

AIRWAY INFLAMMATORY RESPONSES TO OXIDATIVE STRESS INDUCED BY LOW-DOSE DIESEL EXHAUST PARTICLE EXPOSURE DIFFER BETWEEN MOUSE STRAINS

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□ *Low-dose diesel exhaust particle (DEP) exposure induces airway inflammation and exaggerates asthmatic responses in mice, but it is unclear whether strains differ in their susceptibility to adverse effects from low-dose DEP exposure. The authors used BALB/c and C57BL/6 mouse strains to search for genetically based differences in response to low-dose DEP (100 µg/m³) exposure in terms of airway inflammatory response. The macrophage count in bronchoalveolar lavage (BAL) fluid soon after DE exposure began was significantly greater in C57BL/6 mice ($P < .05$) than that in BALB/c mice. The count did not increase significantly in BALB/c mice until later. Heme oxygenase-1 (HO-1) mRNA expression and protein production in lung tissues soon after exposure began were more marked in BALB/c mice than in C57BL/6 mice, but the reverse was true later on. The increases in interleukin (IL)-1β and interferon (IFN)-γ levels in BAL fluid after DE exposure were significant only in BALB/c mice; there were significant increases in monocyte chemoattractant protein (MCP)-1, IL-12, IL-10, IL-4, and IL-13 in both strains, but these were more marked in C57BL/6 mice. These interstrain differences in airway inflammatory response after DE exposure were significantly attenuated by antioxidant N-acetylcysteine (NAC) treatment. Changes in airway hyperresponsiveness were independent of the airway inflammation induced by low-dose DEP. Thus, in BALB/c mice, innate immunity may play a central role in DE exposure response, whereas in C57BL/6 mice Th2-dominant responses*

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play a central role. Low-dose DEP exposure induces airway inflammatory responses that differ among strains, and these differences may be caused by differences in sensitivity to oxidative stress.

Keywords airway inflammation, low-dose DEP, mouse strain, oxidative stress

Previous animal experimental studies have shown that diesel exhaust particles (DEP) are involved in the increasing prevalence of allergic diseases, including asthma, and DEP inhalation enhances airway hyperresponsiveness and/or airway inflammatory changes in experimental animal models [1–3]. These animals were exposed to high-dose DEP (i.e., 1–3 mg/m³) by inhalation or by nonphysiological routes (i.e., intratracheal instillation). In reality, DEP exist in air at low concentrations and are inhaled by animals over long periods of time.

Host responses to DEP are regulated by a balance between antioxidant defenses and proinflammatory responses [4]. Recent investigations have started to recognize that susceptibility to the adverse risks posed by pollutants is an intrinsic trait most probably related to genotype [5].

Although it has been reported that low-dose DEP exposure induces airway inflammation and exaggerates asthmatic responses in mice [6–8], it is unclear whether there are intraspecies differences in susceptibility to the oxidative stress caused by low-dose DEP.

Major histocompatibility complex (MHC) genes in both mice and humans code for proteins involved in nearly every aspect of the immune response and immune regulation, including the complement cascade, cytokine production, and antigen presentation. BALB/c (MHC H2-d) mice readily show increases in Th2 cytokine responses. In contrast, C57BL/6 (MHC H2-b) mice readily show increases in Th1 cytokine responses [9, 10].

We attempted to determine whether there were differences between BALB/c and C57BL/6 mouse strains in terms of their airway hyperresponsiveness and inflammatory response to exposure to low-dose DEP (i.e., at concentrations similar to those we inhale outdoors). We also attempted to identify susceptibility factors characterizing the host response to low-dose DEP exposure.

METHODS

Animals

Nine-week-old female BALB/c and C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). The mice were randomly classified into each experimental group, and housed in a plastic cage with plywood chips. The cages were placed in an exposure chamber. All experiments used six mice per group, 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.

DE Exposure

The *in vivo* diesel exhaust (DE) exposure system has been described previously [6]. The concentration of DEP was monitored and kept at low levels (approximately $100 \mu\text{g}/\text{m}^3$). The concentrations of CO, NO₂, and SO₂ were measured with an infrared gas analyzer (Ultramat-S; Fuji Electric, Tokyo, Japan). The average concentrations of CO, NO₂, SO₂, and DEP were 3.5 ± 0.1 ppm, 2.2 ± 0.3 ppm, <0.01 ppm, and $103.1 \pm 9.2 \mu\text{g}/\text{m}^3$, respectively. BALB/c and C57BL/6 mice were exposed to DE for 7 hours daily and 5 days weekly.

Experimental Protocol 1

Both strains were exposed to low-dose DEP or clean air (control) for a maximum of 8 weeks. Airway hyperresponsiveness was measured immediately at day 0 and after 1, 4, and 8 weeks of DE exposure or clean air in all experimental groups. BALB/c and C57BL/6 mice were sacrificed at these same time points in all experimental groups. We examined the histopathology of the lung tissues, and we analyzed the cell populations and the concentrations of cytokines (monocyte chemoattractant protein [MCP]-1, interleukin [IL]-1 β , IL-12, interferon [IFN]- γ , IL-10, IL-4, and IL-13) in the bronchoalveolar lavage (BAL) fluid by enzyme-linked immunosorbent assay (ELISA). We also examined the expression of heme oxygenase (HO)-1 and nuclear factor (NF)-E₂-related factor-2 (Nrf2) mRNA in the lung tissues by reverse transcriptase–polymerase chain reaction (RT-PCR) and

