Diesel Exhaust Particles Enhance Antigen-induced Airway Inflammation and Local Cytokine Expression in Mice

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Previous experimental studies have suggested that nasal instillation of diesel exhaust particles (DEP) can enhance nasal IgE response and cytokine production. However, there is no experimental evidence for the relation of DEP to allergic asthma. We investigated the effects of DEP inoculated intratracheally on antigen-induced airway inflammation, local expression of cytokine proteins, and antigen-specific immunoglobulin production in mice. DEP aggravated ovalbumin-induced airway inflammation characterized by infiltration of eosinophils and lymphocytes and an increase in goblet cells in bronchial epithelium. DEP with antigen markedly increased interleukin-5 (IL-5) protein levels in lung tissue and bronchoalveolar lavage supernatants compared with either antigen or DEP alone. The combination of DEP and antigen induced significant increases in local expression of IL-4, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-2, whereas expression of interferon-gamma was not affected. In addition, DEP exhibited adjuvant activity for the antigen-specific production of IgG and IgE. These results provide the first experimental evidence that DEP can enhance the manifestations of allergic asthma. The enhancement may be mediated mainly by the increased local expression of IL-5, and also by the modulated expression of IL-4, GM-CSF, and IL-2. Takano H, Yoshikawa T, Ichinose T, Miyabara Y, Imaoka K, Sagai M. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice.

AM J RESPIR CRIT CARE MED 1997;156:36–42.

A steady increment of diesel-engine-powered cars has increased the concentration of diesel exhaust particles (DEP) in the atmosphere of urban areas. Because DEP contain carbon nuclei that absorbed a variety of toxic compounds, including polyaromatic hydrocarbons, quinones, and aldehydes (1, 2), estimating the risk of DEP has recently become a major worldwide health issue. DEP remain buoyant in the atmosphere, with a certain fraction being carried into lungs (3). Previous experimental studies have indicated that diesel exhaust, particularly DEP, may cause lung cancer, pulmonary fibrosis, edematous changes, chronic alveolitis, and bronchitis (4–6). However, few studies have investigated the relationship of DEP to bronchial asthma, which has recently shown increasing prevalence in urban areas of the world (7, 8).

Recently, bronchial asthma has been recognized as chronic airway inflammation that is characterized by an increase in the number of activated lymphocytes and eosinophils. A previous report from our laboratory showed that the intratracheal instillation of DEP once a week for 16 wk induced hypersecretion of mucus and airway inflammation associated with eosinophils and lymphocytes (9).

Bronchial asthma is usually divided into allergic (extrinsic) and nonallergic (intrinsic) forms. Epidemiologic investigations and experimental studies on the nose suggest that DEP may be implicated in the increasing severity and prevalence of allergic diseases. The prevalence of allergic rhinitis among schoolchildren in a district polluted by automobile exhaust is reported to be significantly higher than that in nonpolluted districts (10). The intranasal inoculation of DEP along with the allergen enhances the allergen-specific IgE response (11), and the intraperitoneal injection of DEP increases the IgE response to ovalbumin and Japanese cedar pollen (12). However, it remains to be elucidated whether DEP may enhance the manifestations of allergic asthma.

Although IgE may be pivotal in allergic asthma, some observations have suggested the existence of alternative or additional pathways of hypersensitivity reactions, including IgG, recruitment of lymphocytes and eosinophils, and local cytokine expression. Late asthmatic reactions are associated with IgG antibody rather than IgE antibody (13, 14). Recent studies have shown a strong correlation between the level of activated $CD4^+$ T-cells and disease severity (15). The relative proportions of cells expressing T helper (Th)2 cytokines in bronchoalveolar lavage (BAL) fluid or biopsy specimens are increased in patients with symptomatic versus those with asymptomatic asthma (16) and in patients with allergic asthma

(*Received in original form October 16, 1996 and in revised form March 11, 1997*)

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after segmental allergen challenge (17, 18). The concentration of granulocyte macrophage-colony stimulating factor (GM-CSF) in BAL supernatants is correlated with the percentage of BAL eosinophils and severity of the late asthmatic response (19).

We conducted the present study to evaluate the effects of DEP on the manifestations of allergic asthma, with emphasis on antigen-induced airway inflammation, the local expression of interleukin-5 (IL-5), IL-4, GM-CSF, IL-2, and interferon (IFN)-gamma and the production of antigen-specific IgE and IgG.

