

RESEARCH ARTICLE

The effects of oxidative stress induced by prolonged low-dose diesel exhaust particle exposure on the generation of allergic airway inflammation differ between BALB/c and C57BL/6 mice

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Abstract

We have recently reported that airway inflammatory responses to the oxidative stress induced by prolonged low-dose diesel exhaust particle (DEP) exposure differ markedly between BALB/c and C57BL/6 mice. In the present study, the effects of genetic differences in the response to prolonged low-dose DEP exposure on the generation of ovalbumin-induced allergic airway inflammation were further explored using the same mouse strains. In BALB/c mice, eosinophils and mucous goblet cells in histopathological pulmonary specimens increased significantly after DEP exposure, and were more marked than in C57BL/6 mice. Interleukin (IL)-5 and IL-13 levels in bronchoalveolar lavage (BAL) fluid were increased significantly by DEP exposure only in BALB/c mice. The DEP-induced increases in peribronchial eosinophils and mucous goblet cells in the lung tissues, and of IL-5 and IL-13 in the BAL fluid, were significantly attenuated by the antioxidant N-acetylcysteine. Thus, the effects of prolonged low-dose DEP exposure on the generation of allergic airway inflammation differed markedly between the mouse strains. These differences may be caused by different antioxidant responses to the oxidative stress induced by DEP exposure. Our results contribute more information to the search for genetic susceptibility factors in the response to DEP, and may thus assist in the discovery of new biomarkers for DEP-related disease.

Keywords: Prolonged low-dose DEP exposure; oxidative stress; mouse strain; allergic airway inflammation

Introduction

Epidemiologic studies conducted throughout the world have demonstrated an important association between ambient levels of motor vehicle traffic emissions and increased symptoms of asthma and rhinitis. 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)

Since diesel exhaust particles (DEP) are the major components of ambient fine particulate matter (i.e., particulate matter less than 2.5 μm , PM_{2.5}), especially in urban areas, many *in vivo* studies have been performed to clarify the association between DEP and these disorders.^(3–5) We have previously reported that continuous low-dose DEP exposure (i.e., at concentrations similar to those inhaled outdoors) exaggerates asthmatic responses in mice.⁽⁶⁾ Moreover, adjuvant effects caused by DEP exposure result in allergic airway inflammation.⁽⁷⁾

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Recent investigations indicate that susceptibility to the adverse effects of pollutants is an intrinsic trait most probably related to genotype.⁽⁸⁾ It has also been reported that the airway inflammatory response to DEP^(7,9) varies greatly among mouse strains with different genetic backgrounds. In pilot experiments for this study, we found that low-dose DEP exposure induces airway inflammatory responses that differ among strains, and that these differences may be caused by differences in susceptibility to the oxidative stress induced by DEP.^(10,11) These initial results suggested that C57BL/6 mice were more sensitive to DEP than BALB/c mice.

On the other hand, it has been reported that there are also differences among mouse strains as regards allergic airway inflammation, and that BALB/c mice are more sensitive to allergens than C57BL/6 mice, as measured by their airway inflammatory responses.^(12,13) However, the effects of mouse strain differences in airway inflammatory responses to prolonged low-dose DEP exposure (i.e., at concentrations similar to those inhaled outdoors) on the generation of allergic airway inflammation have not been reported.

Based on previously obtained results, we selected BALB/c and C57BL/6 mice, which differ in their genetic backgrounds,^(11,14,15) for our present study. A feature of our experimental design was that the mice were born in DEP-polluted areas, and thus DEP exposure started at birth (day zero). Genetically based differences in the responses to prolonged (6 months) low-dose (ca. 100 µg/m³) DEP exposure on the generation of ovalbumin (OVA)-induced allergic airway inflammation were then examined to explore the factors underlying susceptibility to DEP exposure, and hence to discover new biomarkers.

