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Repeated exposure to low-dose diesel exhaust after allergen challenge exaggerates asthmatic responses in mice

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KEYWORDS

Diesel exhaust;
Low dose;
Asthma;
Mice model

Abstract

Background: In conjunction with allergens, diesel exhaust particles act as an adjuvant to enhance IgE responses, inducing expression of cytokines/chemokines and adhesion molecules, and increasing airway hyper-responsiveness (AHR). As most studies were designed to expose animals to diesel exhaust throughout the periods of both sensitization and allergen challenge, it remains unclear whether diesel exhaust (DE) exposure exaggerates airway responses in asthmatic animals. **Objective:** To study effects of exposure to low-dose DE on AHR and allergic airway inflammation in asthmatic mice.

Methods: BALB/c mice were sensitized by intraperitoneal injection of ovalbumin and challenged by intranasal administration with ovalbumin. They were exposed to low-dose DE for 7 h/day, 5 days/week, for up to 12 weeks. AHR to methacholine was evaluated by whole-body plethysmography as well as bronchoalveolar lavage cell analysis and cytokine gene expression in lungs.

Results: Repeated exposure of asthmatic mice to low-dose DE resulted in increased AHR and gene expression of several pro-asthmatic cytokines/chemokines, but these effects rapidly subsided with continued exposure to DE.

Conclusion: Repeated exposure to low-dose DE after ovalbumin challenge exaggerates allergic responses in mice, but effects are not prolonged with continuous DE exposure.

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Introduction

The incidence of allergic diseases has increased in most industrialized countries of the world. Persistent exposure to particulate air pollution from motor vehicles has been implicated as one of the factors responsible for the increased prevalence of allergic diseases [1,2]. Epidemiologic studies conducted in different parts of the world have demonstrated an important association between ambient levels of motor vehicle traffic emissions and increased symptoms of asthma and rhinitis.

Since diesel exhaust particles (DEPs) are the major components of ambient fine particulate matter (i.e., particulate matter less than 2.5 μm in diameter: PM_{2.5}), especially in urban areas, many *in vivo* and *in vitro* studies have been performed to clarify the association between DEP and these disorders [3,4]. The results obtained so far strongly suggest that DEP may induce pro-inflammatory mediators that are involved in airway inflammation [5–7]. Exposure of experimental animals to diesel exhaust (DE) enhances airway hyper-responsiveness (AHR) and allergic airway inflammation [8,9]. In conjunction with allergens, DEPs act as an adjuvant to enhance IgE antibody responses [10], inducing the expression of cytokines/chemokines [11] and adhesion molecules [12], and increasing airway hyper-responsiveness (AHR) [13].

However, in previous studies conducted to clarify the potential of DEPs or DE to induce toxicological effects, animals were generally exposed to a high dose (i.e., ~3 mg/m³) [8,9], or via non-physiological routes (i.e., intratracheal injection of suspended particles) [7,11]. Moreover, since most of the previous experimental studies were designed to expose animals to DE throughout the periods of both sensitization and allergen challenge [8,9,14], it still remains unclear whether DE exposure actually exaggerates airway responses in animals that have already developed asthmatic status.

In the present study, we attempted to examine the effects of exposure to low-dose DE on AHR and allergic airway inflammation in asthmatic mice.

Materials and methods

Animals

Specific pathogen-free female BALB/c mice (6 weeks old), weighing 15 to 20 g, were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in cages (6 to 8 mice per cage) in a clean room and maintained on autoclaved food and water. They were fed according to the guidelines of the institution.

Study design

The study design is shown in Fig. 1. Allergic airway inflammation associated with AHR was generated in mice by intraperitoneal sensitization with ovalbumin (OVA) followed by intranasal challenge, as described below. On the following day, exposure to DE or clean air was started for up to 8 weeks. Each of the study groups consisted of 46 mice. Since our preliminary experiments had shown that the degrees of allergic inflammation, AHR and cytokine gene

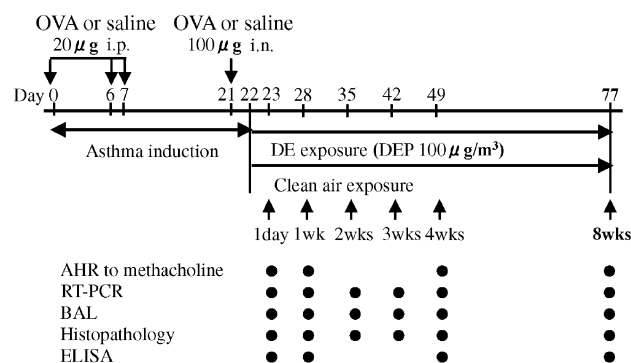


Figure 1 Experimental design. OVA, ovalbumin; DEP, diesel exhaust particles; AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage.

expression peaked at 12 h after the last exposure to DE, we assessed the various parameters at this time point. The degree of AHR was measured in six mice of each group after various periods of DE exposure: 1 day, and 1, 4 and 8 weeks. Bronchoalveolar lavage (BAL), lung histology and RT-PCR were performed at 1 day, and 1, 2, 3, 4 and 8 weeks after DE exposure in three mice of each group.

