was operated using standard diesel fuel at a speed of 1,500 rpm under a load of 10 torque (kg/m) [8]. DE was diluted and cooled by clean air, and regulated in stable concentrations of 1 and 3 mg DEP/m³. Atmospheric concentrations of major components were monitored continuously by a particle counter (Shibata Science Technology, Tokyo, Japan) and portable gas tester (CGT-10-3-A, Shimadzu, Kyoto, Japan). NO and NO₂ concentrations were measured by chemiluminescence NO-NO₂-NO_x analyzer (model 43; Thermo Environmental Instruments, Franklin, Mass., USA). SO₂ concentration was determined by a fluorescent SO₂ analyzer (model 8850; Monitor Labs., Englewood, Colo., USA). The concentrations of NO, NO₂, SO₂ and CO₂ in separate chambers are shown in table 1.

Study Protocol

78 C3H/HeN mice were divided into six groups. These groups of mice were exposed to clean air (control group: Air), DE containing 1 mg DEP/m³ (DE-1) or DE containing 3 mg DEP/m³ (DE-3) in separated chambers (2.2 m³) for 12 h per day (10 p.m. to 10 a.m.) during 12 weeks, respectively. Before clean air or DE exposure, 10 μ g of OVA dissolved in 0.1 ml of saline solution was injected intraperitoneally as a sensitization. After 3 weeks of air or DE exposure, these mice were

Table 1. The concentrations of gaseous components in separate chambers (means $\pm$ SEM)

Chamber	NO ppm	NO ₂ ppm	SO ₂ ppm	CO ₂ ppm
Air	0.04 $\pm$ 0.02	0.05 $\pm$ 0.01	0.01 $\pm$ 0.00	245 $\pm$ 33
DE-1	8.85 $\pm$ 0.85	1.42 $\pm$ 0.19	0.87 $\pm$ 0.04	1,477 $\pm$ 146
DE-3	21.34 $\pm$ 2.38	4.02 $\pm$ 0.28	1.83 $\pm$ 0.10	2,916 $\pm$ 220

challenged with OVA every 3 week (total 4 times). The challenge was conducted by exposure to an aerosol of 1% OVA in saline administered through an ultrasonic nebulizer (NE-U07; Omron, Tokyo, Japan) in a chamber of 2,000 cm³ for 6 min. Three groups of nonsensitized mice were injected with saline instead of OVA, and were challenged with a saline aerosol. Twenty-four hours following the last challenge of OVA, 8 and 5 mice were sacrificed to study airway responsiveness and to analyze bronchoalveolar lavage (BAL), respectively. After measurement of airway responsiveness, lungs were removed for determinations of histology.

Measurement of Airway Responsiveness

Respiratory resistance (Rrs) was measured according to the method of Sorkness et al. [9], with a minor modification. In brief, each mouse was anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and a tracheostomy was performed with an 18-gauge cannula. Each mouse was mechanically ventilated with a rodent respirator (model 683; Harvard Apparatus, South Natick, Mass., USA) in a plethysmograph box with a pneumotachometer (BUXCO Electronics, Sharon, Conn., USA) at a constant tidal volume (0.3 ml) and at a rate of 120 breaths/min. Spontaneous respiration was inhibited by pancuronium bromide (1 mg/kg, i.m.). Continuous measurements of endotracheal pressure, flow, respiratory resistance, and dynamic lung compliance were recorded on a six-channel recorder (BUXCO Electronics). A 4-second average of Rrs and dynamic lung compliance was also recorded. An ACh challenge was performed with ACh solution (0.313–10 mg/ml) inhaled for 2 min. The solution was aerosolized by an ultrasonic nebulizer (NE-U07; Omron), which generates aerosol particles of approximately 5 μ m in diameter.