Methods

Animals

Pregnant BALB/c and C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan) and newborn mice from these strains were used for the experiments. The mice were randomly grouped, and were housed in

plastic cages with plywood chips. The cages were placed in DEP exposure chambers. All experimental groups contained 6 female mice, unless otherwise noted in the figure legends. All procedures conformed to National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Experimental design (Figure 1)

The study design is shown in Figure 1. Both mouse strains were exposed to low-dose DEP (DEP+OVA- and DEP+OVA+N-acetylcysteine [NAC]-treated groups) or clean air (OVA group) for a maximum of 24 weeks. After 21 weeks of DEP exposure, allergic airway inflammation was generated in the mice by intraperitoneal sensitization with OVA followed by intranasal challenge. The DEP+OVA+NAC group (BALB/c mice) was treated with NAC from week 19 of DEP exposure (2 weeks before OVA sensitization) to week 23 of DEP exposure (1 week before OVA challenge). (INDENT) Twenty-four hours after the OVA challenge, we assessed the mice using various parameters. Airway hyperresponsiveness was measured in all experimental groups immediately. BALB/c and C57BL/6 mice were sacrificed at the same time in all experimental groups. The histopathology of the lung tissues, the cell populations in bronchoalveolar lavage (BAL) fluid, the concentrations of inflammatory cytokines in the BAL fluid, immunoglobulin (Ig)E, IgG₁, and IgG₂ levels in the serum and the expression of antioxidant enzyme mRNA in the lung tissues were examined.

DEP exposure

The *in vivo* DEP exposure system has been described previously.⁽¹⁶⁾ The concentration of DEP was monitored and kept at a low level (Ca. 100 µg/m³). BALB/c and C57BL/6 mice were exposed to DEP for 7 h per day and 5 days per week.

OVA sensitization and provocation procedures

Allergic airway inflammation was generated in the mice as described previously.^(6,17) Briefly, the mice were

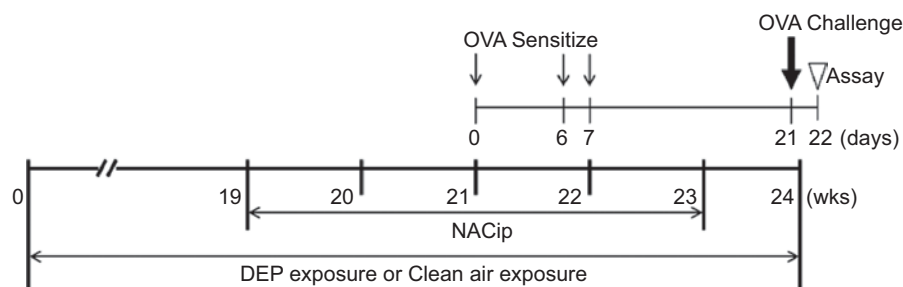


Figure 1. Experimental design. Further details are described in the METHODS section. DEP, diesel exhaust particle; OVA, ovalbumin; NAC, N-acetylcysteine.

injected intraperitoneally with 20 µg OVA (Sigma, St. Louis, MO, USA), adsorbed onto 2 mg alum and diluted with 0.5 ml physiological saline, on days 0 (after 21 weeks of DEP exposure), 6 and 7. Two weeks later (i.e., on day 21), the mice were challenged intranasally with OVA 0.1 mg in 0.1 ml physiological saline.

Treatment with NAC

NAC (Sigma, St. Louis, MO, USA) was dissolved in distilled water (3 mmol/kg body weight, pH 7.0) and administered intraperitoneally to the DEP+OVA+NAC group (BALB/c mice) on alternate days.

Assessment of airway hyperresponsiveness

Airway hyperresponsiveness was assessed by whole-body plethysmography with free moving application (Buxco Electronics, Troy, NY, USA) as described previously.⁽¹⁰⁾

Histological analysis

For the histological examination, 10% formalin-fixed lung tissues were embedded in paraffin and sliced into sections. The sections were stained with hematoxylin and eosin (HE), and systematically examined using a light microscope (Eclipse E800, Nikon, Tokyo, Japan). The tissue sections were also stained with periodic acid-Schiff (PAS) stain to identify mucous goblet cells.

