

Mouse Strain Differences in Eosinophilic Airway Inflammation Caused by Intratracheal Instillation of Mite Allergen and Diesel Exhaust Particles

Takamichi Ichinose,¹ Hirohisa Takano,² Kaori Sadakane,¹ Rie Yanagisawa,² Toshikazu Yoshikawa,³ Masaru Sagai⁴ and Takayuki Shibamoto^{5,*}

¹ Department of Health Sciences, Oita University of Nursing and Health Sciences, 2944-9 Notsuharu, Oita 870-1201, Japan

² Research Team for Health Effects of Air Pollutants, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-0053, Japan

³ First Department of Internal Medicine, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602, Japan

⁴ Faculty of Health Sciences, Division of Nursing/Human Sciences, Aomori University of Health and Welfare, 58-1 Aza-Mase Oaza-Hamadate, Aomori, Japan

⁵ Department of Environmental Toxicology, University of California, Davis, CA 95616, USA

Key words: eosinophilic airway inflammation; cytokines; diesel exhaust particles; intratracheal instillation; house-dust mite; mouse strain.

Response differences by different strains of mice towards house dust mites (*Dermatophagoides farinae*) or diesel exhaust particles (DEP) were investigated. Mouse strains BALB/c, ICR and C3H/He received 1 µg of *D. farinae* or 1 µg of *D. farinae* + 50 µg of DEP intratracheally four times at 2-week intervals. *Dermatophagoides farinae* treatment caused the recruitment of eosinophils and lymphocytes. The order of magnitude of the eosinophilic airway inflammation was BALB/c < ICR < C3H/He mice. The protein levels of eotaxin and IL-5 in lung tissues correlated with the manifestations of eosinophilic airway inflammation by *D. farinae* administration. Diesel exhaust particles aggravated the manifestation of the eosinophilic inflammation through goblet cell proliferation in the airway and enhanced the local expression of eotaxin and IL-5 in all three strains of mice. The levels of eotaxin and IL-5 in lung tissues corresponded to the pathological changes caused by *D. farinae* + DEP. The increasing order of production levels of antigen-specific IgG1 by *D. farinae* or *D. farinae* + DEP was BALB/c < ICR < C3H/He mice. The significant adjuvant effect of DEP on IgG1 production was observed in the C3H/He mice ($P < 0.05$). These results suggest that the murine strain differences in the production of eosinophilic airway inflammation by *D. farinae* + DEP are related to differences in local expression of IL-5 and eotaxin. The enhancing effects of DEP may be mediated by a cytokine increase in the local expression. Antigen-specific IgG1 may be an important immunoglobulin in the pathogenesis of allergic asthma enhanced by DEP. Copyright © 2004 John Wiley & Sons, Ltd.

INTRODUCTION

Allergic asthma is a chronic disease with airway obstruction. It is clinically characterized by eosinophilic bronchitis and airway hyperresponsiveness. The number of school-children with allergic asthma has been increasing steadily in the world (Bjorksten *et al.*, 1998; Japan Ministry of Education, Science and Culture, 2000; Shamssain and Shamssian, 2001). The increase of indoor allergens such as dust mites or mould in Japan seems to be due to changes in the Japanese lifestyle. House-dust mites and mold are increasing in airtight buildings with high humidity (Custovic *et al.*, 1996; Etzel and Rylander, 1999), the conditions that tend to cause allergic asthma and atopic dermatitis (Custovic *et al.*, 1996; Etzel and Rylander, 1999; Scalabrin

et al., 1999; Huss *et al.*, 2001). In particular, house-dust mites are known as the predominant cause of the current increase in asthma (Huss *et al.*, 2001).

Airway allergic diseases have also been increasing, especially in urban areas polluted by automobile exhaust (Kaneko *et al.*, 1980; Nishima, 1993). Epidemiological studies show that ambient particulate matter (PM10, PM2.5) is associated with an increase of asthma. The development of chronic respiratory diseases increases cardiopulmonary mortality and childhood asthma (Pope *et al.*, 1991, 1995; Schwartz *et al.*, 1993; Romieu *et al.*, 1996). Results of several experimental studies suggest that automobile exhaust, such as diesel exhaust or diesel exhaust particles (DEP), enhances the onset of allergic airway diseases. These diseases include allergic rhinitis and induced bronchial asthma (Diaz-Sanchez *et al.*, 1996, 2000a,b; Ichinose *et al.*, 1997, 1998; Takano *et al.*, 1997, 1998; Miyabara *et al.*, 1998a,b).

In a model study involving murine asthma by ovalbumin (OVA) and DEP, DEP enhanced hyperresponsiveness and

* Correspondence to: T. Shibamoto, Department of Environmental Toxicology, University of California, Davis, CA 95616, USA.
E-mail: tshibamoto@ucdavis.edu

the manifestations of eosinophilic inflammation in the airway as well as OVA-specific IgG1 production (Miyabara *et al.*, 1998b; Takano *et al.*, 1998). This inflammation was not induced by DEP alone (Miyabara *et al.*, 1998b; Takano *et al.*, 1998). Thus, the effects of DEP on allergies were clarified in an asthma model used for OVA. However, the experiment using OVA is not realistic. A model study in murine asthma used for house-dust mites as the predominant cause of asthma is needed. When CBA/JN and C57BL/6N mice were treated intratracheally with DEP and *Dermatophagoides farinae*, which is a major allergen of house-dust mites, DEP enhanced the *D. farinae*-induced eosinophilic airway inflammation in the CBA/JN mice. However, the exact effect of DEP towards eosinophilic inflammation could not be estimated in C57BL/6 mice because of the severe inflammation enhanced by DEP (Sadakane *et al.*, 2002). Therefore, a mouse is needed in which the exact effect of DEP towards eosinophilic airway inflammation can be estimated. The airway of C3H/He mice was susceptible to allergic reaction induced by OVA, whereas the airway of BALB/c mice was less susceptible (Miyabara *et al.*, 1998b). The C3H/He mouse showed the highest IgG1 responsiveness to OVA, the ICR mice were less responsive (Ichinose *et al.*, 1997) and the BALB/c mice showed the least response.

In the present study, differences in the development of eosinophilic airway inflammation induced by mite allergen and DEP in BALB/c, ICR and C3H/He mouse strains were investigated in order to extend our previous findings.