METHODS

Collection of DEP

A 4JB1-type, light-duty (2,740 ml), four-cylinder diesel engine (Isuzu Automobile Co., Tokyo, Japan) was connected to an EDYC dynamometer (Meiden-sya, Tokyo, Japan). The engine was operated using standard diesel fuel at a speed of 1,500 rpm under a load of 10 torque (kg/m). DEP were collected as previously described (20). The mean diameter of the particles was 0.4 μm. Most of the particles were globular in shape.

Animals

Male ICR mice 6 to 7 wk of age and weighing 29 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea Co.) and given water ad libitum. Mice were housed in an animal facility that was maintained at 24 to 26° C with 55 to 75% humidity and a 14-h/10-h light/dark cycle. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by the Institutional Review Board.

Study Protocol

Mice were divided into four groups (Figure 1). The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) once a week for 6 wk. The ovalbumin (OVA) group received 1 μg of OVA (Sigma Chemical, St. Louis, MO) dissolved in the same vehicle every 3 wk for 6 wk. The DEP group received 100 μg of suspended DEP in the same vehicle every week for 6 wk. The suspension was sonicated for 3 min using an Ultrasonic disrupter (UD-201; Tomy Seiko, Tokyo, Japan). The OVA+DEP group received the combined treatment in the same protocol as the OVA and the DEP groups, respectively. In each group, vehicle, OVA, DEP, or OVA+DEP was dissolved in 0.1-ml aliquots, and inoculated by the intratracheal route through a polyethylene tube under anesthesia with 4% halothane (Hoechst, Japan, Tokyo, Japan). The animals were studied 24 h after the last intratracheal administration, with lung histology, bronchoalveolar lavage (BAL), protein levels of cytokines in BAL and lung tissue supernatants, and with OVA-specific immunoglobulins.

In a separate series of animals, treatment with vehicle, OVA, DEP, or OVA+DEP was conducted at the same intervals as described above for 9 wk. OVA-specific immunoglobulins were studied 24 h after the last intratracheal administration.

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 assayed for OVA-specific IgE, IgG_1, and IgG_{2a}.

Histologic Evaluation of Eosinophils, Lymphocytes, Neutrophils, and Goblet Cells in Lung

After exsanguination, the lungs were fixed by intratracheal instillation with 10% neutral phosphate-buffered formalin at a pressure of 20 cm H_2O for at least 72 h. Slices 2 to 3 mm thick of all pulmonary lobes were embedded in paraffin. Sections 3 μm thick were stained with Diff-Quik (International Reagents Co., Kobe, Japan) or periodic acid-Schiff (PAS) and examined by two of us (HT, TI) in a blind fashion.

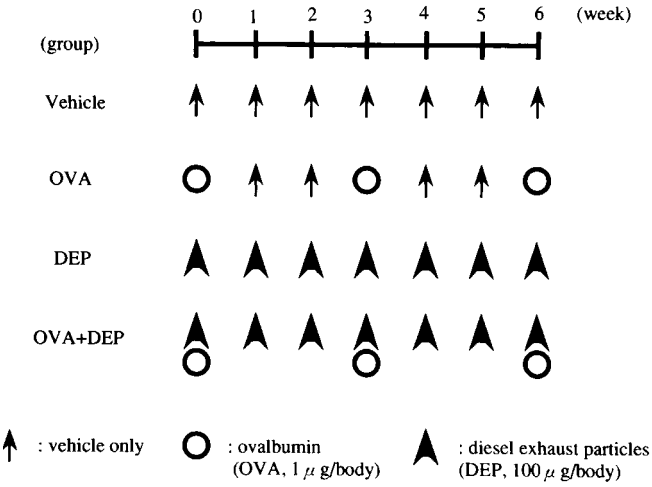

Figure 1. Experimental design for intratracheal instillation.

Measurement of Eosinophils, Lymphocytes, Neutrophils, and Goblet Cells

Sections were stained with Diff-Quik to quantitate the numbers of infiltrated eosinophils, neutrophils, and lymphocytes. The length of the basement membrane of the airways was measured by videomicrometer (Olympus, Tokyo, Japan) in each sample slide. The number of eosinophils, neutrophils, and lymphocytes around the airways were counted with a micrometer under oil immersion. Results were expressed as the number of inflammatory cells per millimeter of basement membrane.