BAL and cell differentials in BAL fluid

BAL was performed as described previously.^(10,11) Briefly, the lungs were lavaged three times with 1.0 ml physiological saline via a tracheal cannula. The ratios of the recovered lavage fluid volumes ranged from 85% to 90% and did not differ significantly among the groups. The recovered BAL fluid was immediately centrifuged, and the total number of cells in the BAL fluid was counted using a hemocytometer. Differential cell counts were obtained from cytospin smear preparations (Lab Systems; Tokyo, Japan) using Diff-Quick Romanowski staining (Muto Kagaku Co., Tokyo, Japan). Differential counts were performed on 200 cells per smear.

Measurement of cytokines in BAL fluid

Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-12, interferon(IFN)-γ, IL-4, IL-5, and IL-13 levels in the BAL fluid supernatants were measured in triplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Inc., Camarillo, CA, USA). ELISA was carried out according to the manufacturer's instruction sheet.

Measurement of IgG₁, IgG_{2a} and IgE in serum

IgG₁, IgG_{2a} (Cygnus Technologies, Inc., Southport, NC, USA), and IgE (Bethyl Laboratories, Inc., Montgomery, TX, USA) levels in the serum were measured in triplicate using an ELISA kit. ELISA was carried out according to the manufacturer's instruction sheet.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed as described previously.⁽¹¹⁾ Briefly, total RNA was extracted from the lung tissues (n = 3) with TRIzol Reagent (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. cDNA generation and PCR were performed as described previously.⁽¹⁶⁾ The cDNA was quantified using a sequence detector (7900HT Sequence Detection System, Applied Biosystems) using PCR Master Mix (TaqMan Universal PCR Master Mix, Applied Biosystems) and primer sets of the heme-oxygenase-1 (HO-1), glutathione-S-transferase (GST)-M1, and β-actin genes (the assay IDs of the primers were Mm00516004_m1, Mm00833915_g1, and Mm00607939_s1, respectively, Applied Biosystems).

Statistical analysis

All results are shown as means ± SD. Differences between groups were evaluated using the unpaired Student's *t* test with Stat Mate III software (ATMS Digital Medical Station, Tokyo, Japan). *P* values less than 0.05 were considered significant.

Results

Airway hyperresponsiveness to methacholine

Airway hyperresponsiveness (expressed in Penh values) was assessed by whole-body plethysmography. In the OVA group (OVA-sensitized and -challenged mice), the BALB/c mice were more sensitive to methacholine than the C57BL/6 mice. However, there were no marked changes in Penh values after DEP exposure in either the BALB/c or the C57BL/6 mice compared with their counterparts in the OVA group (Figure 2).

Lung histopathology

Tissue sections were prepared from the dissected mouse lungs and histopathological changes were examined by light microscopy. In the OVA group, there was peribronchial and perivascular infiltration of inflammatory cells, particularly eosinophils, in both the BALB/c and C57BL/6 mice. Infiltration was more markedly

increased by DEP exposure in the BALB/c mice than in the C57BL/6 mice (Figure 3A). Furthermore, mucin-producing cells (PAS-positive cells) were apparent in the airways of both the BALB/c and C57BL/6 mouse strains in the OVA group, and their numbers were increased more markedly after DEP exposure in the BALB/c mice than in the C57BL/6 mice (Figure 3B).

BAL cell differentials

Total cell and macrophage counts in the BAL fluid after DEP exposure were significantly higher in C57BL/6 mice and lower in BALB/c mice. The numbers of neutrophils and lymphocytes in the BAL fluid after DEP exposure

were significantly higher in the BALB/c mice, while no changes were seen in the C57BL/6 mice. The number of eosinophils in the BAL fluid was significantly higher in both mouse strains after DEP exposure (Figure 4).