BAL

The trachea was cannulated after blood collection. Lungs were lavaged with 1.2 ml of sterile saline at 37°C instilled bilaterally by syringe. The lavage fluid was harvested by gentle aspiration. This procedure was conducted two more times. Average volume retrieved was 90% of the 3.6 ml instilled; the amounts did not differ among groups. The fluid collections were combined, and cooled to 4°C. The lavage fluid was centrifuged at 300 g for 10 min, and the total cell counts were determined on a fresh fluid specimen using a hemocytometer. Differential cell counts were assessed on cytologic preparations. Slides were prepared using a Cytospin (Tomy Seiko, Tokyo, Japan) and stained with Diff-Quik. A total of 300 cells was counted under oil immersion microscopy.

Histologic Evaluation of Eosinophils, Mast Cells and Goblet Cells in Lungs

After exsanguination, the lungs were removed and fixed by intratracheal instillation with 10% neutral phosphate-buffered formalin at a pressure of 20 cm H₂O. After at least 72 h, slices (4–5 mm thick) of all pulmonary lobes were embedded in paraffin. Sections (3 µm thick) were prepared and stained with Diff-Quik to quantitate the number of infiltrated eosinophils. The length of the basement membrane of all airways in each specimen was measured by video micrometer (VM-30; Olympus, Tokyo, Japan). In each sample, the numbers of eosinophils under the bronchial epithelium were counted with a micrometer (AX80; Olympus) under oil immersion. Results were expressed as the number of inflammatory cells per millimeter of basement membrane. To quantitate mast cells, sections were stained with toluidine blue. The number of mast cells under the bronchial epithelium was counted by the micrometer. Results were expressed as the number of mast cells per millimeter of basement membrane in the same manner. To quantitate goblet cells, sections were stained with periodic acid Schiff. The number of goblet cells also stained red in the bronchial epithelium was counted by the micrometer. Results were expressed as the number of goblet cells per millimeter of basement membrane in the same manner.

Blood Retrieval and Analysis

Mice were anesthetized with diethyl ether. The chest and abdominal walls were opened, and blood was retrieved by cardiac puncture. Plasma was prepared and frozen at –80°C until assay for OVA-specific IgE and IgG1.

OVA-Specific IgE Determination

The titer of OVA-specific IgE antibody was measured by IgE-capture enzyme-linked immunosorbent assay (ELISA) [10]. In brief, microplate wells (Dynatech, Chantilly, Vt., USA) were coated with an anti-mouse IgE rat monoclonal antibody at 37°C for 3 h, and incubated at 37°C for 1 h with PBS containing 1% BSA and 0.01% thimerosal. After washing with PBS containing 0.05% Tween 20 (PBST), diluted serum samples were added to the wells and incubated overnight at 4°C. After washing with PBST, biotinylated OVA was added to each well and incubated for 1 h at room temperature. After washing, wells were incubated for 1 h at room temperature with β-D-galactosidase-conjugated streptavidin. After the final washing, wells were incubated with 4-methylumbelliferyl-β-galactoside as the enzyme substrate at 37°C for 2 h. The enzyme reaction was stopped with 0.1 M glycine-NaOH buffer solution (pH 10.3). The fluorescence intensity was read by a microplate fluorescence reader (Fluoroskan Flow Laboratories, Costa Mesa, Calif., USA). Each plate included a previously screened standard plasma that contained a high titer of anti-OVA antibodies. For standardization, 1 titer was defined as twice the fluorescence units of preimmune plasma (blank) reaction. OVA-specific IgE titer in the well of each plate was calculated using a standard curve made by serial dilution of the standard plasma and dilution factor. The diluted plasma sample, which is lower than 0.1 titer, was expressed as negative. ELISA antibody titer was expressed as the highest plasma dilution giving the positive reaction.