EXPERIMENTAL

Animals

The BALB/c mice (5 weeks of age), ICR mice (5 weeks of age) and C3H/He mice (5 weeks of age) were purchased from Charles River (Kanagawa, Japan). After body weight and infection were checked for 1 week, the mice were used at 6 weeks of age in this experiment. Commercial animal food (CE-2, CLEA Japan, Tokyo, Japan) and water were accessible *ad libitum*. The mice were housed in a plastic cage with plywood chips. The cages were placed in a conventional room and temperature and humidity were kept at 23 °C and 55–70%, respectively. The room light was kept on a 12-h light/dark cycle. The study adhered to the US National Institutes of Health guidelines for the use of experimental animals. The animal care method was approved by the Animal Care and Use Committee at Oita University of Nursing and Health Sciences in Oita, Japan.

Immunization and DEP administration

Diesel exhaust particles (0.4 µm aerodynamic diameter) were prepared from a light-duty (2740 cc), four-cylinder diesel engine as described previously (Ichinose *et al.*, 1997; Miyabara *et al.*, 1998b; Takano *et al.*, 1998). The crude extract of *D. farinae* was purchased from LSL (Tokyo, Japan). Mice of each strain were divided into two groups. Each group was intratracheally instilled with *D. farinae* alone or *D. farinae* with DEP. *Dermatophagoides farinae* (1 µg) was dissolved in a phosphate-buffered saline (PBS, pH 7.4; Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) and

0 or 50 µg of DEP. The samples were instilled intratracheally into 32 or 33 mice four times at 2-week intervals. This intratracheal instillation can estimate the effect of DEP without gaseous components, such as NO_x, CO₂ or CO, and can instill a known concentration of DEP. Three days after the last instillation, the mice were sacrificed by exsanguination under deep ethyl ether anaesthesia. Plasma prepared from the sacrificed mice was stored at –80 °C until assay for *D. farinae*-specific IgG1, IgE and cytokines. The lungs were removed, snap-frozen in liquid nitrogen and then stored at –80 °C until analysis for cytokines.

Histological analysis

Ten animals in each group were used for histological examination. The lungs were fixed by intratracheal instillation of 10% neutral phosphate-buffered formalin at a pressure of 20 cmH₂O. After separation of each lobe, 2-mm thick sections were taken for paraffin embedding. Embedded blocks were sectioned at a width of 3 µm and then stained with haematoxylin and eosin (H&E) to evaluate the degree of inflammation of the eosinophils or lymphocytes in the airway from the proximal to the distal airway. The sections were stained with periodic acid–Schiff (PAS) to evaluate the degree of proliferation of goblet cells in the bronchial epithelium. The histological analyses of inflammatory cells and epithelial cells in the airway were performed using an Olympus BH-2 light microscope (Olympus, Tokyo, Japan). The degree of infiltration of eosinophils and lymphocytes in the airway and the proliferation of goblet cells in the bronchial epithelium were graded: 0 = not present; 1 = very slight; 2 = slight; 3 = moderate; 4 = moderate to marked; 5 = marked. 'Very slight' was defined as an eosinophilic inflammatory reaction affecting <10% of the airways (<10% goblet cells stained with PAS); 'slight,' 20–30% of the airways are affected; 'moderate,' 40–50%; 'moderate to marked,' 60–70%; 'marked,' >80% of the airways are affected (Ichinose *et al.*, 1997, 1998; Miyabara *et al.*, 1998a; Takano *et al.*, 1998).

Immunohistochemistry for eotaxin

The slides were deparaffinized with Auto Dewaxer (Res Gen, Huntsville, AL). Endogenous peroxidase activity was blocked by a 60% methanol solution containing 2% hydrogen peroxide for 2 h at room temperature. The slides were subsequently soaked in 0.2% sodium azide for 20 min. After hydration and a rinse in Universal buffer (Res Gen), non-specific antigens were blocked by exposure to 2% normal rabbit serum for 30 min at room temperature. Endogenous biotin and avidin binding were sequentially blocked using a Vector Blocking Kit (Vector Lab, Burlingame, CA). After thorough washing in a Universal buffer, the tissue sections were incubated with 2.5 µg ml^{–1} of rat monoclonal anti-eotaxin mouse IgG2a (R&D, Systems, Minneapolis, MN) in PBS containing 0.1% bovine serum albumin (BSA) for 1 h at room temperature. The sections were then rinsed in Universal buffer and incubated in 0.5% biotinylated anti-rat IgG (Vectastain Elite ABC Kit, Vector Lab) for 30 min at room temperature. After being rinsed in Universal buffer, the sections were incubated in avidin–biotin complex for 30 min at room temperature. After the sections were rinsed in PBS, they were stained with 1% 3,3'-diaminobenzidine solution (Dako, Carpinteria, CA, USA) containing an imidazol-

HCl-buffered substrate (pH 7.5). The sections were then washed with water, lightly counterstained with Myer's haematoxylin (Wako, Tokyo, Japan), dehydrated and mounted with Pristin Mount (Res Gen).

Controls were prepared using the conditions described above, except that the non-immune rat IgG2a (Zymed Laboratories, San Francisco, CA) was used instead of a primary antibody. See Figure 1.

Measurement of IgG1 and IgE antibodies

Dermatophagoides farinae-specific IgG1 in the plasma of the 10 animals from each group used for histological analysis was subsequently measured using enzyme-linked immunosorbent assays (ELISA) with solid-phase antigen. Briefly, a microtitre plate was coated with *D. farinae* at a concentration of $2 \mu\text{g ml}^{-1}$ overnight at 4°C according to a method reported previously (Ichinose *et al.*, 1997, 1998; Takano *et al.*, 1997, 1998; Miyabara *et al.*, 1998a,b). The plate was incubated with a 1% BSA-PBS solution (pH 7.4) containing 0.01% thimerosal for 1 h at room temperature. After washing, 100 μl of each diluted sample (1 : 50, 1 : 500, 1 : 5000 and 1 : 50 000) was placed on the

microplate well and incubated for 1 h at room temperature. After additional washing with a PBS solution containing Tween 20, the plate was incubated with biotinylated rabbit anti-mouse IgG1 at a concentration of $1.67 \mu\text{g ml}^{-1}$ (Zymed Laboratories) for 1 h at room temperature. After the second washing, wells were incubated with horseradish peroxidase-conjugated streptavidin (Sigma, St. Louis, MO) for 1 h at room temperature. The wells were washed and incubated with *o*-phenylenediamine and hydrogen peroxide for 30 min at room temperature in the dark. The enzyme reaction was stopped with 4 N H_2SO_4 and the absorption at 492 nm was measured using a microplate reader (SPECTRAFluor; Tecan, Salzburg, Austria). Each plate contained previously screened standard plasma with a high titre of anti-*D. farinae* antibodies to calculate titres by standard curve. The results were expressed in titres, which were calculated based on the titres of the standard plasma. Cut-off values for antibody-positive plasma were set at twice the mean absorbance of preimmune plasma.