Cytokine levels in BAL fluid

Levels of IL-5 and IL-13 in the BAL fluid after DEP exposure were significantly higher only in the BALB/c mice

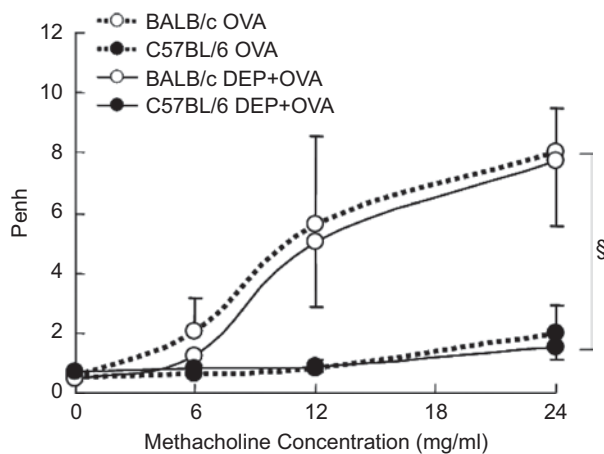


Figure 2. Airway hyperresponsiveness to inhaled aerosolized methacholine (0, 6, 12, and 24 mg/ml) was assessed in both BALB/c and C57BL/6 mice by whole-body plethysmography with free moving application. The results are expressed as means $\pm$ SD (in Penh values) for each group ($n = 6$). $\$P < 0.05$, BALB/c versus C57BL/6 mice at each methacholine concentration level (6, 12, and 24 mg/ml).

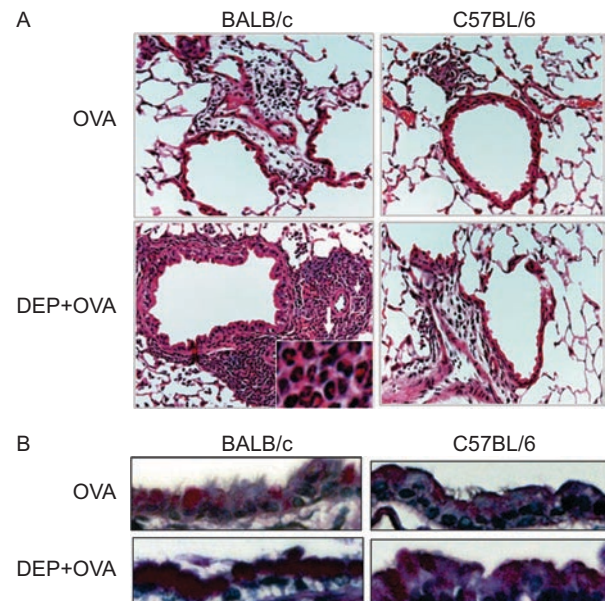


Figure 3. Histopathology of the lung tissues. Lung tissue sections from each experimental group (consisting of 3 BALB/c and 3 C57BL/6 mice) were stained with HE (3A) and PAS stain (3B), and examined by light microscopy (original magnification, 200x). Inset is a magnification of the area within the square on the photomicrograph for the BALB/c DEP + OVA group. Eosinophils are stained red with HE stain.

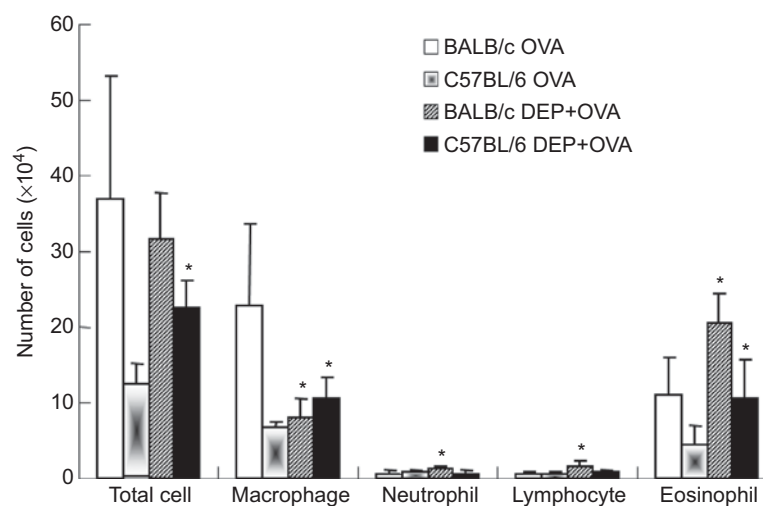


Figure 4. Changes in total and differential cell counts in BAL fluid. The results are expressed as means $\pm$ SD for each group ($n = 6$). $*P < 0.05$, compared with the OVA group.