OVA-Specific IgG1 Determination

OVA-specific IgG1 was measured by ELISA with solid phase antigen. In brief, microplate wells were coated with OVA overnight at 4°C. Then the plate was incubated at room temperature for 1 h with 1% BSA-PBS containing 0.01% thimerosal. After washing with PBST, di-

luted serum samples were added to the wells and incubated at room temperature for 1 h. After one more washing with PBST, the plate was incubated at room temperature for 1 h with biotinylated rabbit anti-mouse IgG1. After another washing, wells were incubated with horseradish peroxidase-conjugated streptavidin at room temperature for 1 h. Then, wells were washed and incubated with *o*-phenylenediamine and H₂O₂ in the dark at room temperature for 30 min. The enzyme reaction was stopped with 4 N H₂SO₄. Absorbance at 490 nm was read by a microplate reader. Each plate included standard serum that contained a high titer of anti-OVA IgG1 antibodies. For standardization, 1 titer was defined as twice the absorbance of preimmune plasma (blank) reaction. OVA-specific IgG1 titer in the well of each plate was calculated using a standard curve made by serial dilution of the standard plasma and dilution factor. The diluted plasma sample, which is lower than 1 titer, was expressed as negative. ELISA antibody titer was expressed as the highest plasma dilution giving the positive reaction.

Statistical Analysis

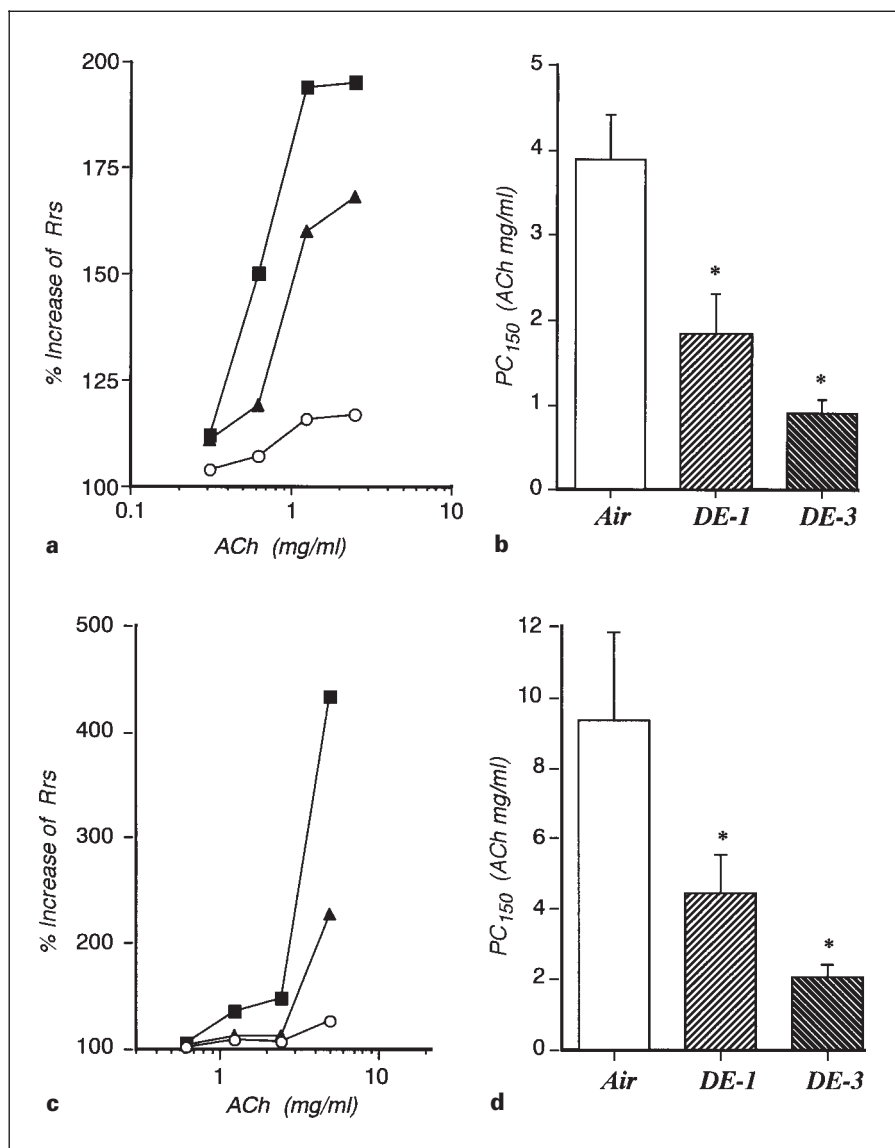
Data are reported as means ± SEM. Differences in the numbers of infiltrated inflammatory cells and goblet cells, in airway hyperresponsiveness, and in immunoglobulin titers among groups were determined using analysis of variance (Statview; Abacus Concepts, Berkeley, Calif., USA). If differences among groups were significant ($p < 0.05$), Fisher's protected least significant difference test was used.