The assay protocol for *D. farinae*-specific IgE was a slight modification of that for *D. farinae*-specific IgG1. The microtitre plate was coated with *D. farinae* at a concentration of $2 \mu\text{g ml}^{-1}$ overnight at 4°C . The plate was incubated with

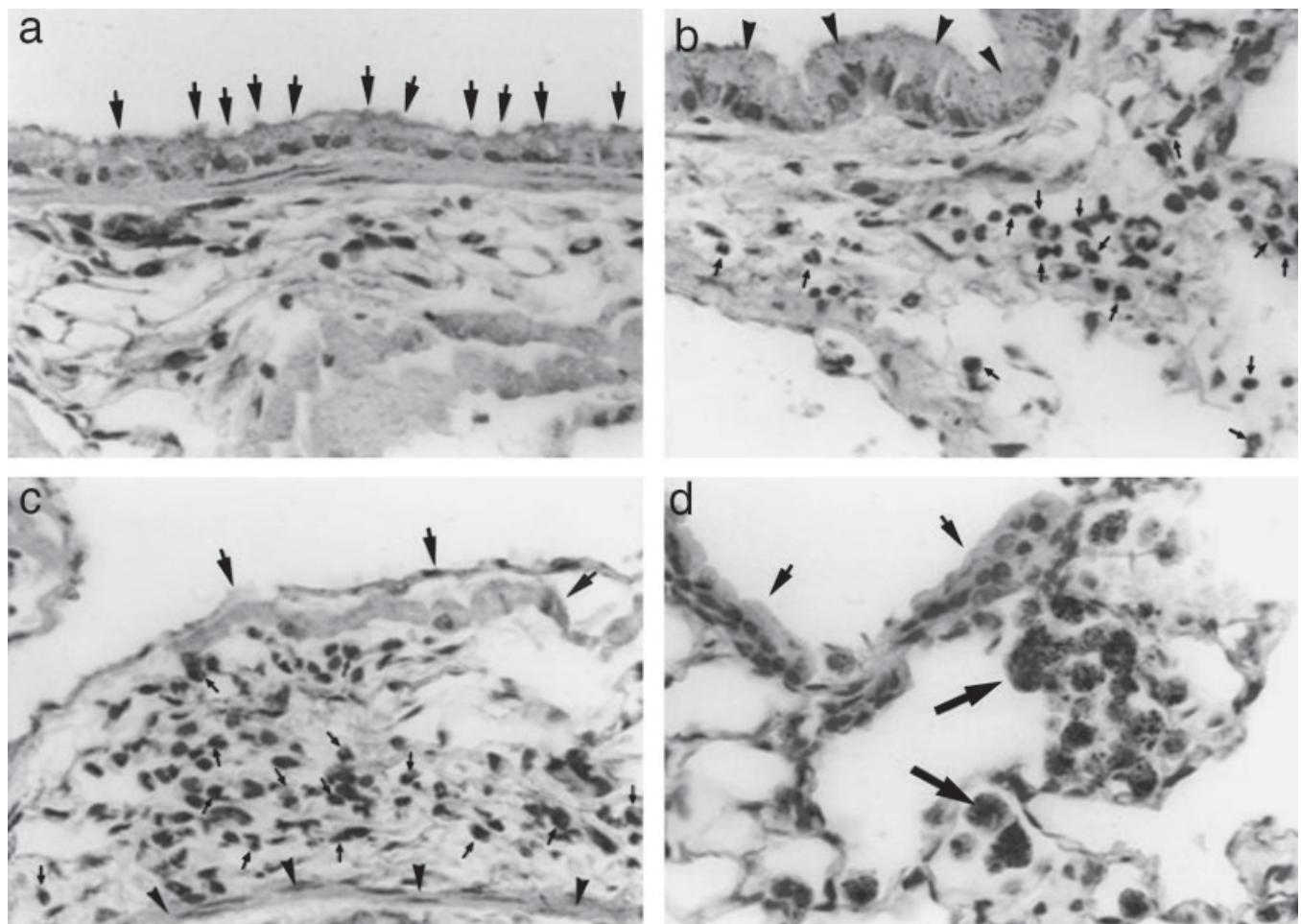


Figure 1. Immunohistochemical localization of eotaxin. (a) Light micrograph of the airway of ICR mouse treated with Der f alone (Magnification $\times 150$); eotaxin positive ciliated cells (arrows) were observed. (b) Light micrograph of the airway of C3H/He mouse treated with Der f (Magnification $\times 150$); eotaxin negative goblet cells (arrows) in the airway epithelium and positive eosinophils (small arrows) in the submucosal layer can be seen. (c) Light micrograph of the airway of BALB/c mouse treated with Der f + DEP (Magnification $\times 150$); eotaxin positive eosinophils (small arrows) in the submucosal layer of the airway, and positive smooth muscles of vessel (arrows) and bronchus (arrow heads) can be observed. (d) Light micrograph of the airway of a C3H/He mouse treated with Der f + DEP (Magnification $\times 150$); eotaxin positive nonciliated cells and clara cells in the airway epithelium and eotaxin positive soot-laden macrophages (big arrows) in the alveoli can be observed.

a 1% BSA–PBS solution (pH 7.4) containing 0.01% thimerosal for 1 h at room temperature. After the samples were diluted twofold, they were placed on the microtitre plates and incubated for 1 h at room temperature. After washing, the plates were reacted with biotinylated rabbit anti-mouse IgE at a concentration of $1 \mu\text{g ml}^{-1}$ (Biosource International, Camarillo, CA). The results were expressed as absorbance (blank value was subtracted).