(Fig. 5). The levels of IL-1 β , TNF- α , IL-12, IFN- γ , and IL-4 in the BAL fluid after DEP exposure did not differ significantly between the C57BL/6 and BALB/c mouse strains (data not shown).

IgG₁, IgG_{2a}, and IgE levels in serum

Levels of IgG₁, IgG₂, and IgE in the sera did not change markedly in either the C57BL/6 or the BALB/c mouse strain in response to DEP exposure (data not shown).

Induction of antioxidant enzyme-related genes in the lung tissues

The expression of GST-M1 mRNA in the lung tissues, expressed as a percentage of that in the respective OVA-treated group, was significantly lower in the BALB/c mice (mean $\pm$ SD: 68% $\pm$ 34%) than in the C57BL/6 mice (mean $\pm$ SD: 130% $\pm$ 43%). There was no statistically significant difference between the two mouse strains in the expression level of HO-1 mRNA in the lung tissues (Figure 6).

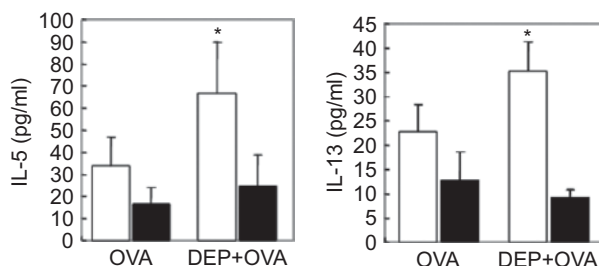


Figure 5. Changes in cytokine levels in BAL fluid. The white bar indicates BALB/c mice; the black bar indicates C57BL/6 mice. The results are expressed as means $\pm$ SD for each group ($n = 6$). * $P < 0.05$, compared with the OVA group.

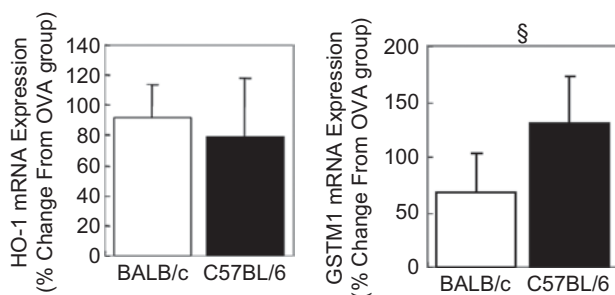


Figure 6. HO-1 and GST-M1 mRNA expression in the lung tissues, as assessed by real-time PCR. The percentage changes in the expression levels of mRNA in the BALB/c and C57BL/6 mice after DEP exposure, compared with the OVA group, are shown. The results are expressed as means $\pm$ SD for 3 mice in each group. § $P < 0.05$, BALB/c vs C57BL/6 mice.

Effects of NAC on the responses to DEP exposure in BALB/c mice

The effects of NAC on the allergic airway inflammation induced by OVA sensitization and -challenge in DEP-exposed BALB/c mice were examined. The intense peribronchial and perivascular infiltration of eosinophils was markedly inhibited by NAC treatment, and the number of PAS-positive cells in the airways was also markedly reduced (Figure 7A). The increases in the numbers of eosinophils, neutrophils and lymphocytes in the BAL fluid induced after DEP exposure was significantly decreased by NAC treatment (Figure 7B). The increases in the levels of IL-5 and IL-13 in the BAL fluid induced by DEP exposure were also significantly lowered by NAC treatment (Figure 7C).

Discussion

In the present study, we used BALB/c and C57BL/6 mouse strains, which have different genetic backgrounds,^(11,14,15) to examine the impact of genetic differences on the generation of allergic airway inflammation as a response to prolonged low-dose DEP exposure. Our results suggest that the role of long-term, low-dose DEP exposure in the generation of allergic airway inflammation differs markedly between these mouse strains, and that the difference may be caused by different responses to the oxidative stress induced by DEP exposure.