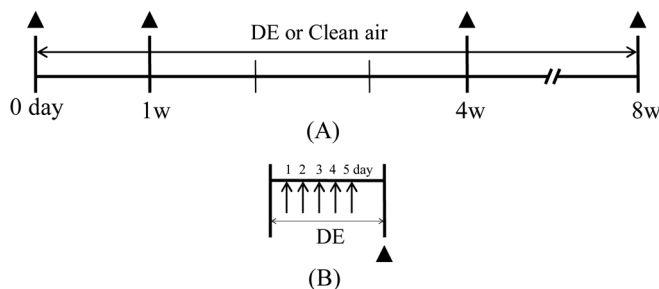


FIGURE 1 Experimental protocol. (A) BALB/c and C57BL/6 mice were exposed to low-dose DEP or clean air (control) for a maximum of 8 weeks. ▲: Analysis time-points (refer to Materials and Methods). (B) BALB/c and C57BL/6 mice were exposed to low-dose DEP for 1 week and given NAC on day 1 to day 5. ↑: Time-points of administration of NAC (320 mg/kg body weight IP).

the expression of HO-1 protein in the lung tissues by Western blot at each time point after DE exposure (Figure 1A).

Experimental Protocol 2

The experimental groups were divided into the DE exposure alone (DE) group and DE exposure plus treatment with *N*-acetylcysteine (NAC) (DE + NAC) group. DE + NAC group mice were treated with 320 mg/kg NAC (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally (IP) [11] from days 1 to 5 of DE exposure. After 1 week of DE exposure, we immediately measured airway hyperresponsiveness, performed BAL, and analyzed cell populations in the BAL fluid (Figure 1B).

Assessment of Airway Hyperresponsiveness

Airway hyperresponsiveness was assessed by whole body plethysmography with free moving application (Buxco Electronics, Troy, NY) and evaluated by the Penh value. Penh is equal to $\text{Pause} \times \text{PIF} / \text{PEF}$, where $\text{Pause} = (\text{TE} - \text{RT}) / \text{RT}$ (PIF, peak inspiratory flow; PEF, peak expiratory flow; TE, expiratory time; RT, relaxation time). The mice were given step-wise doses of 0 (normal saline), 6, 12, and 24 mg/mL methacholine (Sigma-Aldrich) by aerosol for 3 minutes.

Histological Analysis

For histologic examination, 10% formalin-fixed lung tissues were embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin and systematically scanned under a light microscope (Eclipse E800; Nikon, Tokyo, Japan).

BAL and Cell Differentials in BAL Fluid

BAL fluid was obtained by injecting 1 mL of saline solution (3 times; total, 3 mL) and then gently aspirating the fluid from the lungs after securing an intratracheal catheter within the trachea. With the use of this catheter, recovery ratios of lavage fluids ranged from 80% to 90% and did not differ significantly among the groups. The total number of cells in BAL fluid was counted with a hemocytometer. For differential counts of leukocytes in BAL fluid, cytospin smear slides (Lab Systems, Tokyo, Japan) were prepared. The smears were stained with Diff-Quick Romanowski stain (Muto Kagaku, Tokyo, Japan). Differential counts of 200 cells per smear were performed.

TABLE 1 RT-PCR Primer Sequences

Primer	Sequences	Product size
HO-1	Sense: 5'-GAGCAGAACCAGCCTGAACTA-3' antisense: 5'-GGTACAAGGAAGCCATCACCA-3'	200 bp
Nrf2	sense: 5'-CTCGCTGGAAAAAGAAGTGG-3' antisense: 5'-CCGTCCAGGAGTTCAGAGAG-3'	240 bp
β -Actin	sense: 5'-TGTGATGGTGGGAATGGGTCA-3' antisense: 5'-TTTGATGTCACGCACGATTTCG-3'	514 bp

Measurement of Cytokines in BAL Fluid

Immunoreactivity for MCP-1, IL-1 β , IL-12, IFN- γ , IL-10, IL-4, and IL-13 in the BAL fluid supernatants was measured with an ELISA kit (Biosource International, Camarillo, CA). ELISA was carried out in accordance with the manufacturer's instruction sheet. Each sample was assayed in triplicate.

RT-PCR

HO-1 and Nrf2 mRNA levels in lung tissues were evaluated by RT-PCR. Total RNA was extracted from each lung tissue specimen by using TRIzol Reagent (Gibco BRL, Gaithersburg, MD) in accordance with the manufacturer's instructions. cDNA and PCR were synthesized as previously described [12]. The primer construction program Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), was used with mouse mRNA sequence information collected by the sequence retrieval system at the Center for Information Biology and DNA Data Bank of Japan (DDBJ), National Institute of Genetics. PCR primer sets for murine β -actin and HO-1 were synthesized by Sigma (Genosys, Sapporo Japan). The sequences of the primer sets are shown in Table 1. The PCR mixture was amplified with a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA) under the conditions shown in Table 2. The optimum PCR cycles were determined by preliminary experiments that showed linear amplification. The PCR products underwent electrophoresis on 4% (*w/v*) SeaKem GTG agarose and NuSieve GTG (1:3) gels. They were stained with ethidium bromide and then observed under ultraviolet transillumination. Intensity analysis of the bands was performed with Adobe Photoshop 5.0 (Adobe Systems,

TABLE 2 PCR Conditions

Primers	Cycle parameters	Cycles
HO-1	94°C 5 s; 60°C 5 s; 72°C 10 s	30
Nrf2	94°C 5 s; 60°C 5 s; 72°C 10 s	25
β -Actin	94°C 5 s; 65°C 5 s; 72°C 10 s	25

Tokyo, Japan), and expression of the mRNA of each factor was indicated by the number of white pixellated areas.