Quantitation of cytokine protein levels in lung supernatants and plasma

The cytokine protein levels of interleukin (IL)-2, IL-4, IL-5, granulocyte–macrophage colony-stimulating factor (GM-CSF), eotaxin and RANTES (regulated on activation, normal T cell expressed and presumably secreted) were examined in lung tissue supernatants from six or seven animals from each group, IL-4, IL-5 and RANTES were also examined in plasma. The lungs were homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing $0.1 \mu\text{M}$ ethylenediamine tetraacetic acid (Sigma, St. Louis, MO), $0.1 \mu\text{M}$ phenylmethane sulphonyl fluoride (Nacalai Tesque, Kyoto, Japan), $1 \mu\text{M}$ pepstatin (Peptide Institute, Osaka, Japan) and $2 \mu\text{M}$ leupeptin (Peptide Institute, Osaka, Japan) according to a previously reported method (Sadakane *et al.*, 2002).²³ The homogenate was then centrifuged at $105\,000 \text{ g}$ for 1 h at 4°C . The lung supernatants were stored at -80°C until analysis. Both IL-5 and GM-CSF were analysed using commercial ELISA kits from Endogen (Cambridge, MA) and IL-4, IL-2 (Amersham, Little Chalfont, UK), RANTES and eotaxin (R&D Systems) were also analysed using commercial ELISA kits. Using a microplate reader (Model 3550, Bio-Rad Laboratories, Hercules, CA), subtractive reading at 550 from the readings at 450 nm were converted to pg ml^{-1} using values obtained from the standard curves. The standard curves were prepared using varying concentrations of recombinant IL-2, IL-4, IL-5, GM-CSF, RANTES and eotaxin. The detection limits of these assays were <3 , 5, 5, 5, 7.8 and 3 pg ml^{-1} , respectively.

Statistical analysis

Statistical analysis of the histological evaluation of inflammation and epithelial cells in the airway was performed

using the ANOVA test. The values of cytokine levels were expressed as mean $\pm$ SEM. Statistical analysis of cytokine proteins and *D. farinae*-specific IgG or IgE was conducted using Student's *t*-test. Differences among groups were considered statistically significant at a level of $P < 0.05$.

RESULTS

Histological changes in murine airway

The histological changes of lungs from three murine strains treated with *D. farinae* or *D. farinae* + DEP are shown in Table 1. *Dermatophagoides farinae* treatment produced very slight to moderate recruitment of eosinophils in three strains. The order of potency was BALB/c $<$ ICR $<$ C3H/He mice. The combination of DEP and *D. farinae* induced a significant recruitment of eosinophils in all three strains compared with mice treated with *D. farinae* alone. Similar changes of lymphocytes were observed among all three strains treated with *D. farinae* alone or *D. farinae* + DEP. The combination of DEP and *D. farinae* induced a significant recruitment of lymphocytes in BALB/c and ICR mice compared with mice treated with *D. farinae* alone.

Dermatophagoides farinae treatment induced a slight proliferation of goblet cells in the airway epithelium of BALB/c and ICR mice, and a moderate proliferation in C3H/He mice. *Dermatophagoides farinae* + DEP caused more proliferation of goblet cells in all three strains than *D. farinae* alone did. The increased level in ICR mice treated with DEP + *D. farinae* was statistically significant ($P < 0.001$) compared with that in mice treated with *D. farinae* alone.

Figure 1 shows the results of immunohistochemical staining for eotaxin. Immunohistochemical staining was found in ciliated and non-ciliated airway epithelial cells, including clara cells. It was also observed in smooth muscle and endothelial cells of the vessel as well as the bronchus. No eotaxin-positive goblet cells were observed in the bronchial epithelium. The positive protein was frequently observed in inflammatory cells, such as alveolar macrophages. It was also found in lymphocytes and eosinophils recruited into the submucosal layer or interstitium around peribronchial

Table 1—Estimation of histopathologic changes in the murine airway

Mouse strain	Group	Histopathologic changes		
		Proliferation of Goblet cells	Eosinophils	Lymphocytes
BALB/c	Der f	2.00 ± 0.78	0.75 ± 0.92	1.00 ± 1.00
	Der f + DEP	2.45 ± 0.37	1.60 ± 0.84^a	1.95 ± 0.28^b
ICR	Der f	1.80 ± 1.01	1.00 ± 0.82	1.00 ± 0.78
	Der f + DEP	3.25 ± 0.49^c	2.55 ± 0.50^c	2.55 ± 0.83^c
C3H/He	Der f	2.95 ± 0.26	2.35 ± 0.53	2.55 ± 0.44
	Der f + DEP	3.30 ± 0.24	3.10 ± 0.82^a	3.05 ± 0.68

Mice were treated intratracheally with Der f ($1 \mu\text{g}$) or Der f ($1 \mu\text{g}$) + DEP ($50 \mu\text{g}$) four times at 2-week intervals. All values were expressed as mean $\pm$ SD ($n = 10$). Data were shown as the histopathologic values calculated by the ANOVA test. The degree of histopathologic changes in the airway was estimated: (0), none; (1), very slight; (2), slight; (3), moderate; (4), moderate to marked; (5), marked.

^a Significant difference from Der f by ANOVA test, $P < 0.05$.

^b Significant difference from Der f by ANOVA test, $P < 0.01$.

^c Significant difference from Der f by ANOVA test, $P < 0.001$.

Table 2—Protein levels of cytokines in murine lung tissue supernatants

Mouse strain	Group	Local cytokine expressions (pg protein/total lung tissue supernatants)					
		IL-2	IL-4	IL-5	GM-CSF	RANTES	Eotaxin
BALB/c	Der f	ND	503.4 ± 44.3	ND	ND	171.4 ± 13.1	188.4 ± 10.2
	Der f + DEP	7.0 ± 3.9	387.3 ± 32.6	46.3 ± 6.6 ^b	ND	208.4 ± 19.1	471.1 ± 31.2 ^b
ICR	Der f	ND	183.5 ± 25.5	ND	ND	264.5 ± 28.2	199.3 ± 13.7
	Der f + DEP	2.5 ± 1.6	212.2 ± 26.8	54.0 ± 7.5 ^b	2.6	236.1 ± 18.2	1271.9 ± 219.1 ^b
C3H/He	Der f	ND	64.9 ± 12.1	76.3 ± 14.5	ND	169.3 ± 8.7	559.7 ± 56.2
	Der f + DEP	ND	90.4 ± 12.8	94.2 ± 20.8	ND	188.4 ± 30.6	1409.1 ± 187.0 ^a

Mice were treated intratracheally with Der f (1 µg) or Der f (1 µg) + DEP (50 µg) four times at 2-week intervals. All values were expressed as mean ± SEM. ($n = 6$ for Der f group and $n = 7$ for Der f + DEP group). ND: not detected.

^a Significant difference from Der f by Student *t* test, $P < 0.01$.