To quantitate goblet cells, sections were stained with PAS. The number of goblet cells in the bronchial epithelium was counted by micrometer. Results were expressed as the number of goblet cells per millimeter of basement membrane.

BAL

The trachea was cannulated after the collection of blood. The lungs were lavaged with 1.2 ml of sterile saline at 37° C, instilled bilaterally by syringe. The lavaged fluid was harvested by gentle aspiration. This procedure was conducted two more times. Average volume retrieved was 90% of the 3.6 ml that was instilled; the amounts did not differ among treatments. 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 count was 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) and stained with Diff-Quik. A total of 300 cells were counted under oil immersion microscopy. Aliquots of the supernatants were stored without further treatment at −80° C until analyzed for cytokines.

Quantitation of Cytokine Protein Levels in BAL Supernatants

Enzyme-linked immunosorbent assays (ELISA) for IL-5, GM-CSF, and IL-2 were conducted using matching antibody pairs (Endogen, Cambridge, MA). The following antibody pairs were used for detection of IL-5, GM-CSF, and IL-2, respectively: TRFK5 and TRFK4; MP1-22E9 and MP1-31G6; S4B6 and 5H 4.1.1. The ELISA for IL-4 and IFN-gamma were conducted using matching antibody pairs (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to picograms per milliliter using values obtained from standard curves generated with varying concentrations of recombinant IL-5, GM-CSF, IL-2, IL-4, and IFN-gamma. The limits of detection in these assays were less than 5 pg/ml, 5 pg/ml, 3 pg/ml, 5 pg/ml, and 15 pg/ml, respectively.

Quantitation of Cytokine Protein Levels in Lung Tissue Supernatants

The lungs were removed after retrieval of blood, snap-frozen in liquid nitrogen, and stored at −80° C. Later, the lungs were homogenized with 10 mM potassium phosphate buffer (pH, 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma), 0.1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque), 1 μM pepstatin (Peptide Institute, Osaka, Japan) and 2 μM leupeptin (Peptide Institute). The homogenates were then centrifuged at 105,000 *g* for 1 h. The supernatants were stored at −80° C. In the manner described above for BAL supernatants, tissue supernatant was assayed for the protein levels of IL-5, GM-CSF, IL-2, IL-4, and IFN-gamma.

OVA-specific IgE Determination

OVA-specific IgE antibody was measured by IgE-capture ELISA (21). In brief, microplate wells (Dynatech, Chantilly, VA) were coated with an antimouse IgE rat monoclonal antibody (Yamasa Syoyu Co., Chiba, Japan) at 37° C for 3 h and then incubated at 37° C for 1 h with PBS containing 1% bovine serum albumin (BSA; Sigma) and 0.01% thimerosal (Nacalai Tesque). After washing with PBS containing 0.05% Tween 20 (PBST; Nacalai Tesque), diluted samples were introduced to the microplate 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, the wells were incubated for 1 h at room temperature with β-D-galactosidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA). After the final washing, the wells were incubated with 4-methylumbelliferyl-β-galactoside (Sigma) as the enzyme substrate at 37° C for 2 h. The enzyme reaction was stopped with 0.1 M glycine-NaOH (pH, 10.3). The fluorescence intensity was read by a microplate fluorescence reader (Fluoroskan Flow Laboratories, Costa Mesa, CA). Each plate included a previously screened standard plasma that contained a high titer of anti-OVA antibodies. The results were expressed in titers, calculated based on the titers of the standard plasma. Cutoff values for antibody-positive plasma were set two hold as mean fluorescence units of preimmune plasma.

OVA-specific IgG_1 and IgG_{2a} Determination

OVA-specific IgG_1 and IgG_{2a} antibodies were measured by ELISA with solid-phase antigen. In brief, microplate wells were coated with OVA overnight at 4° C and then incubated at room temperature for 1 h with 1% BSA-PBS containing 0.01% thimerosal. After washing, diluted samples were introduced to the microplate and incubated at room temperature for 1 h. After another washing, the wells were incubated at room temperature for 1 h with biotinylated rabbit antimouse IgG_1 or IgG_{2a} (Zymed). After yet another washing, the wells were incubated with horseradish-peroxidase-conjugated streptavidin (Sigma) at room temperature for 1 h. The wells were then washed and incubated with *o*-phenylenediamine and H_2O_2 in dark at room temperature for 30 min. The enzyme reaction was stopped with 4 N H_2SO_4. Absorbance was read at 492 nm. Each plate included a previously screened standard plasma that contained a high titer of anti-OVA antibodies. The results were expressed in titers, calculated based on the titers of the standard plasma. Cutoff values for antibody-positive plasma were set to hold as the mean value of absorbance of preimmune plasma.