A negative control group consisting of 12 mice that were sham-sensitized and subsequently challenged with saline intranasally was used for assessment of AHR only.

Sensitization and provocation procedures

Allergic airway inflammation associated with AHR was generated in mice as described previously [15,16]. Briefly, we developed a murine model of lung inflammation and AHR, which consists of an initial phase of sensitization and a second phase of induction of the allergic response. Ovalbumin (OVA) was purchased from Sigma (St. Louis, MO). Mice were injected intraperitoneally with 20 μg of OVA adsorbed to 2 mg alum diluted with 0.5 ml saline on days 0, 6 and 7. Two weeks later, the mice were challenged with OVA 0.1 mg in 0.1 ml saline intranasally (on day 21). The negative control group received 0.5 ml of saline intraperitoneally, and received 0.1 ml of saline intranasally on day 21.

DE exposure system

The *in vivo* DE exposure system has been described previously [17,18]. A 2369-cm³ diesel engine manufactured by Isuzu Motor (Tokyo, Japan) was operated at 1050 rpm and 80% load with commercial light oil. The engine exhaust was introduced into a dilution tunnel where it was diluted with clean air that was passed through a high-efficiency particulate air filter and a charcoal filter. The diluted DE was introduced into the exposure chamber. The concentration of DEP was determined by a mass monitoring system (TEOM; Rupprecht and Patashnick Co. Inc., Albany, NY) and regulated to supply low levels, being adjusted to approximately 100 $\mu\text{g}/\text{m}^3$. The concentrations of CO, SO₂ and NO₂ were measured by an infrared gas analyzer (ULTRAMAT-S; Fuji Electric Co. Ltd., Tokyo, Japan). The average levels of CO, NO₂, SO₂ and DEP were 3.5 $\pm$ 0.1 ppm, 2.2 $\pm$ 0.3 ppm, <0.01

ppm and $103.1 \pm 9.2 \mu\text{g}/\text{m}^3$, respectively. The mice were exposed to DE for 1 day, or 1, 2, 3, 4 or 8 weeks (7 h per day, 5 days a week). Control mice were housed in a clean room for the same periods of time.

Determination of AHR

Total pulmonary airflow in unrestrained conscious mice was estimated with a whole-body plethysmography (WBP) device (Buxco Electronics, Troy, NY) [19,20]. Mice were challenged for 3 min with a series of aerosolized methacholine (MCh, 6–48 mg/ml; Sigma) administrations. Pressure differences between the chambers containing individual animals and a reference chamber were used to extrapolate minute volume, tidal volume, breathing frequency, and enhanced pause (PenH) [20]. Airway reactivity was monitored for 5 min after each aerosol challenge. PenH is a function of total pulmonary airflow during the respiratory cycle and is described by the

equation: $\text{PenH} = (\text{PEP}/\text{PIP}) \times \text{pause}$, where PEP is the peak expiratory pressure, PIP is the peak inspiratory pressure, and pause is a component of expiration time. This parameter is dependent on the breathing pattern and correlates with airway resistance as measured by traditional invasive techniques in ventilated mice [20].

From these dose–response curves the provocative concentration of methacholine causing a 200% increase of the baseline PenH (PC200) was calculated.

Bronchoalveolar lavage (BAL)

BAL was performed as described previously [18,21]. Briefly, the lungs were lavaged with 1.0 ml of physiological saline via a tracheal cannula three times, using three mice from each group. The recovered BAL fluid was immediately centrifuged, and differential cell counts were obtained from cytospin preparations with Diff-Quick staining (Muto Kagaku Co., Tokyo, Japan).