^b Significant difference from Der f by Student *t* test, $P < 0.001$.

vessels. The number of eotaxin positive macrophages in the alveoli of mice treated with *D. farinae* + DEP was much greater than that of mice treated with *D. farinae* alone.

Local cytokine expression in lung tissue supernatant

Table 2 shows the cytokine protein levels of IL-2, IL-4, IL-5, GM-CSF, RANTES and eotaxin in the lung tissue supernatants from each mouse strain treated with *D. farinae* or *D. farinae* + DEP. The protein of IL-5 in the lungs was not detected in BALB/c and ICR mice treated with *D. farinae* alone, whereas the mean protein level in C3H/He mice showed 76.3 ± 1.45 pg in lung tissue. *Dermatophagoides farinae* + DEP induced significant elevation ($P < 0.001$) of local IL-5 in ICR and C3H/He mice compared with treatment with *D. farinae* alone. The local levels of eotaxin in C3H/He mice were the highest among the mice tested. They were 3 times and 2.8 times higher than that of BALB/c and ICR mice, respectively. *Dermatophagoides farinae* + DEP also caused significant elevation of eotaxin in three strains compared with treatment with *D. farinae* alone. The increased levels of eotaxin in BALB/c, ICR and C3H/He mice treated with *D. farinae* + DEP were 2.5 times, 5.6 times and 2.5 times higher than those in the groups treated with *D. farinae* alone. The production levels of IL-5 and eotaxin in the lung tissues correlated with the recruitment of eosinophils by *D. farinae* alone or *D. farinae* + DEP treatment. The expression order of RANTES protein in lung tissues of mice treated with *D. farinae* alone was ICR < BALB/c < C3H/He mice. These protein levels were unaffected by DEP. The expression order of IL-4 protein in lung tissues of mice treated with *D. farinae* was C3H/He < ICR < BALB/c mice. The changes of protein levels by DEP were not significant. The production levels of IL-4 and RANTES in lung tissues did not correlate with the manifestations of allergic airway inflammation induced by *D. farinae* treatment with or without DEP. The IL-2 and GM-CSF concentrations in lung tissues were lower than the detection limit in all groups.

Cytokine expression in plasma

Table 3 shows the results of cytokine expression analysis in plasma. The protein levels of IL-5 in plasma of ICR mice treated with *D. farinae* alone or *D. farinae* + DEP were below the detection limits. The protein levels of

Table 3—Protein levels of cytokines in murine plasma

Mouse strain	Group	Plasma (pg/ml)	
		IL-5	RANTES
BALB/c	Der f	10.5 ± 3.2	10.1 ± 0.8
	Der f + DEP	6.0 ± 2.1	10.4 ± 0.8
ICR	Der f	ND	8.5 ± 0.9
	Der f + DEP	ND	11.2 ± 1.2
C3H/He	Der f	5.0 ± 3.1	7.8 ± 0.5
	Der f + DEP	14.2 ± 3.2 ^a	7.1 ± 0.6

Mice were intratracheally injected with 1 µg Der f per animal or 1 µg Der f + 50 µg DEP per animal four times during an interval of 2 weeks. Results are expressed as Mean ± SEM of 6 or 7 per group. Significant difference was evaluated by Student's *t* test. ND: Not detected.

^a Significant difference from Der f by Student *t* test, $P < 0.05$.

IL-5 in plasma of BALB/c and C3H/He mice treated with *D. farinae* alone or *D. farinae* + DEP were slightly higher than the detection limits. The level of IL-5 in C3H/He mice treated with *D. farinae* + DEP was significantly higher ($P < 0.05$) than that in mice treated with *D. farinae* alone. RANTES in the plasma of all three strains treated with *D. farinae* alone or *D. farinae* + DEP were slightly higher than the detection limits, whereas the levels were unaffected by DEP treatment alone. The protein levels of IL-4 in the plasma of all three strains were below the detection limits (data not shown).

Dermatophagoides farinae-specific immunoglobulin production in plasma

The titres of *D. farinae*-specific-IgG1 in plasma of the mice are shown in Fig. 2. The production order of antigen-specific IgG1 antibody in mice treated with *D. farinae* alone or *D. farinae* + DEP was BALB/c < ICR < C3H/He mice. The adjuvant effect of DEP on IgG1 production was observed in all three strains. Increased *D. farinae*-specific-IgG1 production in C3H/He mice was statistically significant ($P < 0.05$) compared with that in the mice treated with *D. farinae* alone. The production levels of *D. farinae*-specific IgG1 in plasma correlated with the manifestations of eosinophilic airway inflammation by *D. farinae* alone or *D. farinae* + DEP treatment. Figure 3 shows the absorbance of *D. farinae*-specific IgE in the plasma of the three strains. The results are shown in absorbance units as

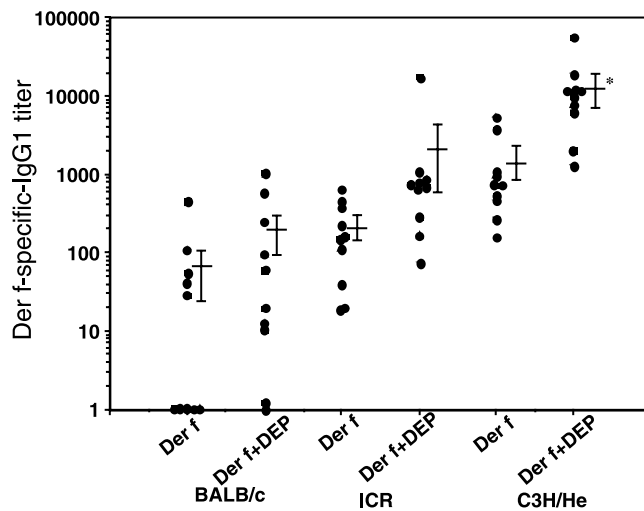


Figure 2. Antigen-specific IgG1 responses in plasma. Results are expressed as mean $\pm$ SEM ($n = 10$) and individual values. The mean values of antigen-specific IgG1 titer in BALB/c, ICR, and C3H/He mice treated with Der f were 69 ± 44 , 219 ± 66 , and 1452 ± 37 , respectively. The mean values of antigen-specific IgG1 titer in BALB/c, ICR, and C3H/He mice treated with Der f + DEP were 205 ± 108 , 2237 ± 1641 , and 12635 ± 5080 , respectively. The results were expressed in titers with calculation based on the titers of the standard plasma. Cut-off values for antibody-positive plasma were set two holds as mean absorbance of preimmune plasma. Significant difference was Student's t test. * $P < 0.05$ compared with group treated with Der f alone.