Statistical Analysis

Data are reported as mean ± SE. Differences in the numbers of infiltrated inflammatory cells and goblet cells, cytokine protein levels, and immunoglobulin titers between groups were determined using analysis of variance (Statview; Abacus Concepts, Inc., Berkeley, CA). If differences between groups were significant ($p < 0.05$), Fisher's protected least significant difference test or Scheffe's F test was used to distinguish between pairs of groups.

RESULTS

DEP Accelerate Antigen-induced Airway Inflammation

To evaluate the effect of DEP on antigen-induced airway inflammation, we investigated the cellular profile of BAL fluid and lung histology in four groups of mice.

As shown in Table 1, the total number of cells and the number of macrophages in BAL fluid were significantly greater in the OVA ($p < 0.05$) and OVA+DEP group ($p < 0.001$ for total cells, $p < 0.05$ for macrophages) than in the vehicle group. A 20-fold or greater increase in the number of BAL eosinophils was observed in the OVA+DEP group compared with the OVA group or the DEP group ($p < 0.001$). The vehicle group showed no eosinophils in BAL fluid. Combined OVA+DEP instillation caused a 10-fold increase in BAL neutrophils ($p < 0.0001$) and a 4-fold increase in BAL lymphocytes ($p < 0.05$) compared with OVA instillation. The OVA+DEP group demonstrated a 3-fold elevation in BAL neutrophils ($p < 0.01$) and a 10-fold elevation in BAL lymphocytes ($p < 0.01$) compared with the DEP group. In the vehicle group, BAL fluid contained few neutrophils ($p < 0.0001$ versus the OVA+DEP group) and no lymphocytes ($p < 0.01$ versus the OVA+DEP group).

The magnitude and cellular profiles of airway inflammation were also evaluated in lung specimens stained with Diff-Quik. Intratracheal instillation of DEP provided diffuse deposition of the particles into the bilateral lungs, including the bronchi and alveolar spaces. The particles were occasionally present within the subepithelial neutrophils and the alveolar

TABLE 1

CELLULAR PROFILE IN BRONCHOALVEOLAR LAVAGE FLUID*

Group	Animals (*n*)	Total Cells ($\times 10^4$/*total BAL*)	Macrophages ($\times 10^4$/*total BAL*)	Eosinophils ($\times 10^4$/*total BAL*)	Neutrophils ($\times 10^4$/*total BAL*)	Lymphocytes ($\times 10^4$/*total BAL*)
Vehicle	12	31.6 ± 3.22	31.2 ± 3.13	0	0.391 ± 0.169	0
OVA	13	49.4 ± 4.16†	48.7 ± 4.25†	0.167 ± 0.116	0.385 ± 0.189	0.157 ± 0.114
DEP	13	43.9 ± 4.21	42.4 ± 3.88	0.185 ± 0.148	1.297 ± 0.417	0.057 ± 0.032
OVA+DEP	13	57.2 ± 3.85§	48.3 ± 3.36†	4.23 ± 1.21§**§§	4.04 ± 0.787‖††‡‡	0.598 ± 0.163‡¶‡‡

* Four groups of mice were intratracheally administered vehicle, ovalbumin (OVA), diesel exhaust particles (DEP), or a combination of OVA and DEP for 6 wk. Bronchoalveolar lavage (BAL) was conducted 24 h after the last intratracheal instillation. Total cell counts were determined on fresh BAL fluid, and differential cell counts were assessed with Diff-Quik-staining. Results are presented as mean ± SE.

† $p < 0.05$ versus vehicle.
‡ $p < 0.01$ versus vehicle.
§ $p < 0.001$ versus vehicle.
‖ $p < 0.0001$ versus vehicle.
¶ $p < 0.05$ versus OVA.
** $p < 0.001$ versus OVA.
†† $p < 0.0001$ versus OVA.
‡‡ $p < 0.01$ versus DEP.
§§ $p < 0.001$ versus DEP.