Table 1 RT-PCR Primer sets and amplification conditions

Primer	Sequences		
IL-4	5' -CTAGTTGTCATCCTGCTCTTCTTT-3'	(Sense)	
	5' -CTTTAGGCTTTCCAGGAAGTCTTT-3'	(Antisense)	
IL-5	5' -GTGAAAGAGACCTTGACACAGCT-3'	(Sense)	
	5' -CACACCAAGGAACCTTGCAGGT-3'	(Antisense)	
IL-13	5' -CTCCCTCTGACCCTTAAGGAG-3'	(Sense)	
	5' -GAAGGGGCGGTGGCGAAACAG-3'	(Antisense)	
RANTES	5' -TGCCCTCACCATCATCCTCA-3'	(Sense)	
	5' -GGACCGGAGTGGGAGTAGGG-3'	(Antisense)	
Eotaxin	5' -TCCACAGCGCTTCTATTCCT-3'	(Sense)	
	5' -CTATGGCTTTTCAGGGTGCAT-3'	(Antisense)	
MCP-1	5' -AAGCCAGCTCTCTCTTCCTC-3'	(Sense)	
	5' -CCTCTCTCTTGAGCTTGGTG-3'	(Antisense)	
MDC	5' -TCCCACTCCTGGTGGCTCTC-3'	(Sense)	
	5' -CCCTGGACCACAACCAGACC-3'	(Antisense)	
TARC	5' -CAGGAAGTTGGTGAGCTGGTATA-3'	(Sense)	
	5' -TTGTGTTTCGCCTGTAGTGCATA-3'	(Antisense)	
IL-12 p40	5' -ATCTCCTGGTTTGCCATCGTTTTG-3'	(Sense)	
	5' -TCCCTTTGGTCCAGTGACCTTC-3'	(Antisense)	
INF- γ	5' -TACTGCCACGGCACAGTCATTGAA-3'	(Sense)	
	5' -GCAGCGACTCCTTTTCGCTTCCT-3'	(Antisense)	
IL-10	5' -CTGGACAACATACTGCTAACCGAC-3'	(Sense)	
	5' -ATTCATTATGGCCTTGTAGACACC-3'	(Antisense)	
β -actin	5' -TGTGATGGTGGGAATGGGTCAG-3'	(Sense)	
	5' -TTTGATGTACGCACGATTTC-3'	(Antisense)	
	Cycle parameters	Cycles	Product size (bp)
IL-4	94°C 10 s; 62°C 10 s; 72°C 15 s	40	378
IL-5	94°C 10 s; 65°C 10 s; 72°C 15 s	40	288
IL-13	94°C 10 s; 65°C 10 s; 72°C 15 s	40	309
RANTES	94°C 10 s; 65°C 10 s; 72°C 15 s	30	371
Eotaxin	94°C 10 s; 65°C 10 s; 72°C 15 s	35	178
MCP-1	94°C 10 s; 65°C 10 s; 72°C 15 s	35	247
MDC	94°C 10 s; 65°C 10 s; 72°C 15 s	30	305
TARC	94°C 10 s; 68°C 10 s; 72°C 15 s	40	301
IL-12p40	94°C 10 s; 65°C 10 s; 72°C 15 s	35	527
INF- γ	94°C 10 s; 65°C 10 s; 72°C 15 s	35	405
IL-10	94°C 10 s; 65°C 10 s; 72°C 15 s	40	301
β -actin	94°C 10 s; 65°C 10 s; 72°C 15 s	25	514

Enzyme-linked immunosorbent assay (ELISA)

The levels of cytokines in the BAL fluids were determined by ELISA. BAL fluids were collected after 1 day, and 1, 4 and 8 weeks after DE exposure. BAL fluids concentrated by lyophilization were assayed using commercially available ELISA kits for IL-4 and RANTES (Biosource International, Inc., Camarillo, CA). Absorbance values were converted to the concentration of each cytokine in the BAL fluid (pg per ml) by interpolation to the respective standard curve.

Flow cytometric analysis

BALF suspensions were centrifuged, and the pellets were used for flow cytometry to detect CD3, CD4, CD8, and NK1.1 cell expression. After blocking with 5% bovine serum albumin, the cells were stained with purified anti-mouse CD3 (PharMingen International, San Diego, CA, USA), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (L3T4), anti-mouse CD8 (Ly-2), and R-phycoerythrin (R-PE)-conjugated anti-mouse NK1.1 (NKR-P1B and NKR-P1C) monoclonal antibodies (BD Biosciences Pharmingen, USA) for 20 min at 4°C. Thereafter, the cells were fixed in 2% paraformaldehyde/PBS, examined with by FACS (FACScan, BD, San Jose, CA), and analyzed with CELLQUEST software (PharMingen, San Jose, CA, USA). The results were expressed as the mean fluorescence intensity (MFI).

Histological examination

The mice were sacrificed by intraperitoneal administration of pentobarbital. Two mice were used from each group. The lungs were surgically removed, fixed with 20% formalin

and embedded in paraffin. Three-micrometer-thick sections were stained with hematoxylin and eosin (H-E) and periodic acid-Schiff (PAS), as reported previously [18,21]. Histopathological changes were examined under by light microscopy. The presence of peribronchial inflammatory cells and PAS-positive cells in airway tissue was expressed as follows: –, no evidence of discrete inflammation; +, mild, occasional infiltration of inflammatory cells along the airways; ++, moderately dense infiltrates of inflammatory cells; +++, moderate to severe infiltration of inflammatory cells; +++, severe, diffuse infiltration of inflammatory cells and airway obstruction.