Western Blot

Western blot was performed to evaluate HO-1 protein levels in the lung tissues after DE exposure. Total protein was extracted from each lung tissue specimen by using Reagent (BioRad, CA). The total protein concentration in the lysates was measured by dye protein assay (BioRad). Samples containing 100 µg of protein were boiled in sample buffer. The denatured samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gel (BioRad) with a buffer for electrophoresis and then transferred to a nitrocellulose membrane (BioRad). After blocking had been performed with 5% skim milk (Snow Brand Milk Production, Japan), the membrane was incubated with rabbit anti-heme oxygenase-1 polyclonal antibody (1:500; Stressgen Bioreagents, BC, Canada) for 1 hour. The membrane was washed, and then primary antibody was detected by using goat anti-rabbit immunoglobulin (IgG) antibody conjugated to biotin (Histofine, Nichirei, Tokyo, Japan) (10 µg/mL) and incubated for 1 hour. Next, the membrane was washed and incubated for 1 hour with streptavidin (100 µg/mL) conjugated to horseradish peroxidase (Histofine, Nichirei, Tokyo, Japan). The HO-1 protein band was visualized by the addition of diaminobenzidine (0.2 mg/mL) and 0.06% H₂O₂. Intensity analysis of the bands was performed with Adobe Photoshop 5.0 (Adobe Systems, Tokyo, Japan), and protein production was indicated by the number of black pixellated areas.

Statistical Analysis

Statistical analysis of data was performed with Stat Mate III software (ATMS Digital Medical Station, Tokyo, Japan). Groups were compared by 1-way analysis of variance (ANOVA) followed by the Newman-Keuls test. *P* values less than .05 were considered significant.

RESULTS

Assessment of Changes in Airway Hyperresponsiveness to DE Exposure

To examine interstrain variations in response to low-dose DEP exposure, we first assessed airway hyperresponsiveness (as expressed in Penh) by using whole body plethysmography. BALB/c mice were more sensitive to methacholine (as determined by hyperresponsiveness

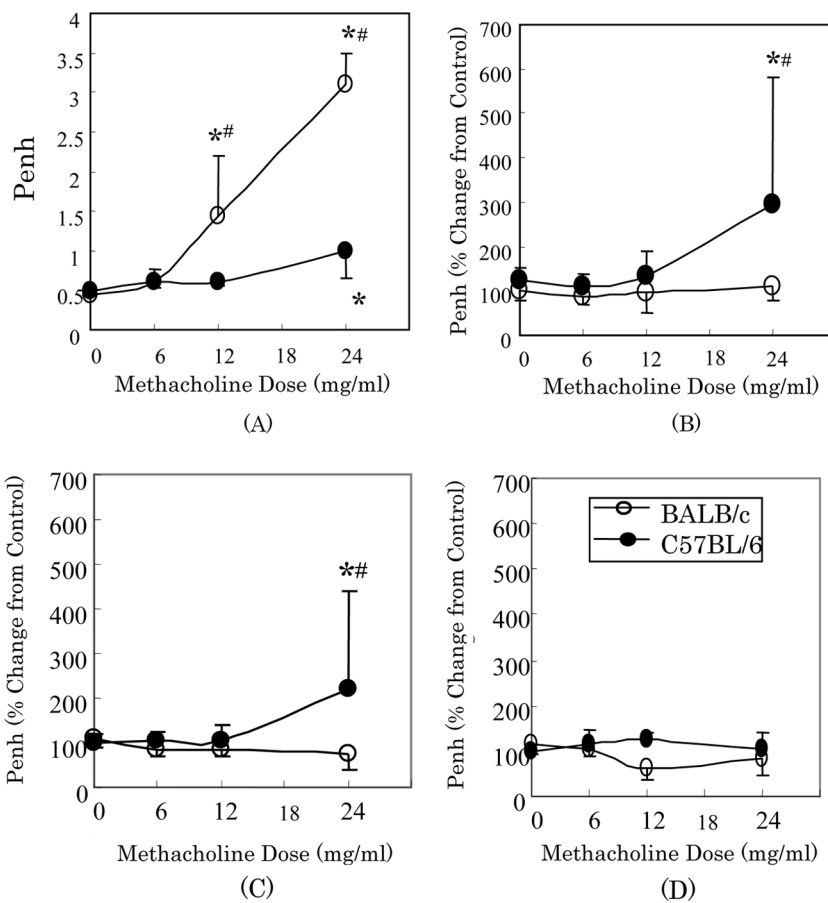


FIGURE 2 Airway hyperresponsiveness was assessed by whole-body plethysmography with free moving application and then evaluated by Penh values. Changes in Penh induced by inhaled aerosolized methacholine (0, 6, 12, 24 mg/mL) in BALB/c and C57BL/6 mice at the baseline level (0 day) are shown (A). The percentage changes in Penh from baseline in response to inhaled aerosolized methacholine after 1 (B), 4 (C), and 8 (D) weeks of DE exposure are shown. Results are means $\pm$ SD of data on 12 mice in each group. * $P < .05$ compared with methacholine 0 mg/mL (normal saline) in the respective mouse strain; # $P < .05$, BALB/c versus C57BL/6 mice at each methacholine concentration point.

expressed in Penh) than were C57BL/6 mice at the baseline level (day 0) (Figure 2A). Percentage changes from the respective control (data not shown) in Penh with methacholine 24 mg/mL were significantly greater in C57BL/6 mice (mean $\pm$ SD: 1 week: 296% $\pm$ 284%; 4 weeks: 222% $\pm$ 216%) than in BALB/c mice (mean $\pm$ SD: week 1: 110% $\pm$ 30%; week 4: 64% $\pm$ 32%) (Figures 2B, C). In C57BL/6 mice, the percentage changes in Penh had recovered to the baseline level by week 8 of DE exposure (Figure 2D). In BALB/c mice, no remarkable percentage changes from baseline were seen in Penh after DE exposure.