TABLE 2

NUMBERS OF INFLAMMATORY CELLS AND GOBLET CELLS IN LUNG TISSUE*

Group	Animals (*n*)	Eosinophils (*n/mm*)	Neutrophils (*n/mm*)	Lymphocytes (*n/mm*)	Goblet Cells (*n/mm*)
Vehicle	7	0.016 ± 0.010	0.038 ± 0.010	0.060 ± 0.013	0.309 ± 0.117
OVA	9	0.744 ± 0.569	0.348 ± 0.172	1.30 ± 0.529	0.957 ± 0.377
DEP	9	0.150 ± 0.087	0.428 ± 0.117	1.32 ± 0.209	3.92 ± 1.10
OVA+DEP	9	5.24 ± 1.74†§**	1.85 ± 0.706†	8.11 ± 1.76‡‖††	13.0 ± 2.23‡¶†††

* Animals received intratracheal instillation of vehicle, OVA, DEP, or OVA+DEP for 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Sections were stained with Diff-Quik for measurement of inflammatory cells around the airways or with PAS for goblet cells in the bronchial epithelium. Results are expressed as number of cells per length of basement membrane of airways. Values are mean ± SE.

† $p < 0.05$ versus vehicle.
‡ $p < 0.0001$ versus vehicle.
§ $p < 0.05$ versus OVA.
‖ $p < 0.001$ versus OVA.
¶ $p < 0.0001$ versus OVA.
** $p < 0.01$ versus DEP.
†† $p < 0.001$ versus DEP.

macrophages. The combined instillation of OVA+DEP for 6 wk led to a marked infiltration of eosinophils and lymphocytes around the bronchi and bronchioles. Either OVA or DEP alone resulted in slight recruitment of these cells, and vehicle administration caused little infiltration of inflammatory cells.

To quantitate the infiltration around the airways, we expressed the number of inflammatory cells per length of basement membrane of the airways (Table 2). The combined instillation of OVA+DEP produced a 7-fold increase in the number of infiltrated eosinophils compared with OVA exposure ($p < 0.05$), and a 35-fold increase compared with DEP exposure ($p < 0.01$). Although the combined OVA+DEP instillation induced a 4- to 5-fold elevation of infiltrated neutrophils as compared with either OVA or DEP given alone, these effects did not achieve statistical significance. A 6-fold increment in infiltrated lymphocytes was observed in the OVA+DEP group compared with the OVA ($p < 0.001$) and DEP ($p < 0.001$) groups.

DEP Increase Goblet Cells in Airways after Antigen Challenge

To evaluate airway epithelial injury and hypersecretion of mucus, lung sections were stained with PAS. As shown in Table 2, the OVA+DEP group revealed a 13-fold increase in the number of goblet cells compared with the OVA group ($p < 0.0001$) and a 3-fold increase compared with the DEP group ($p < 0.001$). Vehicle instillation resulted in few goblet cells.

DEP Increase the Local Expression of Th2 Cytokines in the Presence of Antigen

To explore the role of local expression of Th2 cytokines in the enhancing effects of DEP on antigen-induced airway inflammation, we quantitated protein levels of IL-5 and IL-4 in lung tissue and BAL supernatants (Table 3). The OVA+DEP group showed a 7- to 8-fold increase in IL-5 in lung tissue supernatants compared with other groups ($p < 0.01$ versus each other group). Instillation of OVA alone or with DEP resulted in significant elevation of the IL-4 concentration in lung tissue supernatants compared with vehicle or DEP treatment ($p < 0.01$ for OVA, $p < 0.05$ for OVA+DEP). No significant difference in the level of IL-4 in lung tissue was found between OVA and OVA+DEP treatment.

In BAL supernatants, IL-5 was significantly increased in the OVA+DEP group compared with the vehicle ($p < 0.001$) and DEP ($p < 0.01$) groups. There were no significant differences in IL-4 in BAL supernatants between the four treatment groups.

DEP Modulate Local Expression of GM-CSF and IL-2

To investigate the local expression of GM-CSF, IL-2 and IFN-gamma, we measured protein levels of these cytokines in lung

TABLE 3

PROTEIN LEVELS OF TH2 CYTOKINES IN LUNG TISSUE AND BAL SUPERNATANTS*

		Lung Tissue Supernatants		BAL Supernatants	
Group	Animals (*n*)	IL-5 (*pg/total lung tissue supernatants*)	IL-4 (*pg/total lung tissue supernatants*)	IL-5 (*pg/total BAL supernatants*)	IL-4 (*pg/total BAL supernatants*)
Vehicle	12	11.3 ± 5.46	123.1 ± 20.5	21.2 ± 7.62	79.4 ± 17.2
OVA	12	12.4 ± 4.52	391.3 ± 88.0‡**	48.7 ± 15.9	76.7 ± 18.0
DEP	12	11.1 ± 3.71	112.1 ± 15.3	23.8 ± 5.92	95.3 ± 17.2
OVA+DEP	12	90.7 ± 33.8‡‖**	308.5 ± 63.5†¶	86.4 ± 18.8§**	78.9 ± 14.9