Analysis of cytokine and chemokine mRNA expression in the lung

Reverse transcription-polymerase chain reaction (RT-PCR) was utilized to examine the expression levels of interleukin [IL]-4, IL-5, IL-10, IL-12p40, IL-13, interferon-gamma [IFN- γ], regulated upon activation, normal T expressed and secreted [RANTES], eotaxin, thymus and activation-regulated chemokine [TARC], macrophage-derived chemokine [MDC] and macrophage chemotactic protein-1 [MCP-1] messenger RNA (mRNA) in the lung tissues. Lung tissues were obtained from three mice in each group, rapidly dissected, snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted using TRIzol Reagent (Invitrogen Carlsbad, CA) according to the manufacturer's instructions. Preparation of complementary DNA (cDNA) and PCR were performed as described previously [21–23]. After RNA isolation, total RNA was reverse-transcribed into cDNA with M-MLV reverse transcriptase (Gibco BRL) after measurement of the total

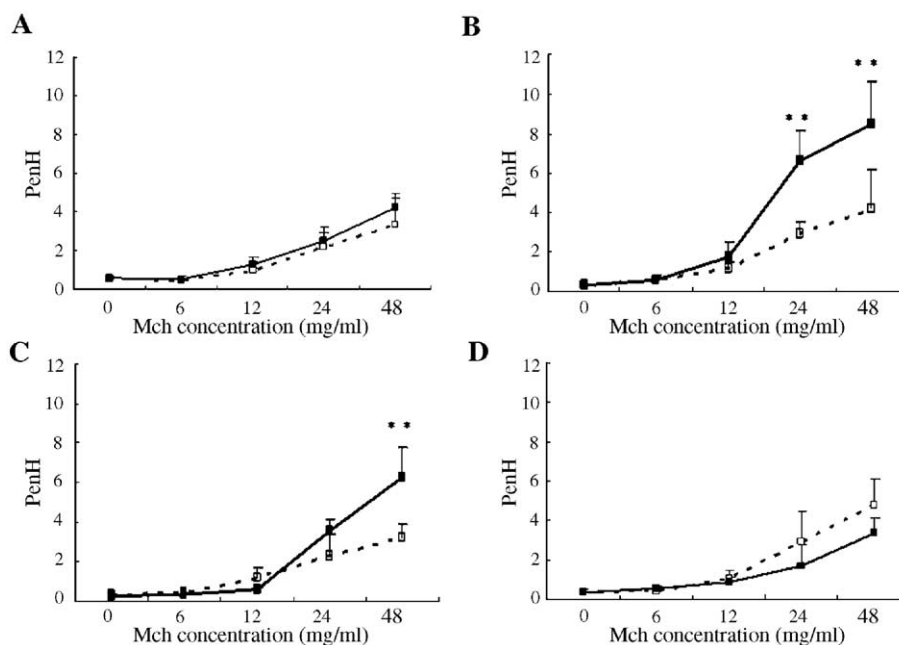


Figure 2 Airway responsiveness to methacholine assessed by whole-body plethysmography. Dose–response curves after 1-day-exposure (A), 1-week exposure (B), 4-week exposure (C) and 8-week exposure to DE (E) are presented. Horizontal axis; concentration of methacholine challenged (mg/ml), and vertical axis; airway reactivity shown in PenH. OVA–challenged DE exposure group (—■—) showed a significantly increased bronchoconstriction, when compared with OVA–challenged, clean air exposure group (—□—). ** $p < 0.001$, OVA–clean air vs. OVA–DE.

RNA concentration. The sequences of the primer sets and PCR conditions are shown in Table 1. Amplification was carried out with a DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, CT, USA). The PCR products were subjected to 4% agarose and NuSieve GTG (1:3) gel electrophoresis in $1\times$ TAE (Tris-HCl, acetic acid, EDTA) buffer at 135 V for 30 min, and visualized by fluorescence under ultraviolet (UV) light after staining with 5.0 $\mu\text{g}/\text{ml}$ ethidium bromide. The expression level of the β -actin gene was used as an internal control in RT-PCR analysis. Thereafter, we conducted densitometric analysis of the electrophoretic RT-PCR results using Beta 4,0,2 Scion Image software (which is based on NIH Image version 1.62). Relative densitometric ratios were determined with β -actin mRNA as an internal control, as reported previously [24,25].

Statistical analysis

The data were expressed as the mean $\pm$ standard deviation (SD) and analyzed for between-group differences by Newman-Keuls test. Differences at $p < 0.05$ were considered significant.

Results

DE increase AHR in OVA-induced asthma models

The magnitude of AHR was evaluated by increase in PenH as described in the Methods and two kinds of parameters were used; the airway reactivity to the documented dose of methacholine, and airway sensitivity as PC200 calculated by dose-response curves. Exposure to DE significantly increased the airway reactivity to methacholine (24 and 48 mg/ml) after 1 and 4 weeks, as shown in Fig. 2 and Table 2. The airway sensitivity as evaluated by PC200 was significantly increased after 1 week of DE exposure, but not after 4 and 8 weeks (Table 2).