Results

DE Exposure with OVA Sensitization Enhances Airway Hyperresponsiveness in Mice

We measured Rrs in mice that inhaled ACh cumulatively to compare the effect of DE on the airway hyperresponsiveness. Rrs was significantly greater in the DE-1 and DE-3 groups compared with the Air group by ACh inhalation in both the sensitized and nonsensitized mice (fig. 1a, c). We estimated the provocative concentration of ACh causing a 50% increase in Rrs (PC₁₅₀) values to compare the effects of DE exposure on Rrs. A decrease in PC₁₅₀ represents an increase in Rrs. The PC₁₅₀ values of the sensitized and nonsensitized mice are shown in figures 1b and 1d, respectively. Both the values of PC₁₅₀ in the DE-1 and the DE-3 groups were significantly decreased compared with the Air group in both the sensitized and nonsensitized mice ($p < 0.005$). The PC₁₅₀ value of the DE-3 group (sensitized) was 0.903 ± 0.155 mg ACh/ml, and it was lower than in other groups (PC₁₅₀: 1.848 ± 0.469 and 3.880 ± 0.537 mg ACh/ml). On the other hand, the PC₁₅₀ value of the DE-3 group (nonsensitized) was 2.046 ± 0.373 mg ACh/ml, and it was also lower than in other groups (PC₁₅₀: 4.416 ± 1.109 and 9.375 ± 2.467 mg ACh/ml). These results show DE enhanced airway hyperresponsiveness dose dependently with or without OVA sensitization.

Fig. 1. Airway responsiveness expressed in Rrs to ACh. **a** Percent increase in Rrs in sensitized mice. **b** Provocative concentration of ACh causing a 50% increase in Rrs (PC_{150}) values of sensitized mice. **c** Percent increase in Rrs in nonsensitized mice. **d** PC_{150} of nonsensitized mice. $\circ$ = Air group; $\blacktriangle$ = DE-1 group; $\blacksquare$ = DE-3 group. Results are means SEM of 8 mice per group. * $p < 0.05$, compared to the Air group (Fisher's protected least significant difference test).



DE Exposure Alters Cell Composition in BAL Fluid

Total cell and differential cell counts in BAL fluid of individual mice were measured (fig. 2a, b). The numbers of total cells, macrophages and neutrophils in BAL recovered from DE-exposed groups (DE-1 and DE-3) were higher than those from nonexposed groups (Air) in both the sensitized and nonsensitized mice. The number of eosinophils in BAL fluid from the sensitized mice was increased by DE exposure, but in nonsensitized mice it was not. Although there was no significant difference in the amount of these cells, the number of eosinophils in the DE-3 group (sensitized) was 2.6 times higher than that in the DE-1 group.

Infiltration of Eosinophils and Mast Cells, and Hyperplasia of Goblet Cells in the Lung

The number of eosinophils, mast cells and goblet cells per length of basement membrane of the bronchial epithelium are shown in table 2. DE inhalation caused eosinophilic infiltration, and high-dose DE led to a marked and significant infiltration under bronchial epithelium in sensitized mice. The number of eosinophils in the DE-3 group was 4.6 and 2.3 times higher than those in the Air and the DE-1 groups, respectively. DE exposure alone did not induce an infiltration of eosinophils in murine lungs (data not shown).