Assessment of Changes in Pulmonary Histopathology and in Cell Differentials and Cytokine Induction in BAL Fluid in Response to DE Exposure

To determine the mechanisms behind the interstrain differences in airway hyperresponsiveness to DE exposure, we examined the changes in pulmonary histopathology and in cell differential counts and inflammatory cytokine levels in BAL fluid in response to DE exposure. We performed histological analyses of lung sections taken from BALB/c and C57BL/6 mice after DE exposure. The histological specimens showed no change in response to DE exposure in either of the strains (data not shown).

The total number of cells (Figure 3A) and the number of macrophages (Figure 3B) in the BAL fluid increased significantly after 1 week of DE exposure in C57BL/6 mice than those in BALB/c mice, and increased significantly after 8 weeks of DE exposure in BALB/c mice than those in C57BL/6 mice. The numbers of neutrophils (Figure 3C) and lymphocytes (Figure 3D) in the BAL fluid were significantly increased after DE exposure in the 2 mouse strains. The each control BAL fluid was free of lymphocytes and neutrophils (data not shown). No eosinophils were found in the BAL fluid after DE exposure.

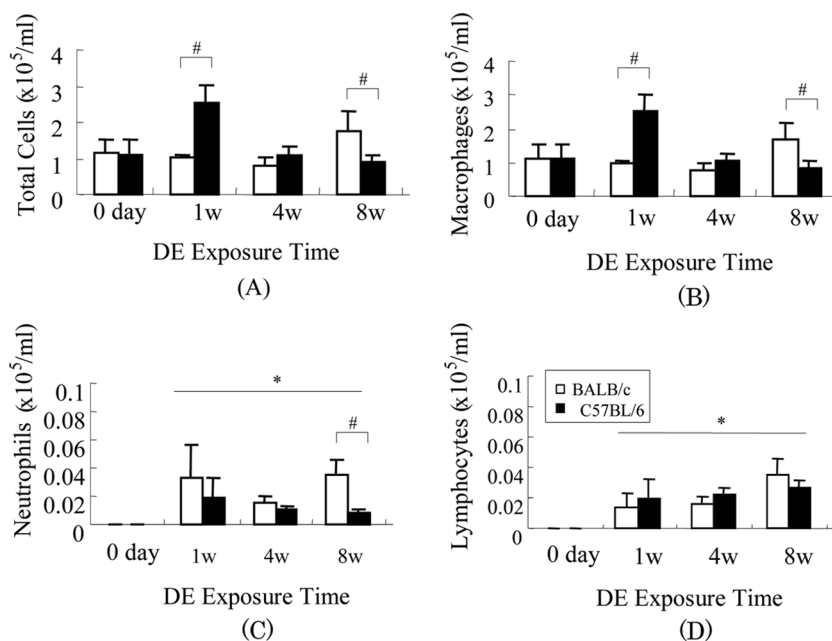


FIGURE 3 Changes in the total number of cells (A) and the numbers of macrophages (B), neutrophils (C), and lymphocytes (D) in BAL fluid after DE exposure. Results are means $\pm$ SD of data from 6 mice in each group. # $P < .05$, BALB/c versus C57BL/6 at each time point.

The concentration of IL-1 β and IFN- γ in BAL fluid increased significantly after DE exposure in BALB/c mice than that in C57BL/6 mice (Figures 4B, D). The concentrations of MCP-1, IL-12, IL-10, IL-4, and IL-13 in BAL fluid increased after DE exposure in both strains, but the changes were more marked in C57BL/6 mice than in BALB/c mice (Figures 4A, C, E, F and G). The control data at 1, 4 and 8 weeks were not changed significantly compared with base line level (day 0) (data not shown).

HO-1 mRNA Expression in Lung Tissues after DE Exposure

As we were pursuing the hypothesis that the DEP-induced proinflammatory effect is mediated through the generation of oxidative stress, we used RT-PCR to examine the expression of mRNA of the antioxidative