Differential cell counts in BAL fluid

In OVA-induced asthmatic mice, the total number of cells in BAL fluid was increased, reaching a peak 7 days after DE exposure (Fig. 3A). This increase was mostly due to the increase in the number of eosinophils (Fig. 3B). DE exposure had no effect on the numbers of total cells or eosinophils in BAL fluid throughout the course ($p > 0.05$). This was also the

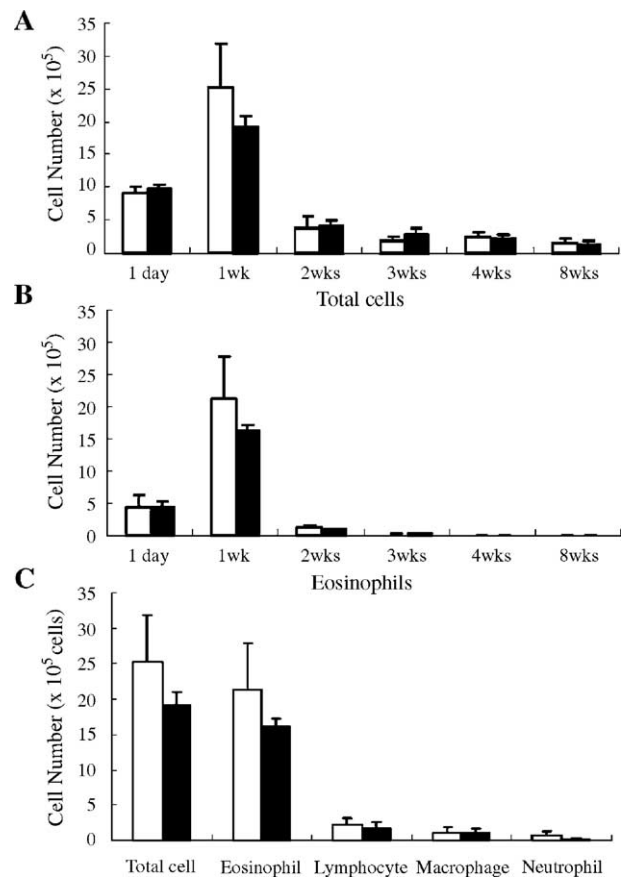


Figure 3 Differential cell counts in BALF. (A) Total number of cells and (B) the number of eosinophils in BAL fluid. Horizontal axis, the periods of DE or clean air exposed, and vertical axis, cell number ($\times 10^5$). Open bars show clean air exposure group, and black bars show DE exposure group. (C) Differential cell count in BALF after 1-week DE exposure. DE exposure had no effect on the number of total cells, eosinophils or other kinds of the cells in BAL fluid at any times ($p > 0.05$).

case for the number of neutrophils, lymphocytes and macrophages (Fig. 3C, after 1 week of DE exposure, $p > 0.05$). Flow cytometric analysis of lymphocyte surface markers revealed that DE exposure did not affect the number of CD3-, CD4-, CD8- or NK1.1-positive cells at any time point (data not shown).

Table 2 PenH and PC200 by Mch Challenge Test ($n=6$, mean $\pm$ SD)

	Group	1 days	1 week	4 weeks	8 weeks
PenH (Mch24 mg/ml)	Clean	2.18 $\pm$ 1.00	2.89 $\pm$ 0.65	2.28 $\pm$ 1.13	2.92 $\pm$ 1.53
	DE	2.50 $\pm$ 0.41 ^{n.s.}	6.66 $\pm$ 1.49*	3.57 $\pm$ 0.54 ^{n.s.}	1.71 $\pm$ 1.06 ^{n.s.}
PenH (Mch48 mg/ml)	Clean	3.34 $\pm$ 1.56	4.19 $\pm$ 2.01	3.19 $\pm$ 0.67	4.77 $\pm$ 1.35
	DE	4.24 $\pm$ 0.46 ^{n.s.}	8.45 $\pm$ 2.23**	6.22 $\pm$ 1.54**	3.36 $\pm$ 0.81 ^{n.s.}
PC200	Clean	14.55 $\pm$ 5.74	8.62 $\pm$ 2.31	9.40 $\pm$ 4.19	7.20 $\pm$ 2.72 ^{n.s.}
	DE	12.78 $\pm$ 3.74 ^{n.s.}	6.00 $\pm$ 0.78*	9.62 $\pm$ 1.10 ^{n.s.}	8.40 $\pm$ 2.72 ^{n.s.}

Clean: OVA+clean air exposure group.

DE: OVA+DE exposure group.

n.s.: not significant.

* $p < 0.01$ OVA+clean air exposure group versus OVA+DE exposure group.