* Four groups of mice were intratracheally inoculated with vehicle, OVA, DEP, or the combination of OVA and DEP for 6 wk. BAL was conducted 24 h after the last intratracheal instillation. In other animals, lungs were removed and frozen 24 h after the last intratracheal administration. Protein levels in BAL and lung tissue supernatants were analyzed using ELISA. Results are shown as mean ± SE.

† $p < 0.05$ versus vehicle.
‡ $p < 0.01$ versus vehicle.
§ $p < 0.001$ versus vehicle.
‖ $p < 0.01$ versus OVA.
¶ $p < 0.05$ versus DEP.
** $p < 0.01$ versus DEP.

TABLE 4

PROTEIN LEVELS OF GM-CSF, IL-2 AND IFN-GAMMA IN LUNG TISSUE AND BAL SUPERNATANTS*

		Lung Tissue Supernatants			BAL Supernatants		
Group	Animals (*n*)	GM-CSF (*pg/total lung tissue supernatants*)	IL-2 (*pg/total lung tissue supernatants*)	IFN-gamma (*pg/total lung tissue supernatants*)	GM-CSF (*pg/total BAL supernatants*)	IL-2 (*pg/total BAL supernatants*)	IFN-gamma (*pg/total BAL supernatants*)
Vehicle	12	6.13 ± 1.30	7.83 ± 2.90	583 ± 123	8.27 ± 3.67	ND	207 ± 27.6
OVA	12	7.39 ± 0.78	15.3 ± 5.16	683 ± 130	14.5 ± 6.26	ND	235 ± 78.6
DEP	12	10.1 ± 2.60	11.4 ± 5.26	619 ± 142	18.7 ± 5.00	ND	239 ± 65.3
OVA+DEP	12	13.7 ± 1.48†	48.1 ± 10.7†§‖	547 ± 142	21.1 ± 10.9	ND	193 ± 25.0

* Four groups of mice received intratracheal administration of vehicle, OVA, DEP, or OVA+DEP for 6 wk. BAL was conducted 24 h after the last intratracheal challenge. In separate animals, lungs were removed and frozen 24 h after the last intratracheal instillation. Protein levels in BAL and lung tissue supernatants were analyzed using ELISA. Results are shown as mean ± SE.

ND: not detected.

† $p < 0.01$ versus vehicle.

‡ $p < 0.001$ versus vehicle.

§ $p < 0.01$ versus OVA.

‖ $p < 0.001$ versus DEP.

tissue and BAL supernatants (Table 4). The combination of OVA+DEP caused significant elevation of GM-CSF in lung tissue supernatants compared with vehicle treatment ($p < 0.01$). The levels of IL-2 in lung tissue supernatants were significantly increased in the OVA+DEP group compared with the vehicle ($p < 0.001$), OVA ($p < 0.01$), and DEP ($p < 0.001$) groups. The levels of IFN-gamma in lung tissue supernatants were not significantly different between the experimental groups.

Results for GM-CSF in BAL supernatants were analogous to those for lung tissue supernatants with respect to overall trends, but the differences among groups were not statistically significant. The IL-2 concentration in BAL supernatants was lower than the detection limit in all groups. No significant difference in the level of IFN-gamma in BAL supernatants was found among the experimental groups.

DEP Have Adjuvant Activity for Antigen-specific Production of IgG and IgE

To examine whether DEP have adjuvant activity on antigen-specific immunoglobulin production, we measured OVA-specific IgE, IgG_1, and IgG_{2a}. The OVA-specific IgG_1 was significantly elevated in the OVA+DEP group compared with the other groups ($p < 0.01$ versus each other group) 6 wk after the first intratracheal administration (Table 5). The combination of OVA plus DEP significantly increased OVA-specific production of IgE ($p < 0.05$), IgG_1 ($p < 0.05$), and IgG_{2a} ($p < 0.01$) as compared with OVA alone 9 wk after the first instillation (Table 6).