The lung specimen was stained with toluidine blue to estimate the infiltration of mast cells in the airways. A marked number of mast cells was observed in the DE-3 group. The

Fig. 2. Cellular profile in BAL fluid (BALF). **a** Sensitized mice. **b** Nonsensitized mice. Results are means $\pm$ SEM of 5 mice per group. * $p < 0.05$, compared to the Air group (Fisher's protected least significant difference test).

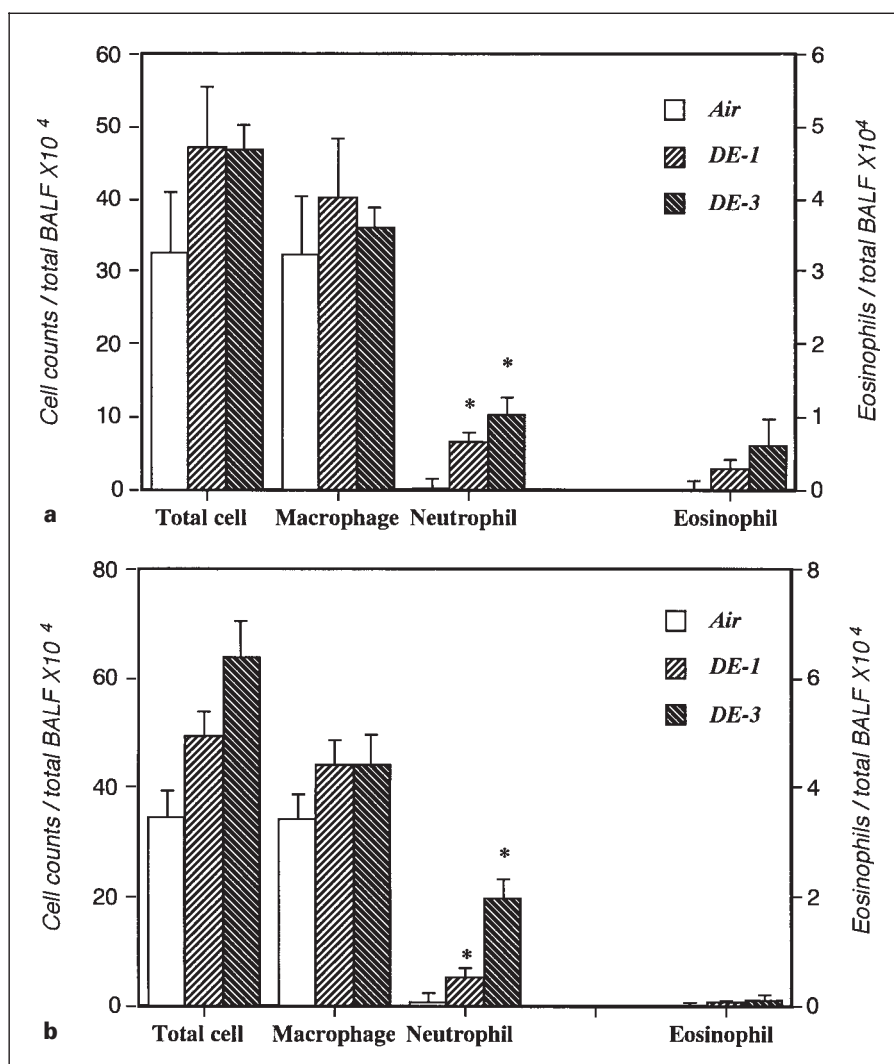


Table 2. Numbers of eosinophils, mast cells and goblet cells in murine lung tissue

Group	Animals n	Eosinophils counts/mm	Mast cells counts/mm	Goblet cells counts/mm
Air	5	0.205 $\pm$ 0.043	0.044 $\pm$ 0.009	9.05 $\pm$ 2.43
DE-1	5	0.411 $\pm$ 0.017	0.096 $\pm$ 0.029	25.66 $\pm$ 4.97
DE-3	5	0.942 $\pm$ 0.176*	0.205 $\pm$ 0.074*	59.75 $\pm$ 12.19*

Results are expressed as means $\pm$ SEM. Significant differences were evaluated by Fisher's protected least significant difference test. * $p < 0.05$, compared to the Air group.