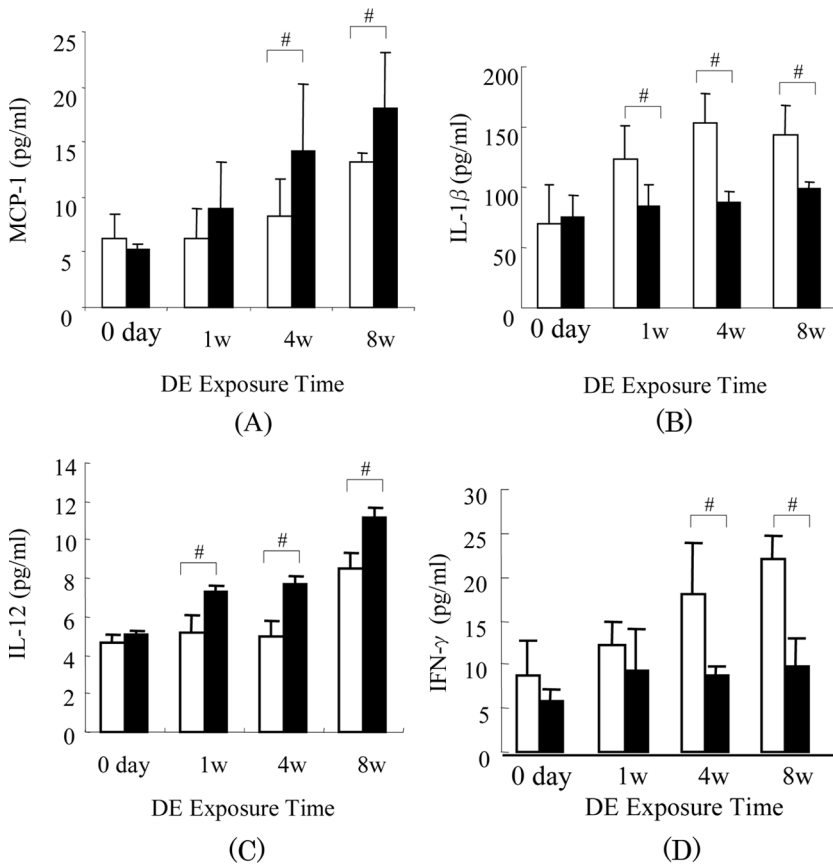


FIGURE 4 Changes in concentrations of cytokine MCP-1 (A), IL-1 β (B), IL-12 (C), IFN- γ (D), IL-10 (E), IL-4 (F), and IL-13 (G) in BAL fluid after DE exposure. Results are means $\pm$ SD of data on 6 mice in each group. # $P < .05$, BALB/c versus C57BL/6 at each time point.

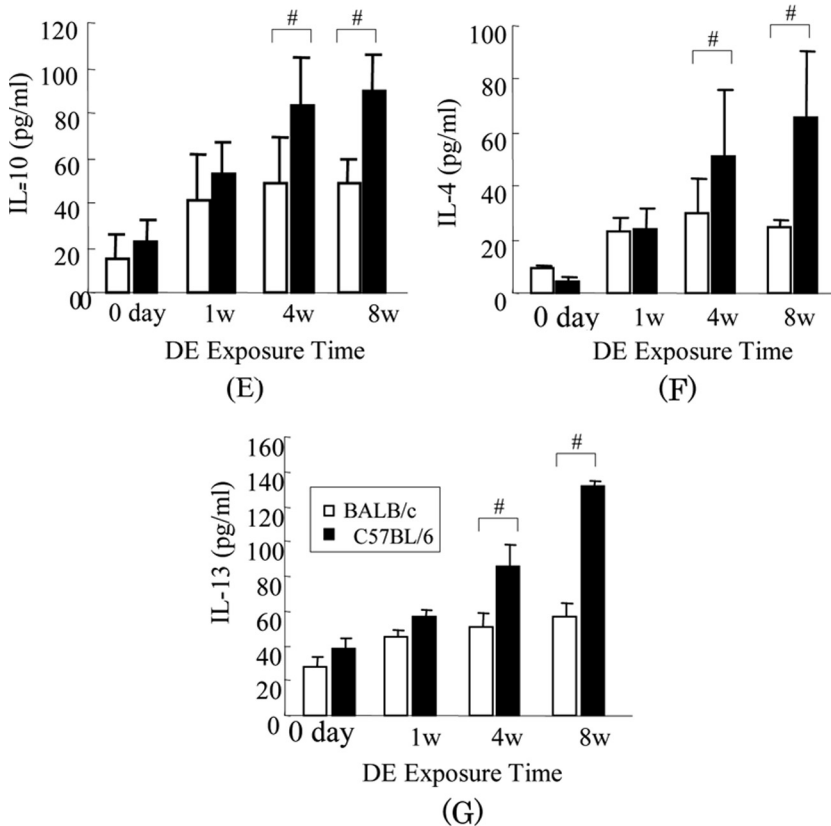


FIGURE 4. (Continued).

enzyme HO-1 and transcription factor Nrf2 in the lung tissues. In BALB/c mice, expression of HO-1 mRNA in the lung tissues after 1 week of DE exposure was more markedly increased than in C57BL/6 mice. In C57BL/6 mice, expression of HO-1 mRNA in lung tissues after 4 and 8 weeks of DE exposure was more marked than in BALB/c mice (Figure 5).

HO-1 Protein Production in Lung Tissues after DE Exposure

Because the expression of the gene encoding the antioxidative enzyme HO-1 showed different patterns of change in BALB/c and C57BL/6 mice exposed to DEP, we used Western blotting to examine the production of HO-1 protein. In BALB/c mice, percentage changes from control (data not shown) in the production of HO-1 protein in the lung tissues after 1 week of DE exposure were significantly ($P < .05$) greater (mean $\pm$ SD: 257% $\pm$ 105%) than in C57BL/6 mice (mean $\pm$ SD: 61% $\pm$ 10%). In C57BL/6 mice, percentage changes from control (data not shown) in the