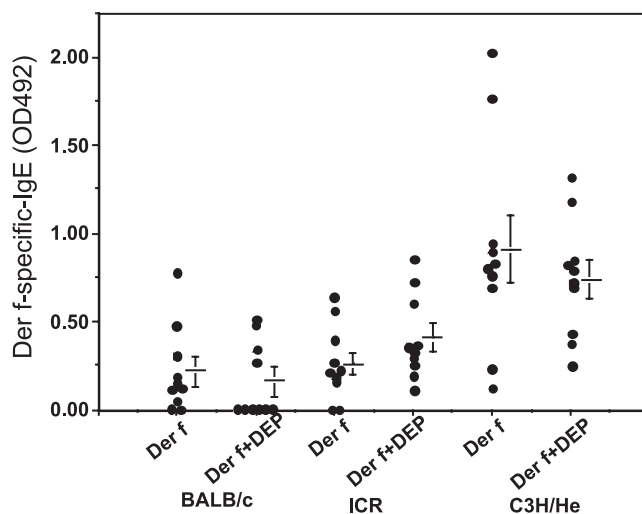


Figure 3. Antigen-specific IgE responses in plasma. The results were expressed as absorbance (blank value was subtracted). Data are expressed as mean $\pm$ SEM ($n = 10$) and individual values. The mean values of antigen-specific IgE titer in BALB/c, ICR, and C3H/He mice treated with Der f were 0.22 ± 0.08 , 0.26 ± 0.06 , and 0.91 ± 0.19 , respectively. The mean values of antigen-specific IgE titer in CBA/JN and C57BL/6N mice treated with Der f + DEP were 0.16 ± 0.07 , 0.41 ± 0.08 , and 0.74 ± 0.11 , respectively.

allergen-specific IgE antibodies, which are <1 titre in all three mice strains. The production order of antigen-specific IgE antibodies in mice treated with *D. farinae* or *D. farinae* + DEP was BALB/c $<$ ICR $<$ C3H/He mice. The adjuvant effect of DEP on IgE production was not observed in all three strains.

DISCUSSION

In the present study, the differences in the development of eosinophilic airway inflammation induced by intratracheal instillation of a mite allergen and DEP among three mouse strains were investigated. The number of eosinophils in the murine airway increased immediately after the last exposure to ovalbumin and then it peaked at 3 days after the exposure (Miyabara *et al.*, 1998a). In the present study, therefore, histological observation and analysis of cytokines and chemokines were performed in the lung samples at 3 days after the last exposure to *D. farinae* or *D. farinae* + DEP. The murine asthma model by intratracheal instillation of mite allergen exhibited symptoms also frequently seen in human allergic asthma, including recruitment of eosinophils and lymphocytes, proliferation of goblet cells and production of a mucus plug in the airway of all three mouse strains. The increasing order of potency was BALB/c $<$ ICR $<$ C3H/He mice. Therefore, among the mice strains tested, the airway of C3H/He mice was most susceptible to the allergic reaction caused by a mite allergen. These histological features in all three strains were enhanced by DEP. Eosinophils are known to play a major role in the pathogenesis of allergic asthma. Eosinophils contribute to airway epithelial injury and hyperresponsiveness by releasing mediators, including the platelet-activating factor, oxygen radicals, leukotrienes and a major basic protein (Gleich *et al.*, 1990).

In the present study, the transformation from airway epithelial cells to goblet cells by mite allergen treatment was dependent on the degree of eosinophil recruitment into the submucosa of the airways in all three strains, suggesting that the airway epithelial injury with goblet cell proliferation is induced by activated eosinophils.

Interleukin 5 is recognized as a key mediator in bronchial asthma, which affects eosinophilic inflammation and airway hyperresponsiveness (Robinson *et al.*, 1993; Foster *et al.*, 1996). Eotaxin is also an eosinophil-specific chemokine, which is implicated in the recruitment of eosinophils in allergic inflammation. A recent report concerning eotaxin-deficient mice suggests a contributory role for eotaxin and eosinophils in the pathogenesis of asthma (Rothenberg *et al.*, 1997). In the present study, the local levels of IL-5 and eotaxin correlated with the manifestations of eosinophilic airway inflammation by *D. farinae* or *D. farinae* + DEP instillation. The increasing order of IL-5 and eotaxin in mice treated with *D. farinae* + DEP was BALB/c $<$ ICR $<$ C3H/He mice. These results suggest that murine strain differences in the production of allergic airway inflammation by *D. farinae* + DEP are related to differences in local expression of IL-5 and eotaxin. The aggravating effects of DEP may be mediated by IL-5 and eotaxin. Based on the results of immunostaining for eotaxin, the source of increased eotaxin may be due to recruited eosinophils, macrophages or other such positive cells, except for eotaxin-negative goblet cells.

RANTES is a potent chemokine that affects the activity of eosinophils (Alam *et al.*, 1993). The protein of RANTES, in the present study, was certainly detected in the lung tissues treated with mite allergen. However,

the production levels of RANTES in lung tissue did not correlate with the manifestations of eosinophilic airway inflammation among the three strains. Furthermore, the clear modulation of RANTES by DEP was not detected. The GM-CSF also has an important role in the recruitment as well as in the activation and degranulation of eosinophils (Hallsworth *et al.*, 1992). The protein of GM-CSF, however, was not detected in this murine asthma model by mite allergen. These results suggest that RANTES and GM-CSF in the lungs may not play an important role in murine strain differences in the induction of eosinophilic airway inflammation by *D. farinae* + DEP and in the enhancing effects of DEP on its inflammation.

Levels of IL-2 in the Th1-type cytokine were not detectable in this asthma model by mite allergen. On the other hand, IL-4, which is secreted from Th2 lymphocytes, is central to the formation of immunoglobulin in B cells and the development of Th2 lymphocytes (Swain *et al.*, 1990). In the present study, the higher expression of IL-4 in the lung tissue of BALB/c mice supposed that the airway inflammation in BALB/c mice is most predominant in Th2 lymphocytes among the three strains, in spite of having the lowest eosinophilic inflammation. The protein levels of IL-4 in the three strains treated with *D. farinae* or *D. farinae* + DEP did not correlate with the production levels of immunoglobulins (IgG1 and IgE) in plasma.