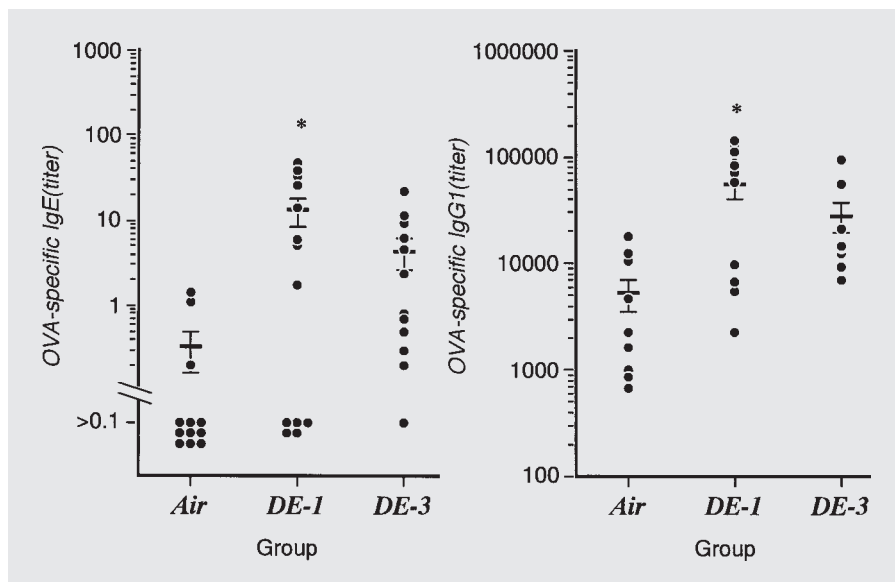
number of mast cells in the DE-3 group was 5.1 and 2.2 times higher than those of the Air and DE-1 groups, respectively.

The lung specimen was stained with periodic acid Schiff to estimate the hypersecretion of mucus in the airways which reflected cell injury. A marked number of goblet cells was observed in the DE-3 group. The number of goblet cells in the DE-3 group was 6.6 and 2.3 times higher than those of the Air and DE-1 groups, respectively. Only a few goblet cells were observed in the nonsensitized groups (data not shown).

OVA-Specific IgE and IgG1 Levels in Serum

The OVA-specific IgE and IgG1 antibodies were measured to examine whether DE affects the antigen-specific immunoglobulin production. Serum OVA-specific antibody

Fig. 3. OVA-specific IgE and IgG1 antibody levels in the serum of sensitized mice. Results are expressed as individual values with means $\pm$ SEM of 13 mice per group. * $p < 0.05$, compared to the Air group (Fisher's protected least significant difference test).



levels were analyzed 1 day after the last OVA nebulization. The titers of OVA-specific IgE and IgG1 in the serum are shown in figure 3.

Although OVA sensitization alone did not induce OVA-specific IgE production, DE exposure with antigen sensitization enhanced IgE production remarkably. The titers of IgE in the DE-1 and DE-3 groups were 41 and 14 times higher than that in the Air group.

On the other hand, OVA-specific IgG1 was elevated by OVA sensitization alone and enhanced by DE exposure. The titers of IgG1 in the DE-1 and DE-3 groups were 10 and 5.5 times higher than that in the Air group ($p < 0.05$), respectively. These results show that DE had an adjuvant effect on the production of IgG1 as well as IgE.

Discussion

DE exposure with OVA sensitization enhanced airway hyperresponsiveness, airway inflammation and hyperplasia of goblet cells in bronchial epithelium, indicating allergic asthma in mice. Although OVA sensitization alone induced the production of OVA-specific IgG1, the intensities of airway inflammation and hyperplasia of goblet cells were weak in this experiment. DE exposure alone also enhanced airway hyperresponsiveness, but did neither induce eosinophilic infiltration nor increase goblet cells.