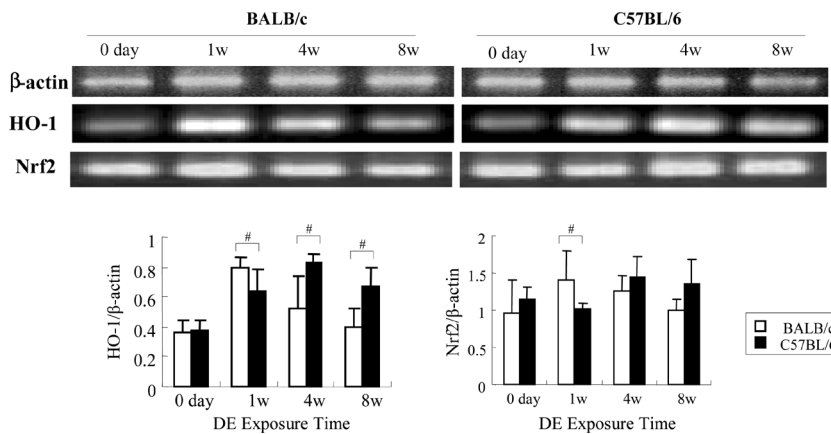


FIGURE 5 Expression of HO-1 and Nrf2 mRNA in lung tissues after DE exposure, by RT-PCR. Photographs show typical results. Each density of PCR product was measured by using Adobe Photoshop 6.0. β-actin was measured as an internal control. The ratio of each HO-1 and Nrf2 molecule against β-actin is shown by a histogram. Results are means $\pm$ SD of data from 3 mice in each group. # $P < .05$, BALB/c versus C57BL/6 at each time point.

production of HO-1 protein in the lung tissues after 8 weeks of DE exposure were more markedly increased (mean $\pm$ SD: 152% $\pm$ 46%) than in BALB/c mice (mean $\pm$ SD: 88% $\pm$ 19%) (Figure 6).

Effects of NAC on Airway Hyperresponsiveness and on Cell Differentials in BAL Fluid after DE Exposure

The antioxidant NAC is a thiol-reducing agent that can antagonize cellular reactive oxygen species (ROS) [13]. To test our hypothesis that the differences between the BALB/c and C57BL/6 strains were caused by differences in response to the antioxidative stress induced by DEP, we performed assays to examine the effects of NAC on airway hyperresponsiveness and on cell differential counts in BAL fluid after DE exposure (Figure 1B). In C57BL/6 mice, the percentage increase in Penh from the baseline (with methacholine 24mg/mL) after 1 week of DE exposure was significantly attenuated by NAC treatment ($P < .05$). Furthermore, the difference between the strains in terms of the percentage change in Penh from baseline after DE exposure was significantly attenuated by NAC treatment, although the value in the C57BL/6 mice still did not return to baseline (Figure 7). The increase in the total number of cells (Figure 8A) and number of macrophages (Figure 8B) in the BAL fluid after 1 week of DE exposure in C57BL/6 mice was significantly inhibited by NAC treatment and returned to the control level. In both mouse strains, the increases in

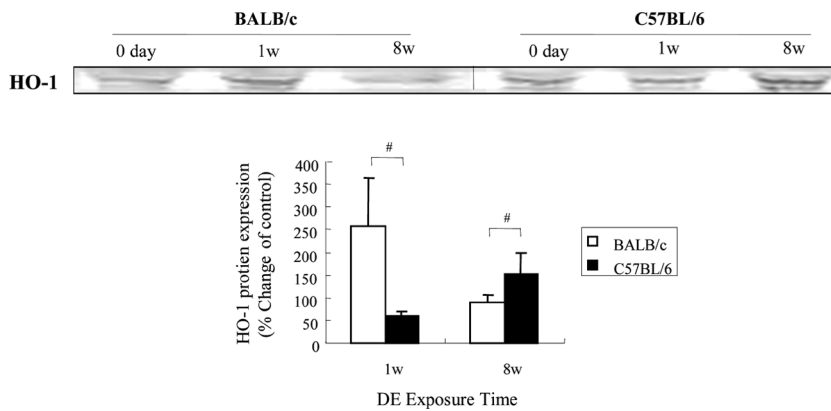


FIGURE 6 Western blot analysis of production of HO-1 protein in lung tissues. Photographs show typical results. Percentage changes from baseline in the production of HO-1 protein after DE exposure are shown as a histogram. Results are means $\pm$ SD of data from 3 mice in each group. # $P < .05$, BALB/c versus C57BL/6 mice.

the numbers of neutrophils (Figure 8C) and lymphocytes (Figure 8D) in the BAL fluid after 1 week of DE exposure were significantly inhibited by NAC treatment and returned to the control level.

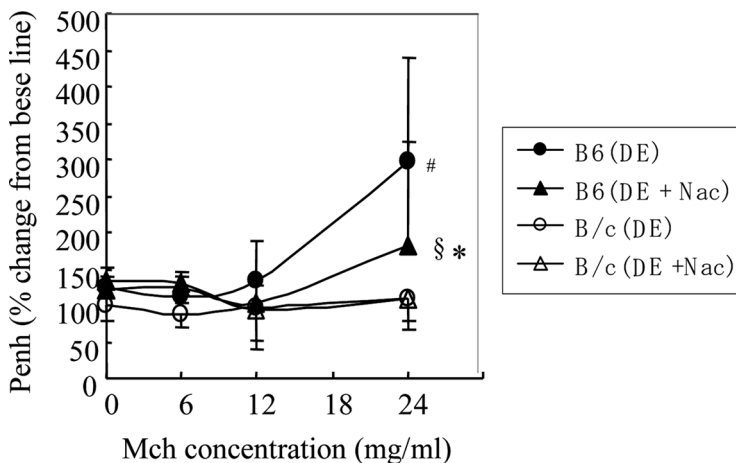


FIGURE 7 Percentage changes in Penh from baseline in response to inhaled aerosolized methacholine after 1 week of DE exposure or after 1 week of DE plus NAC on days 1 to 5. Results are means $\pm$ SD of data from 12 mice in each group. * $P < .05$ compared with the respective mouse strain control (exposed to DE alone); # $P < .01$ for comparison between DE alone-exposed BALB/c and C57BL/6 mouse groups; § $P < .05$ for comparison between DE-exposed plus NAC-treated BALB/c and C57BL/6 mouse groups.