We have recently reported that airway inflammatory responses to prolonged low-dose DEP exposure differ between BALB/c and C57BL/6 mouse strains, and that C57BL/6 mice are more susceptible to DEP exposure than BALB/c mice.⁽¹¹⁾ However, in the present study, it was interesting that BALB/c mice were more susceptible to prolonged low-dose DEP exposure than C57BL/6 mice as regards the generation of OVA-induced allergic airway inflammation.

In vitro experimental studies have focused on the role of reactive oxygen species (ROS) generated by exposure to DEP and the subsequent generation of an oxidative stress response within exposed cells.⁽¹⁸⁻²³⁾ Our pilot study also showed that mouse strain differences in airway responsiveness to low-dose DEP exposure may be caused by oxidative stress induced by DEP.^(10,11) ROS have been reported to be involved in the adjuvant effects of DEP in a murine OVA sensitization model.⁽²⁴⁾ Based on these reports, we designed our experiments to test the hypothesis that ROS is also involved in the different responses of the BALB/c and C57BL/6 strains to long-term, low-dose DEP exposure as regards the generation of allergic airway inflammation. Thus, the effects of the antioxidant NAC on allergic airway inflammation induced in OVA-sensitized and -challenged

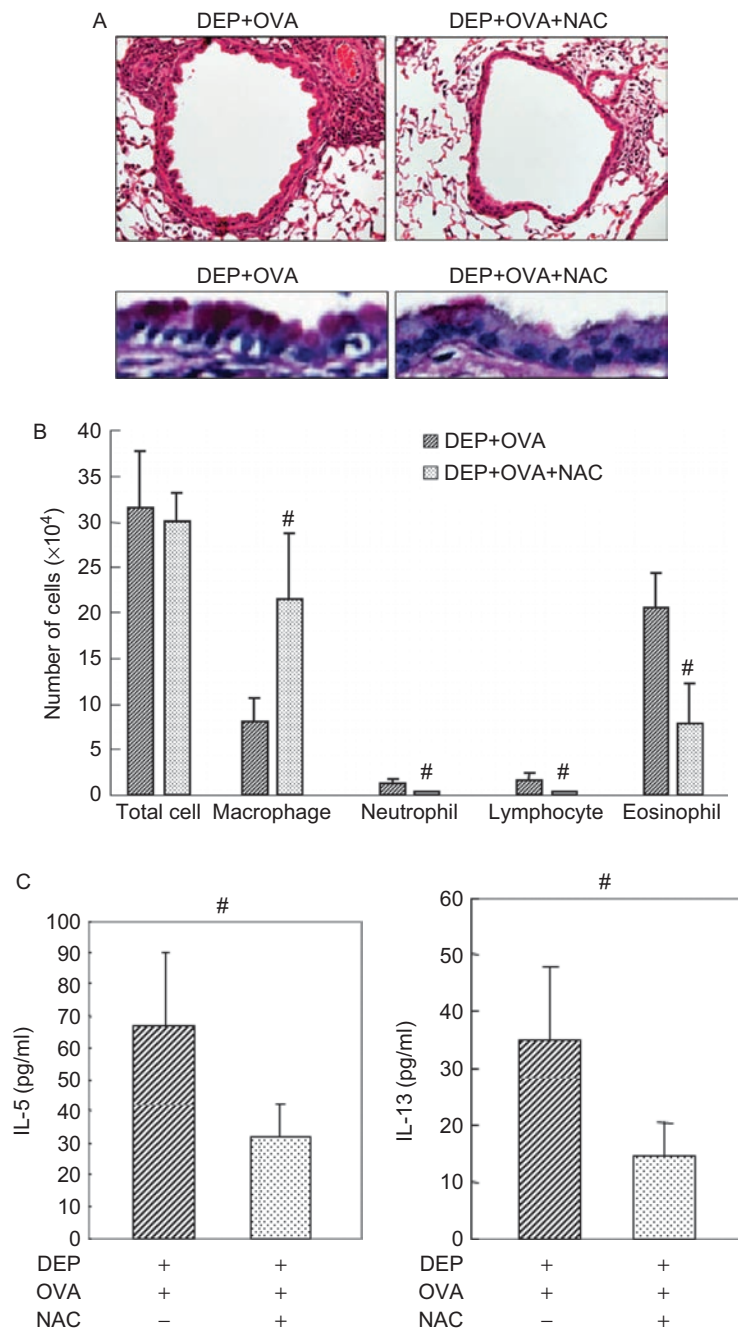


Figure 7. Amelioration of DEP- and OVA-induced airway inflammatory responses in BALB/c mice by NAC treatment. Histopathological changes in the lung tissues after NAC treatment (A). Changes in the total and differential inflammatory cell populations (B) and the levels of cytokines (C) in BAL fluid after NAC treatment. The results are expressed as means $\pm$ SD for each group (n = 6). # $P < 0.05$, compared with the DEP + OVA group.

BALB/c mice which had been exposed to DEP were examined.

Our results showed that NAC treatment significantly reduced the increases in peribronchial eosinophils and mucous goblet cells seen in lung tissues, and the increases in IL-5 and IL-13 seen in BAL fluid, after DEP exposure. (INDENT)There is ample evidence that allergic disorders, including asthma, are mediated by oxidative stress,⁽²⁵⁾ and oxidative stress is thought to play an

important role in the pathogenesis of asthma.⁽²⁶⁾ NAC is also an effective treatment for the pulmonary inflammation seen in experimental asthma. It is most effective when administered 7 days before OVA challenge, with the last dose given 1 h before OVA challenge, in allergen-driven asthmatic inflammation.⁽²⁷⁾ In the present study, to investigate the potential therapeutic effect of NAC after DEP exposure, NAC was given one week before OVA challenge.