** $p < 0.001$ OVA+clean air exposure group versus OVA+DE exposure group.

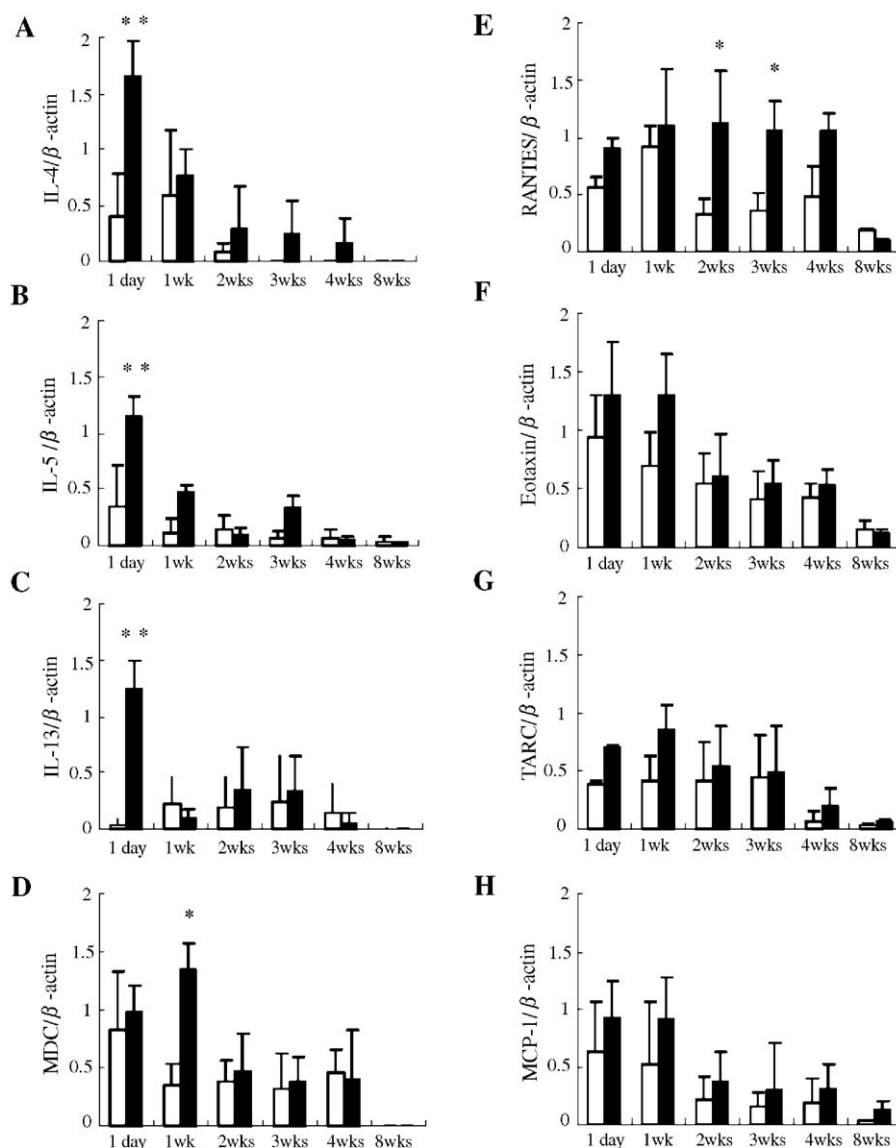


Figure 4 RT-PCR data of cytokine and chemokine mRNA expression in lung tissues. (A) IL-4, (B) IL-5, (C) IL-13, (D) MDC, (E) RANTES, (F) Eotaxin, (G) TARC and (H) MCP-1 mRNA levels are presented. Densitometric ratios of electrophoretic RT-PCR results were standardized with β -actin mRNA as an internal control (vertical axis). Open bars are of clean air exposure group, and closed bars are of DE exposure group. ** $p < 0.001$ OVA—clean air vs. OVA—DE. * $p < 0.05$ OVA—clean air vs. OVA—DE.

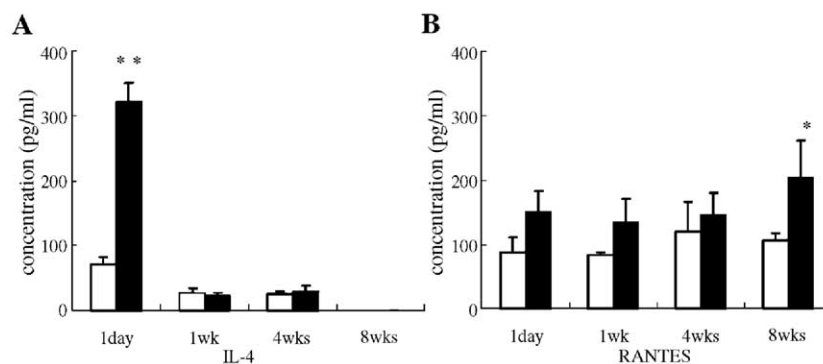


Figure 5 IL-4 (A) and RANTES (B) levels in BAL fluid as evaluated by ELISA. Open bars: clean air exposure group, closed bars: DE exposure group. ** $p < 0.001$ OVA—clean air vs. OVA—DE. * $p < 0.01$ OVA—clean air vs. OVA—DE.

Cytokine and chemokine mRNA levels determined by RT-PCR

After 1 day of DE exposure, the mRNA levels of IL-4, IL-5, and IL-13 were increased significantly compared with those in mice exposed to clean air ($p < 0.001$), but longer periods of DE exposure failed to increase the mRNA levels of these Th-2 cytokines (Figs. 4A–C). The mRNA levels of MDC were significantly increased after 1-week of DE exposure (Fig. 4D), but no significant differences at other time points. In contrast, mRNA levels of RANTES were significantly increased after 2 and 3 weeks of DE exposure ($p < 0.05$) (Fig. 4E). The expression levels of the other cytokines and chemokines studied did not show any significant difference between the two groups (Figs. 4F–H).