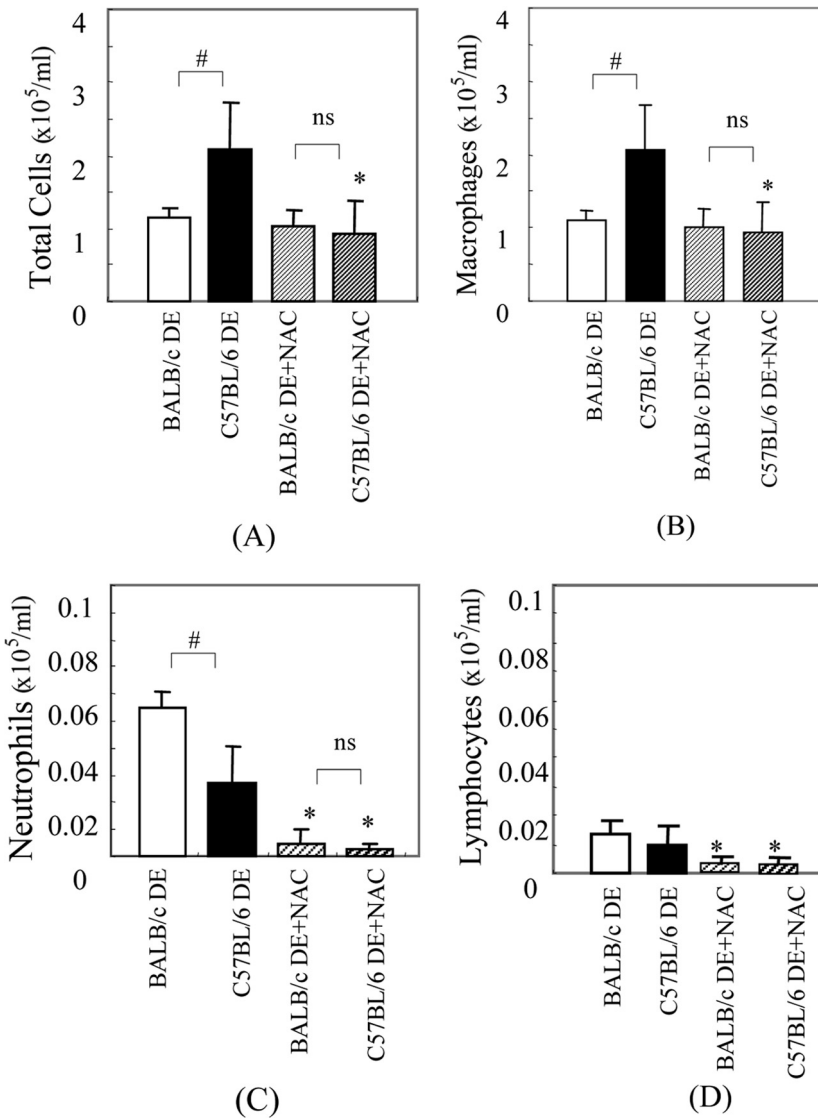


FIGURE 8 Changes in the total number of cells (A) and the numbers of macrophages (B), neutrophils (C), and lymphocytes (D) in BAL fluid after 1 week of DE exposure or after 1 week of DE plus NAC on day 1 to 5. Results are means $\pm$ SD of data from 6 mice in each group. * $P < .05$ compared with the respective mouse strain control (exposed to DE alone). # $P < .05$, BALB/c versus C57BL/6 at each time point.

DISCUSSION

To examine differences between mouse strains in terms of their responses to low-dose DEP exposure, we first assessed airway hyperresponsiveness (as expressed by enhanced Pause values in Penh) by using whole-body plethysmography. The use of Penh as a surrogate for airflow

limitation is currently under debate [14]; nevertheless, it has been reported that Penh is correlated with lung resistance in mice [15]. Penh has also been used in an experimental mouse model in the evaluation of airway hyperresponsiveness [16–18]. Our results showed that, in C57BL/6 mice, the percentage changes in Penh from baseline peaked after 1 week of DE exposure and then recovered to baseline after 8 weeks of DE exposure. In BALB/c mice, Penh showed no marked changes from baseline after DE exposure.

Human challenge studies with DEP have revealed an increase in airway neutrophilic inflammation [19, 20]. We found that after exposure to low-dose DEP ($100\text{ }\mu\text{g}/\text{m}^3$, similar to the level we actually inhale outdoors) the numbers of neutrophils (Figure 3C) and lymphocytes (Figure 3D) in the BAL fluid were increased (but control BAL fluid was lymphocyte- and neutrophil-free) in the 2 mouse strains. The number of macrophages in the BAL fluid of C57BL/6 mice increased significantly in the early stages of DE exposure, but the significant increase occurred later in BALB/c mice.

The underlying mechanism by which DEP exert their biologic effects is an area of ongoing investigation. Most of the focus has been on the role of ROS generated by exposure to DEP and the subsequent generation of oxidative stress within exposed cells [21–24]. Oxidative damage to the airway epithelium also produces airway hyperresponsiveness in human subjects [25], and, in animal models, oxygen radicals can contribute to antigen-induced airway hyperresponsiveness [26]. These previous reports led us to speculate that differences between mouse strains in terms of airway hyperresponsiveness and inflammatory response may be induced by oxidative stress caused by DE exposure.