Host responses to DEP are regulated by a balance between antioxidant defenses and proinflammatory responses.⁽²⁸⁾ Our results showed that the expression of an antioxidative stress-related enzyme, GST-M1, in the lung tissues of BALB/c mice was significantly reduced compared with C57BL/6 mice after DEP exposure, but that the expression of another such enzyme, HO-1, remained unchanged. Thus, HO-1 may contribute only during the early stages of DEP exposure.^(10,11)

Complex host responses to prolonged DEP exposure may be involved in the generation of allergic airway inflammation in this experimental system. It is clear that antioxidative mechanisms involved in the difference between the BALB/c and C57BL/6 mouse strains as regards the generation of allergic airway inflammation as a response to low-dose, long-term DEP exposure.

Although it has been reported that DEP-induced oxidative stress can initiate and exacerbate airway allergic responses through enhanced IgE production,^(24,29) no marked changes in IgE levels in the sera were found after DEP exposure in the low-dose DEP exposure system. IL-5 and IL-13 are thought to play key roles in the pathogenesis of asthma,^(30,31) but our DEP exposure system induced marked oxidative stress that resulted in increased IL-5 and IL-13 in BAL fluid.

Moreover, the number of PAS-positive cells in the lung tissues increased after DEP exposure in BALB/c mice. As oxidative stress stimulates mucin synthesis in the airways,⁽³²⁾ we consider that the increase in PAS-positive cells in the lung tissues after DEP exposure may directly reflect the effect of oxidative stress caused by DEP. From our data, changes in airway hyperresponsiveness are independent of the airway inflammation induced by DEP.

In conclusion, the role of low-dose, long-term DEP exposure in the generation of allergic airway inflammation differs markedly between mouse strains, and these differences may be caused by different host antioxidant responses to the oxidative stress induced by DEP exposure. Our results suggest the existence of susceptibility factors that characterize the host's ability to manufacture antioxidant products as a response to DEP exposure, and that such antioxidant products may be used as biomarkers of susceptibility to DEP in the generation of allergic airway inflammation. If this is indeed the case, it may be possible to use chemo-preventive antioxidants against DEP-induced oxidative stress in people who show a strong response to DEP, similar to that seen in BALB/c mice.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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