It has been reported that antigen-specific IgG1 antibodies might contribute to antigen-specific eosinophil degranulation via Fcγ RII on the eosinophil surface (Kaneko *et al.*, 1995). In the present study, the increasing order of the production levels of antigen-specific IgG1 by *D. farinae* or *D. farinae* + DEP was BALB/c < ICR < C3H/He mice. A significant adjuvant effect of DEP on IgG1 production was observed in the C3H/He mice. The production levels of the IgG1 antibodies correlated with the manifestations of eosinophilic airway inflammation by *D. farinae* + DEP instillation, suggesting that the airway injury enhanced by DEP is induced by the antigen-specific eosinophilic degranulation via increased antigen-specific IgG1 antibodies. Immunoglobulin may play an important role in strain differences of allergic asthma and in the enhancement of DEP.

Allergen-specific IgE was found in very low levels in all three strains. Diesel exhaust particles reportedly enhanced *D. farinae*-specific IgE production in A/J mice. The induction of the specific antibody by *D. farinae* + DEP was dependent on the dose level (Suzuki *et al.*, 1996). The enhancement effect of DEP also has been observed in the

case of ovalbumin (Takafuji *et al.*, 1987). The induction of allergen-specific IgE antibody was changed considerably by the dose of treatment and the routes of administration, such as intranasal, epicutaneous or intraperitoneal (Nelde *et al.*, 2001). The discrepancy of the immune response may be due to differences in the dose of antigen or DEP and in the administration routes, such as intranasal treatment or intratracheal treatment. The differences in the routes of administration may be due to the differences of uptake of an antigen, local number and distribution of allergen-presenting cells (macrophages, dendritic cells) or induction and activation of Th2 lymphocytes in the tissue (nasal or bronchial mucosa) where an antigen was administrated.

The previous studies showed that the eosinophilic inflammation and bronchial hyperreactivity caused by exposure to the allergen occurred even in mice that are deficient in IgE, mast cells, IL-4 or B cells (Oettgen *et al.*, 1994; Brusselle *et al.*, 1995; Korsgren *et al.*, 1997; Mehlhop *et al.*, 1997). In our murine model, therefore, the induction of airway inflammation by mite allergen with or without DEP may not be related to allergen-specific IgE antibodies.

Recent *in vitro* studies suggest that DEP, nitrogen dioxide or ozone modulate the airway sensitivity and allergic airway inflammation via increasing release of specific pro-inflammatory mediators, such as GM-CSF, IL-8, TNF-α, SICAM-1 and RANTES from bronchial epithelial cells in asthmatic patients (Devalia *et al.*, 1999; Bayram *et al.*, 2001). The results of the present study from whole body and whole cells in this murine model suggest that DEP readily induces the production of allergic asthma, even in non-asthmatic subjects, in which it is genetically easy to generate IL-5, eotaxin and *D. farinae*-specific IgG1 induction.

In conclusion, the present study demonstrates that mite allergens from house dust induced manifestations of allergic airway inflammation. The level of this inflammation was different in the three different mouse strains. Diesel exhaust particles enhanced the allergic reaction in this asthma model. The murine strain differences in the production of allergic airway inflammation caused by mite allergen and DEP may be related to differences in local expression of eotaxin, IL-5 and antigen-induced immune responses, especially in terms of IgG1 production. The results of the present study suggest that the cause of individual differences in humans at the onset of allergic asthma is associated with differences in the local expression of eosinophil relevant to cytokines, chemokines and antigen-induced immune responses such as IgG1 and IgE.

REFERENCES

- Alam R, Stafford S, Forsythe P, Harrison R, Faubion D, Lett-Brown MA, Grant JA. 1993. RANTES is a chemotactic and activating factor for human eosinophils. *J. Immunol.* **150**: 3442–3448.
- Bayram H, Sapsford RJ, Abdelaziz MM, Khair OA. 2001. Effect of ozone and nitrogen dioxide on the release of proinflammatory mediators from bronchial epithelial cells of nonatopic nonasthmatic subjects and atopic asthmatic patients *in vitro*. *J. Allergy Clin. Immunol.* **107**: 287–294.
- Bjorksten B, Dumitrascu D, Foucard T, Khetsuriani N, Khaitov R, Leja M, Lis G, Pekkanen J, Priftanji A, Riikjarv MA. 1998. Prevalence of childhood asthma, rhinitis and eczema in Scandinavia and Eastern Europe. *Eur. Respir. J.* **12**: 432–434.
- Brusselle G, Kips J, Joos G, Bluethmann JH, Pauwels R. 1995. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell Mol. Biol.* **12**: 254–259.
- Custovic A, Green R, Smith A, Chapman MD, Woodcock A. 1996. New mattresses: how fast do they become a significant source of exposure to house dust mite allergens? *Clin. Exp. Allergy* **26**: 1243–1245.
- Devalia JL, Bayram H, Abdelaziz MM, Sapsford RJ, Davies RJ. 1999. Differences between cytokine release from bronchial