Measurement of cytokines in BAL fluid by ELISA

The protein levels of IL-4 and RANTES in BAL fluid were measured. After 1 day of DE exposure, the level of IL-4

protein was increased significantly compared with clean air exposure ($p < 0.001$), and then decreased with time (Fig. 5A). The level of RANTES protein increased progressively as DE exposure continued, and after 8 weeks, the level was significantly elevated in comparison with the clean air group ($p < 0.01$) (Fig. 5B).

Lung histopathology

Tissue sections were prepared from dissected mouse lungs and observed for histopathological changes by light microscopy. In OVA-sensitized and challenged mice, there was intense peribronchial and perivascular infiltration of inflammatory cells, which peaked at 1 week after OVA challenge, and then decreased gradually. DE exposure did not significantly increase the number of peribronchial inflammatory cells (Figs. 6A–D and Table 3). In OVA-challenged mice, there was an increased number of cells that were positive for mucin staining (PAS-positive cells) in the airways, reaching a peak 1 week after allergen challenge. DE exposure had no effects on the number of PAS-positive cells, as shown in Figs. 6E–H and Table 3.

Discussion

In the present study, we showed that repeated exposure to low-dose DE in murine asthma models resulted in increased AHR and gene expression of several pro-asthmatic cytokines and chemokines, although these effects gradually subsided despite prolonged exposure to DE. These findings suggest that DE at a low concentration compatible with that in the actual environment may induce acute, transient exacerbation of asthma. Many experimental studies have shown that DE or DEPs aggravate allergic airway inflammation and AHR [13]. However, most of those studies were conducted to demonstrate the potential of DEPs or DE to induce toxicological effects, and therefore the animals were generally exposed to a high dose of DEPs or DE (i.e., $\sim 3 \text{ mg/m}^3$) [26], or via non-physiological routes (i.e., intratracheal injection of suspended particles) [7,11]. Moreover, since most of the previous experimental studies were designed to expose animals to DE throughout the periods of both sensitization and allergen challenge, it still remains unclear whether DE exposure actually exaggerates airway responses in asthmatic animals. We found a significant increase in AHR after exposure to DE at a much lower concentration ($100 \text{ }\mu\text{g/m}^3$) than previously reported. We further studied the effects of prolonged DE exposure for up to 8 weeks, and found that the effects were not persistent, but subsided after a short interval.

Interestingly, the magnitude of allergic airway inflammation in the group exposed to DE, as evaluated by differential BAL cell counts and lung histology, did not differ from that in the control groups. Hao and associates showed that aerosolized DEPs (2 mg/m^3) exerted acute effects on airway inflammation and AHR in murine allergen provocation models [27]. In their study, delayed (i.e., post-allergen challenge) DEP delivery increased AHR, but did not have a significant effect on differential BAL cell counts. Although they used much higher concentration of DE (2 mg/m^3) with different method of administration (nebulizer), their findings suggest that DE exposure could induce AHR without

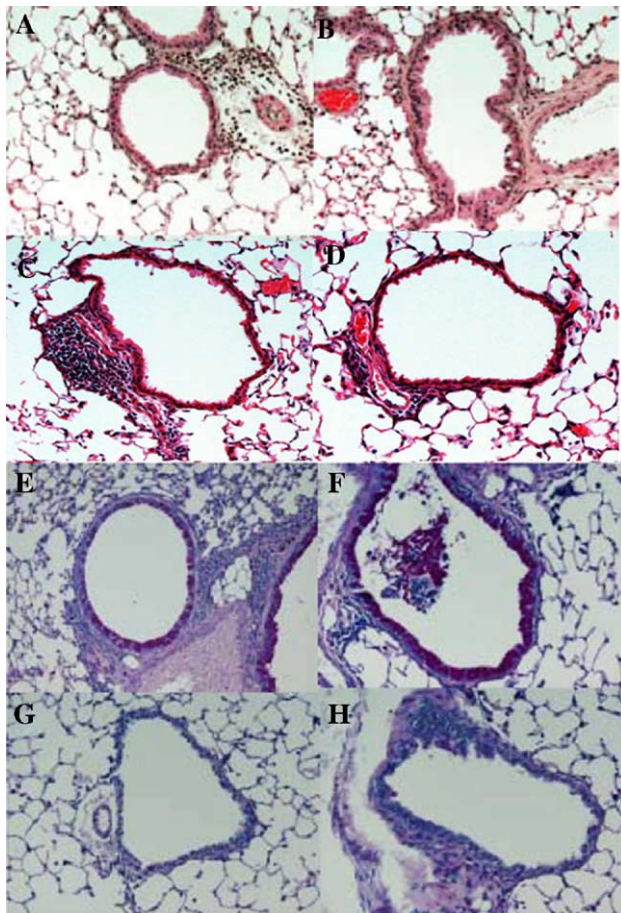


Figure 6 Histopathology of the lung tissues. Lung sections were stained by hematoxylin and eosin staining (A, 1-week clean air-exposed mice; B, 4-week clean air-exposed mice; C, 1-week DE exposed mice; D, 4-week DE exposed mice; original magnification 200 $\times$) and Periodic acid-Schiff staining (E, 1-week clean air-exposed mice; F, 4-week clean air-exposed mice; G, 1-week DE exposed mice; H, 4-week DE exposed mice; original magnification 200 $\times$).