The concentrations of DEP in the present experiment exceeded 10–30 times that of ambient air in districts polluted by automobile exhaust in Japan. Other gaseous components

were also higher in this study than in ambient air. Therefore, to elucidate the effects of DE inhalation on human allergic disease, we need to investigate the inhalation studies at a lower concentration of DE and allergens.

DE consists of particulates (DEP) and gaseous components. Intratracheal instillation of DEP with OVA enhanced airway responsiveness, infiltration of eosinophils, and hyperplasia of goblet cells in mice [5]. NO_2 exposure alone or combined exposure of NO_2 and SO_2 enhanced airway response to inhaled allergens in asthmatics [11, 12]. It is impossible to divide the effects of DEP and gaseous components in this study. However, the effects of DE exposure with OVA sensitization were similar to that of intratracheal instillation of DEP and OVA [5]. DEP in DE inhalation may act in concert with gaseous components.

Intratracheal instillation of DEP with OVA did not induce IgE production in mice [5]. However, DE inhalation enhanced the OVA-specific IgE production in this experiment. Another DE inhalation experiment also showed that DE enhanced OVA-specific IgE production caused by allergen sensitization in mice [13]. Furthermore, intranasal instillation of DEP with antigen also resulted in increased levels of antigen-specific IgE in mice [14] and humans [15–18]. These results seem to be discrepant. However, DEP and/or allergens may stimulate the nasal membrane by intranasal instillation or inhalation, but not by intratracheal instillation. Therefore, the IgE production may depend on the route of administration of DEP and allergen.

The airway inflammation of asthma is characterized by infiltration of T lymphocytes, eosinophils and mast cells.

Each of these cells is thought to contribute to the histologic changes that characterize asthma [1–3]. The mechanism of allergic asthma is complicated, and that of its enhancement by DE inhalation even more. Clearly, in allergic disease, mast cells and allergen-specific IgE play a central role in hypersensitivity reactions, and the IgE-mediated reactions are followed by chronic inflammation leading to airway hyperresponsiveness. Furthermore, eosinophils may also damage bronchial epithelium by its degranulation when they are stimulated by antibodies or cytokines. To our knowledge, this is the first demonstration that infiltration of both mast cells and eosinophils was enhanced by DE inhalation. DE enhanced both IgE and IgG1 production caused by OVA sensitization. Those inflammatory cells and antibodies might lead to the epithelial damage and airway hyperreactivity.

In this experiment, airway resistance, infiltration of inflammatory cells and hyperplasia of goblet cells were enhanced by DE inhalation dose dependently, in contrast to IgE and IgG1 production. Furthermore, evidence suggests the existence of alternative pathways of hypersensitivity reactions without antibody production. The bronchial hyperreactivity and eosinophilic inflammation caused by expo-

sure to the allergen occur even in mice that are deficient in IgE, mast cells or B cells [19–22]. Aeroallergen-induced eosinophilic inflammation, airway damage, and airway hyperreactivity in mice can occur independently of IL-4 and allergen-specific antibodies [23]. These results suggest that the activation and degranulation of eosinophils may play important roles in airway obstruction and airway hyperresponsiveness.

However, DE inhalation alone enhanced airway hyperreactivity without infiltration of eosinophils and immunoglobulin production. Other experiments also showed that airway hypersensitivity occurred independent of eosinophils [24, 25]. These facts suggest that airway hyperresponsiveness is induced by multiple pathways.

In conclusion, DE exposure enhanced airway hyperresponsiveness, eosinophilic inflammation and hyperplasia of goblet cells caused by OVA sensitization in mice. The present experiment demonstrates that infiltration of both eosinophils and mast cells enhanced by DE exposure are closely implicated in allergic reactions. The results support the hypothesis that automobile exhaust might enhance human allergic airway disease.

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