- epithelial cells of asthmatic patients and non-asthmatic subjects: effect of exposure to diesel exhaust particles. *Int. Arch. Allergy Immunol.* **118**: 437–439.
- Diaz-Sanchez D, Tsien A, Casillas A, Dotson R, Saxon A. 1996. Enhanced nasal cytokine production in human beings after *in vivo* challenge with diesel exhaust particles. *J. Allergy Clin. Immunol.* **98**: 114–123.
- Diaz-Sanchez D, Jyrala M, Ng D, Nel D, Saxon A. 2000a. *In vivo* nasal challenge with diesel exhaust particles enhances expression of the CC chemokines rantes, MIP-1 α , and MCP-3 in humans. *Clin. Immunol.* **97**: 140–145.
- Diaz-Sanchez D, Penichet-Garcia M, Saxon A. 2000b. Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J. Allergy Clin. Immunol.* **106**: 1140–1146.
- Etzel R, Rylander R. 1999. Indoor mold and children's health. *Environ. Health Perspect.* **107**: 463.
- Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. 1996. Interleukin 5 deficiency abolishes eosinophilia, airway hyperreactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* **183**: 195–201.
- Gleich GJ. 1990. The eosinophil and bronchial asthma: Current understanding. *J. Allergy Clin. Immunol.* **85**: 422–436.
- Hallsworth MP, Litchfield T, Lee TH. 1992. Glucocorticoids inhibit granulocyte-macrophage colony-stimulating factor-1 and interleukin-5 enhanced *in vitro* survival of human eosinophils. *Immunology* **75**: 382–385.
- Huss K, Jr, Adkinson NF, Eggleston PA, Dawson C, Van Natta ML, Hamilton RG. 2001. House dust mite and cockroach exposure are strong risk factors for positive allergy skin test responses in the Childhood Asthma Management Program. *J. Allergy Clin. Immunol.* **107**: 48–54.
- Ichinose T, Takano H, Miyabara Y, Yanagisawa R, Sagai M. 1997. Murine strain differences in allergic airway inflammation and immunoglobulin production by a combination of antigen and diesel exhaust particles. *Toxicology* **122**: 183–192.
- Ichinose T, Takano H, Miyabara Y, Sagai M. 1998. Long-term exposure to diesel exhaust enhances antigen-induced eosinophilic inflammation and epithelial damage in the murine airway. *Toxicol. Sci.* **44**: 70–79.
- Japan Ministry of Education, Science and Culture (JMESC). 2000. *Report on School Health Survey*. JMESC: Tokyo, Japan.
- Kaneko S, Shimada K, Hirouchi H, Endo A, Kodama M, Shinoda N, Imai T, Takiguchi K. 1980. Nasal allergy and air pollution. *Otorhinolaryngol. Tokyo* **23**: 270–279.
- Kaneko M, Swanson MC, Gleich GJ, Kita H. 1995. Allergen-specific IgG and IgG through Fc γ RII induce eosinophil degranulation. *J. Clin. Invest.* **95**: 2813–2821.
- Korsgren M, Erjefalt JS, Korsgren O, Sundler F, Persson CG. 1997. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J. Exp. Med.* **185**: 885–892.
- Mehlhof PD, van de Rijn M, Goldberg AB, Brewer JP, Kurup VP, Martin TR, Oettgen HC. 1997. Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc. Natl. Acad. Sci. USA* **94**: 1344–1349.
- Miyabara Y, Ichinose T, Takano H, Lim HB, Sagai M. 1998a. Effects of diesel exhaust on allergic airway inflammation in mice. *J. Allergy Clin. Immunol.* **102**: 805–812.
- Miyabara Y, Yanagisawa R, Shimojo N, Takano H, Lim HB, Ichinose T, Sagai M. 1998b. Murine strain differences in airway inflammation caused by diesel exhaust particles. *Eur. Respir. J.* **11**: 291–298.
- Nelde A, Teufel M, Hahn C, Duschl A, Sebald W, Brocker EB, Grunewald SM. 2001. The impact of the route and frequency of antigen exposure on the IgE response in allergy. *Int. Arch. Allergy Immunol.* **124**: 461–469.
- Nishima S. 1993. A study on the prevalence of bronchial asthma in school children in western districts of Japan — comparison between the studies in 1982 and in 1992 with the same methods and same districts. *Arerugi* **42**: 192–204.
- Oettgen HC, Martin TR, Wynshaw-Borls A, Deng C, Drazen JM, Leder P. 1994. Active anaphylaxis in IgE-deficient mice. *Nature* **370**: 367–370.
- Pope III CA, Dockery DW, Spengler JD, Raizenne ME. 1991. Respiratory health and PM10 pollution. A daily time series analysis. *Am. Rev. Respir. Dis.* **144**: 668–674.
- Pope III CA, Thun MJ, Namboodiri MM, Dockery DW, Evans JS, Speizer FE, Heath Jr CW. 1995. Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. *Am. J. Respir. Crit. Care Med.* **151**: 669–674.
- Robinson DS, Ying S, Bentley AM, Meng Q, North J, Durham SR, Kay AB, Hamid Q. 1993. Relationships among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. *J. Allergy Clin. Immunol.* **92**: 397–403.
- Romieu I, Meneses F, Ruiz S, Sienra JJ, Huerta J, White MC, Etzel RA. 1996. Effects of air pollution on the respiratory health of asthmatic children living in Mexico City. *Am. J. Respir. Crit. Care Med.* **154**: 300–403.
- Rothenberg ME, MacLean JA, Pearlman E, Luster AD, Leder P. 1997. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J. Exp. Med.* **185**: 785–790.
- Sadakane K, Ichinose T, Takano H, Yanagisawa R, Yoshikawa T, Sagai M, Shibamoto T. 2002. Murine strain differences in airway inflammation induced by diesel exhaust particles and house dust mite allergen. *Int. Arch. Allergy Immunol.* **128**: 220–228.
- Scalabrini DM, Bavbek S, Perzanowski MS, Wilson BB, Platts-Mills TA, Wheatley LM. 1999. Use of specific IgE in assessing the relevance of fungal and dust mite allergens to atopic dermatitis: a comparison with asthmatic and nonasthmatic control subjects. *J. Allergy Clin. Immunol.* **104**: 1273–1279.
- Schwartz J, Slater T, Larson T, Pierson WE, Koenig JQ. 1993. Particulate air pollution and hospital emergency room visits for asthma in Seattle. *Am. Rev. Respir. Dis.* **147**: 826–831.
- Shamssain MH, Shamssian N. 2001. Prevalence and severity of asthma, rhinitis, and atopic eczema in 13- to 14-year-old schoolchildren from the northeast of England. *Ann. Allergy Asthma Immunol.* **86**: 428–432.
- Suzuki T, Kanoh T, Ishimori M, Ikeda S, Ohkuni H. 1996. Adjuvant activity of diesel exhaust particulates (DEP) in production of anti-IgE and anti-Ig1 antibodies to mite allergen in mice. *J. Clin. Lab. Immunol.* **48**: 187–199.
- Swain SL, Weinderg AD, English M. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* **145**: 3796–3806.
- Takafuji S, Suzuki S, Koizumi K, Tadokoro K, Miyamoto T, Ikemori R, Muranaka M. 1987. Diesel-exhaust particulates inhaled by the intranasal route have an adjuvant activity for IgE production in mice. *J. Allergy Clin. Immunol.* **79**: 639–645.
- Takano H, Yoshikawa T, Ichinose T, Miyabara Y, Imaoka K, Sagai M. 1997. Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. *Am. J. Respir. Crit. Care Med.* **156**: 36–42.
- Takano H, Ichinose T, Miyabara Y, Shibuya T, Lim HB, Yoshikawa T, Sagai M. 1998. Inhalation of diesel exhaust enhances allergen-related eosinophil recruitment and airway hyperresponsiveness in mice. *Toxicol. Appl. Pharmacol.* **150**: 328–337.