TABLE 5

OVA-SPECIFIC IMMUNOGLOBULIN TITERS*

Group	Animals (*n*)	IgG_{2a} (*antibody titers*)	IgG_1 (*antibody titers*)	IgE (*antibody titers*)
Vehicle	36	100.4 ± 0.42	111.3 ± 6.0	16.0 ± 0
OVA	36	161.3 ± 61.3	175.7 ± 24.7	16.3 ± 0.17
DEP	36	100.0 ± 0	131.7 ± 20.7	16.0 ± 0
OVA+DEP	48	100.0 ± 0	1,483 ± 454†‡§	16.0 ± 0

* Four groups of mice were intratracheally administered vehicle, OVA, DEP, or the combination of OVA and DEP for 6 wk. Plasma samples were retrieved 24 h after the last intratracheal instillation. OVA-specific immunoglobulins were analyzed using ELISA. Results are expressed as mean ± SE.

† $p < 0.01$ versus vehicle.

‡ $p < 0.01$ versus OVA.

§ $p < 0.01$ versus DEP.

DISCUSSION

The present study demonstrated that DEP administered by the intratracheal route enhanced the airway inflammation associated with antigen challenge in mice. The inflammatory component was characterized by increased numbers of eosinophils and lymphocytes. Recruitment of these cells was accompanied by an increment in goblet cells in the bronchial epithelium. The airway inflammation induced by the combined administration of DEP and antigen were coincident with the increased protein levels of IL-5 in lung tissue and BAL supernatants. Also, DEP with antigen modulated the local expression of IL-4, GM-CSF, and IL-2. Furthermore, DEP enhanced antigen-specific production of IgG and IgE.

Recently, bronchial asthma has been recognized as an inflammatory disorder of airways accompanied by airway hyperresponsiveness. In our study, lung histology and profiles of BAL cells demonstrated that the combined administration of DEP and antigen resulted in marked pulmonary inflammation characterized by lymphocytes and eosinophils around the airways as compared with the administration of antigen alone. An increase in respiratory resistance in animals that inhaled acetylcholine was significantly greater in the OVA+DEP group than in the other groups (data not shown). To our knowledge, this is the first experimental demonstration of DEP's enhancing effects on allergic asthma.

TABLE 6

OVA-SPECIFIC IMMUNOGLOBULIN TITERS*

Group	Animals (*n*)	IgG_{2a} (*antibody titers*)	IgG_1 (*antibody titers*)	IgE (*antibody titers*)
Vehicle	10	100 ± 0	100 ± 0	16.0 ± 0
OVA	10	293.8 ± 158.4	30,300 ± 13,200†**	23.1 ± 7.09
DEP	10	100.0 ± 0	105 ± 5.0	16.0 ± 0
OVA+DEP	10	1,035 ± 269§¶‡‡	57,200 ± 12,700§‖‡‡	41.6 ± 7.80‡‖††

* Four groups of mice were intratracheally administered vehicle, OVA, DEP, or the combination of OVA and DEP for 9 wk. Plasma samples were retrieved 24 h after the last intratracheal instillation. OVA-specific immunoglobulins were analyzed using ELISA. Results are expressed as mean ± SE.

† $p < 0.05$ versus vehicle.

‡ $p < 0.01$ versus vehicle.

§ $p < 0.0001$ versus vehicle.

‖ $p < 0.05$ versus OVA.

¶ $p < 0.01$ versus OVA.

** $p < 0.05$ versus DEP.

†† $p < 0.01$ versus DEP.

‡‡ $p < 0.0001$ versus DEP.

Lymphocytes and eosinophils are important in airway inflammation. Recent clinical (15) and experimental (22) reports have described the importance of $CD4^+$ T-lymphocytes in allergic asthma. Eosinophils contribute to airway epithelial injury by releasing mediators, including platelet-activating factor, oxygen radicals, leukotrienes, and major basic protein (23). In the present study, the increase in the numbers of infiltrated lymphocytes and eosinophils by the combined administration of DEP and antigen was coincident with the appearance of goblet cells in the bronchial epithelium. An increase in goblet cells can induce mucus hypersecretion and aggravate bronchial obstruction.