DE exposure induces oxidative stress responses and increases production of the antioxidative enzyme HO-1 [23, 24, 27–29] in macrophages and airway epithelial cells. Therefore, we examined mRNA expression and protein production of the antioxidative enzyme HO-1 in the lung tissues. HO-1 mRNA expression and protein production in the lung tissues were more marked in BALB/c mice than in C57BL/6 mice in the early stages of DE exposure, whereas the reverse was true later in the exposure period. Our findings suggest that, in the BALB/c mouse strain, host antioxidant defenses contributed to the response to DEP exposure in the early stages; therefore, airway hyperresponsiveness and the number of macrophages in the BAL fluid showed no remarkable changes. In contrast, in the C57BL/6 mouse strain, host antioxidant defenses were feeble in the early stages of DE exposure, therefore, airway hyperresponsiveness and the number of macrophages in the BAL fluid were markedly elevated.

To demonstrate our hypothesis, we performed assays to examine the effects of NAC on airway hyperresponsiveness and on cell differentials in the BAL fluid after DE exposure. The increase in the number of inflammatory

cells in the BAL fluid after DE exposure was significantly inhibited by NAC treatment in both BALB/c and C57BL/6 mice. The increase in airway hyperresponsiveness to DEP was significantly attenuated by NAC treatment in C57BL/6 mice, but greater airway hyperresponsiveness was still recognized in the NAC-treated C57BL/6 mice than in the BALB/c mice. Therefore, we consider that the changes in airway hyperresponsiveness were independent of the airway inflammation caused by oxidative stress from low-dose DEP exposure. It has been previously shown that ozone-induced airway hyperresponsiveness and airway inflammation in various models are not causally linked. Genetic factors probably account for the observed variability in sensitivity of the airways to ozone [30]. Therefore, we consider that, in this experimental system, the changes in airway hyperresponsiveness caused by low-dose DEP may depend on the mouse genetic type. In C57BL/6 mice, the increase in airway hyperresponsiveness after DE exposure fell as DE exposure persisted. It is conceivable that these responses were a nonspecific physiological response to the oxidative stress caused by low-dose DEP exposure, and that the changes in breathing pattern were dependent on the mouse genetic type.

Recent evidence suggests that Nrf2 is a critical transcription factor for the regulation of antioxidant-responsive element (ARE)-containing genes that encode antioxidant proteins [23, 24, 28]. Nrf2 is a susceptibility gene in hyperoxic lung injury [28]. Cytoprotective pathways are induced by the Nrf2 transcription signal pathway at the lowest levels of oxidative stress, and this may constitute the first tier of a hierarchical oxidative stress response, as is seen in the production of HO-1. If these enzymes fail to neutralize the effects of ROS, proinflammatory effects constitute a second tier or superimposed level of oxidative stress. The final tier or superimposed level of oxidative stress is cytotoxicity, including the initiation of programmed cell death [23].

In the BALB/c mouse strain, production of HO-1 and expression of Nrf2 mRNA were elevated in the early stages of DEP exposure, and macrophage and proinflammatory cytokine (IL-1 β , IFN- γ) concentrations in the BAL fluid increased in the late stages of DEP exposure. This suggests that, in the BALB/c mouse strain, following the induction of host antioxidant defenses as a response to the oxidative stress induced by low-dose DEP exposure, innate immune responses play a central role.

In C57BL/6 mice, production of HO-1 and expression of Nrf2 mRNA were weak and the concentration of macrophages in the BAL fluid was elevated in the early stages of DEP exposure; production of HO-1 was elevated continuously in the late stages. Production of Th2 cytokines (IL-10, IL-4, and IL-13) was markedly increased in the later stages of DEP exposure. We suggest that, in C57BL/6 mice, because the host antioxidant defenses are feeble in the early stages of exposure, the increase in macrophages

levels is induced by the innate immune system response to oxidative stress from low-dose DEP exposure. In this setting, antigen-presenting cells seem to favor the development of T-helper cells [31]. Therefore, after DEP-induced proinflammation-mediated macrophages appear, they may stimulate helper T cells to secrete Th2 cytokines. The concentration of MCP-1 in the BAL fluid was increased in both mice strains after DE exposure, but more markedly in C57BL/6 mice than in BALB/c mice. MCP-1 influences both innate immunity (through effects on monocytes) and adaptive immunity (through the control of Th2 cell polarization) [32]. In C57BL/6 mice, after the innate immune response to oxidative stress induced by low-dose DEP exposure increases the numbers of macrophages in the early stages of exposure, the adaptive immune system may then contribute to the oxidative stress response through the MCP-1-mediated stimulation of Th2 lymphocytes.

BALB/c mice readily show an increased Th2 cytokine response; in contrast, C57BL/6 mice readily show an increased Th1 cytokine response [9, 10], but our results suggested that BALB/c mice showed ready increases in the concentrations of proinflammatory cytokines, and that innate immune responses play a central role in low-dose DEP exposure; in the C57BL/6 mice, Th2-dominant responses played a central role in low-dose DEP exposure. The fact that low-dose DEP induced strain-specific differences in the airway inflammatory response may have been caused by differences in sensitivity to oxidative stress. Our results also suggest that the toxicity and risk associated with low-dose DEP are more marked with continuous, long-time exposure, and there is reason to fear the risk of synergism between DEP and allergens. Mice respond differently from humans to DE, although we suggest that humans, who are highly responsive to DEP, display responses similar to those of C57BL/6 mice; the use of chemopreventive antioxidants against DEP-induced oxidative stress may be possible in such DEP high responders.

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