Table 3 Degree of airway inflammatory changes ($n=2$, mean $\pm$ SD)

	Group	2 days	7 days	28 days
Peribronchial inflammatory cells	Clean air	++	+++	+
	DE	++	+++	+
Mucous cells (PAS-positive cells) (cell No./mm)	Clean air	121.4 $\pm$ 35.0	153.6 $\pm$ 37.5	67.2 $\pm$ 23.1
	DE	100.8 $\pm$ 40.0 ^{n.s.}	180.8 $\pm$ 27.8 ^{n.s.}	74.8 $\pm$ 40.1 ^{n.s.}

n.s.: not significant compared to clean air control.

OVA + clean air exposure group versus OVA + DE exposure group.

affecting the magnitude of airway inflammation, which are consistent with our results.

Our results demonstrated that exposure to DE exaggerated the expression of certain pro-asthmatic cytokines and chemokines in lung tissue, which play an important role in asthmatic airway responses. We found the significant increase in the mRNA levels of IL-4, IL-5, and IL-13, MDC and RANTES, but not of eotaxin, TARC or MCP-1 (Fig. 4). Diaz-Sanchez and associates [28] studied the effects of intranasal administration of DEP on local cytokine production in patients with allergic rhinitis. They found that there was a significant increase in RANTES, MIP-1 α and MCP-3, but not in eotaxin, suggesting that the effects of DE on cytokine/chemokine expression were not global or non-specific. Rao et al. [29] studied the time course of gene expression of a variety of cytokines in rat model. They found that DE exposure upregulated several genes of cytokines, but the each of the time course was quite different; a certain cytokine mRNA levels persist, some showed a acute, mono-phasic change, and the other showed biphasic induction. The molecular mechanisms of these phenomena remain unclear, but they speculated that various post-transcriptional as well as transcriptional regulation could be involved.

It is generally speculated that such an increase in the expression of Th2 cytokines and Th2-directed chemokines is followed by local recruitment of corresponding cells. However, in our studies, DE exposure did not have significant effects on cell infiltration as evaluated by BAL and histology. The reason for this apparent discrepancy is unknown. Kuperman and associates [30] studied the role of IL-13 in the development of asthma by using mice lacking a signal transducer and activator of transcription 6 (STAT6), and showed that reconstitution of STAT6 only in epithelial cells was sufficient for IL-13-induced AHR and mucus production in the absence of inflammation, fibrosis or other lung pathology, and, therefore it is probable that the increased expression of these cytokines induced by DE might have been involved in the increase of AHR we observed in our study. We have shown recently that DEPs directly induce Th2 cytokine predominance such as IL-4 in mice after low-dose exposure (100 μ g/m³ for 1 month) [18]. We further examined the changes in cytokine profiles in the lung after prolonged exposure to DE for 3 months [21]. Levels of mRNA for TNF- α , IL-12 p40, IL-4, IL-10, and further NF- κ B transcription factor were increased, with no significant changes in BAL cell counts. Asthma is a complex condition characterized by variable airflow obstruction, airway hyper-responsiveness (AHR), and Th2-mediated chronic inflammation. However, the relationship between inflammation and AHR is complex, with no good correlation between the

severity of inflammation and the severity of AHR. In addition, there are a number of conditions, such as eosinophilic bronchitis and allergic rhinitis, in which there is a Th2-mediated inflammatory response, but no AHR. Bronchoconstriction can also occur without obvious airway inflammation [31].

Although there is much evidence from animal studies that DE and/or DEPs induce both Th-2-type allergic inflammatory responses and increased airway responsiveness to allergens and non-specific stimuli, there is still some controversy about the impact on the pathogenesis of clinical asthma. In the present study, the degree of AHR as well as airway inflammation subsided spontaneously in animals exposed to both DE and clean air. Therefore, it will be necessary to study the effects of DE in more chronic asthma models receiving allergen challenges intermittently while DE exposure continues [32], and such trials are currently underway.

Another issue of importance is a potential difficulty which exists in extrapolation from animal studies to human pathogenesis. Holgate et al. and Stenfors et al. [33,34] reported that subjects with mild asthma showed an increase in airway resistance of similar magnitude after DE exposure, but DE did not induce a neutrophilic response or exacerbate their pre-existing eosinophilic airway inflammation. These findings raise the possibility of adaptation mechanisms to DE in humans. In contrast, Wiester et al. [35] reported that there exists an adaptation mechanism to ozone in mice and rats, whereas such might not be the case in humans [36]. Therefore, it should be kept in mind to carefully assess the species differences for the evaluation of protection policy for human lung health.

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