Allergic asthma is often associated with activation of IL-4, IL-5, and the GM-CSF gene cluster, a pattern compatible with predominant activation of the Th2-like T-lymphocyte population. Significant associations have been observed between the numbers of cells expressing mRNA for IL-4, IL-5, and GM-CSF, and airflow obstruction, and the asthma score (16). A recent report concerning IL-5-deficient mice supports the concept that IL-5 and eosinophils are central mediators in the pathogenesis of allergic lung diseases (24). Another study with IL-4-deficient mice implied a significant role for IL-4 in antigen-induced eosinophilic airway inflammation (25). Gavett and coworkers (26) have recently provided the first direct evidence that antigen-induced airway inflammation is associated with increases in the protein levels of Th2-type cytokines in murine BAL supernatants. These Th2 cytokines are implicated in the pathogenesis of allergic reactions via their roles in mediating IgE and IgG_1 production, and in differentiation, vascular adhesion, recruitment, activation, and survival of eosinophils. In our study, airway inflammation induced by the combined administration of DEP and antigen were concomitant with the increased protein levels of Th2 cytokines, especially IL-5, which were confirmed in BAL and lung tissue supernatants. These results provide the first evidence that DEP can accelerate antigen-induced IL-5 expression and subsequent eosinophilic inflammation *in vivo*.

Although our profile of GM-CSF in BAL and lung tissue supernatants was analogous to that of IL-5 in overall trend, greater concentrations and larger distinctions among experimental groups were obtained with IL-5 than with GM-CSF. These data imply that IL-5 rather than GM-CSF is a pivotal mediator in the airway inflammation induced by the simultaneous administration of DEP and antigen. It is also notable that the concentration of IL-2, which has been reported to increase OVA-specific IgG and airway inflammation (27), was elevated in lung tissue in the OVA+DEP group. It has been reported that IFN-gamma inhibits the proliferation of Th2 cells *in vitro* (28) and the recruitment of eosinophils and T-lymphocytes *in vivo* (29). In our study, the protein levels of IFN-gamma in lung tissue and BAL supernatants were not significantly different between the experimental groups.

The previous studies on intranasal administration have reported that DEP with antigen demonstrate adjuvant activity for IgE production in mice by passive cutaneous anaphylaxis assay (11) and that those without antigen induce nasal nonspecific IgE response in humans (30, 31). In the present study, the combined intratracheal administration of DEP and antigen induced a significant increase in antigen-specific IgG_1, but not in antigen-specific IgE, 6 wk after the first inoculation. However, the combined administration significantly increased antigen-specific IgE and IgG_1 9 wk after the first inoculation as compared with antigen alone. In this study, it is notable that the combined administration of DEP and antigen established the fundamental traits of asthma, including eosinophilic airway inflammation, airway hyperresponsiveness, and mucus hypersecretion 6 wk after the first inoculation, when antigen-specific IgG_1, but not IgE, was elevated. Although IgE is important in many allergic reactions, antigen-specific IgG antibodies, but not IgE antibodies, in patients' sera contribute to antigen-specific eosinophil degranulation via Fcγ RII on the eosinophil surface (32). IgG_1 with antigen is a strong agonist for eosinophil degranulation *in vitro* (32). Late asthmatic reactions are associated with IgG antibody rather than IgE antibody (13, 14). Mast cells can be sensitized and activated by IgG (33). Significantly elevated levels of IgE and IgG antibodies to inhaled allergens have been detected in children with asthma (34). Immediate cutaneous hypersensitivity and airway hyperresponsiveness are passively transferred by allergen-specific IgE and IgG_1 from B-cell hybridomas in mice (35). Judging from these reports and the present results, antigen-specific IgG_1 appears to be an important immunoglobulin in our murine model as well as IgE. It is likely that DEP with antigen enhance the recruitment of lymphocytes, their cytokine expression, and the subsequent immunoglobulin production and eosinophil activation, inducing airway inflammation with epithelial damage by degranulation of eosinophils.

In conclusion, the present study has shown that DEP enhance the manifestations of allergic airway inflammation. This effect may be mediated mainly by the enhance local expression of IL-5, and also by the modulated expression of IL-4, GM-CSF, and IL-2. Furthermore, DEP enhance antigen-specific production of IgG and IgE. These results suggest that DEP are implicated in the increasing prevalence of allergic asthma in recent years. The effects of the daily inhalation of DEP at lower concentrations and antigen, which occurs in the real world, should be elucidated in future.

***Acknowledgment*:** The writers thank Keiko Miura, Rie Yanagisawa, Setsuko Nagumo, and Yoshiko Sawada in their laboratory for their technical assistance.

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