

Original Article

Diesel exhaust (DE) aggravates pathology of delayed-type hypersensitivity (DTH) Induced by methyl-bovine serum albumin (mBSA) in mice

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(Received June 3, 2009; Accepted June 23, 2009)

ABSTRACT — Diesel exhaust particles (DEP), a well-known air pollutant, exacerbate type I hypersensitivity conditions, such as asthma and pollen allergy. In this study, we examined the effect of diesel exhaust (DE) exposure on delayed-type hypersensitivity (DTH), a type IV hypersensitivity, induced with methyl-bovine serum albumin (mBSA) in C57BL/6 mice. Mice were exposed to DE containing DEP at a dose of 1.78 mg/m³ in an inhalation chamber for 14 days. On Day 7, DTH mice and DE-exposed DTH mice were injected s.c. with 200 μ l of 1.25 mg/ml mBSA emulsified with CFA in the dorsal region as initial sensitization. On Day 14, mice were injected s.c. into one footpad with 20 μ l of 10 mg/ml mBSA dissolved in PBS as challenge. On Day 15, footpad thickness and spleen weight were measured. Significant footpad swelling (%) was observed in DTH mice compared with normal control mice, and this swelling was significantly augmented by DE exposure. The levels of pro-inflammatory cytokines, including IFN- γ , TNF- α , and IL-6, in DTH mice were significantly higher than in normal mice, and were also further enhanced by DE exposure. DE exposure increased production of IL-17, which enhances local tissue inflammation through up-regulation of pro-inflammatory cytokines, while production of IL-10, which inhibits local tissue inflammation through suppression of immune cell proliferation, was unchanged. No change was observed in the percentage of CD4⁺CD25⁺Foxp3⁺ T regulatory (Treg) cells in splenic lymphocytes following DE exposure. IL-6 production was increased by DE, and this would facilitate the differentiation of naïve T cells to IL-17-producing Th17 cells, while concomitantly suppressing the competing differentiation pathway to IL-10-producing Treg cells. Our results indicate that DE inhalation may, in part, exacerbate the pathological symptoms of DTH and induction of pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-6 and IL-17.

Key words: DE, DTH, Exacerbation, Pro-inflammatory cytokines

INTRODUCTION

It is well documented that air pollutants exacerbate respiratory diseases, such as asthma (Brunekreef and Holgate, 2002). Diesel exhaust particles (DEP), which are major contributors to the level of airborne particles less than 2.5 micrometers in aerodynamic diameter (PM_{2.5}) in urban areas, consist of a carbon core upon which are

absorbed various compounds, including polyaromatic hydrocarbons (PAH), nitroaromatic hydrocarbons, heterocyclics, quinones, aldehydes, aliphatic hydrocarbons and heavy metals (Vouk and Piver, 1983; Draper, 1986). As diesel engines have been modified to obtain higher combustion efficiency, the size of the emitted particles has become smaller and the number of particles per unit mass has become larger. Smaller particles have a

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greater surface area per unit mass and therefore can carry more absorbed chemicals. The smallest particles (with a diameter of < 100 nm) can infiltrate the body through the lungs or nasal membrane, and reach the blood circulation, subsequently appearing systemically (Peters *et al.*, 2006). When inhaled, these particles can translocate into liver (Oberdörster *et al.*, 2002), brain (Oberdörster *et al.*, 2004) and fetus (Sugamata *et al.*, 2006). It has recently been reported that DEP or organic DEP extracts induce oxidative stress and adjuvant effects, leading to modulation of cytokine production, in the respiratory tract and these effects can be blocked by thiol-containing antioxidants such as *N*-acetylcysteine (Sagai *et al.*, 1993; Whitekus *et al.*, 2002). DEP has been shown to exacerbate asthma, accompanied with increases of immunoglobulin E (IgE) and several Th 2 cytokines (Takano *et al.*, 1997). Increase of Th 2 cytokines, such as IL-4, IL-5, IL-6 and IL-10, suppresses Th1 cells, while increase of Th 1 cytokines, such as IFN- γ and TNF- β , suppresses Th2 cells, and disturbance of the Th1/Th2 balance is associated with various immune-related diseases (Shirakawa *et al.*, 1997; Nishimura and Ohta, 1999). It has also been shown that DEP facilitates production of IgE antibodies (Diaz-Sanchez *et al.*, 1994; Tsien *et al.*, 1997) and secretion of Th2 cytokines (Diaz-Sanchez, 1997; Polosa *et al.*, 2002). However, its effect on production of IFN- γ is controversial: in cellular studies DEP suppressed production of IFN- γ *in vitro* (Ohtani *et al.*, 2005) and *in vivo* (Finkelman *et al.*, 2004), but it was found to augment production of IFN- γ in collagen-induced arthritis (CIA) mice (Yoshino and Sagai, 1999).

Delayed-type hypersensitivity (DTH) is induced not via antibody-dependent immune response, but via cellular memory immune response involving neutrophils, macrophages and T cells (Black, 1999; Kobayashi *et al.*, 2001). First, T cells sensitized with antigen generate memory T cells. Second, the recall response of T cells is evoked by challenge. IFN- γ and IL-17, produced by antigen-specific CD4 $^{+}$ T cells, play an important role in the development of DTH response, which reaches a peak 24 to 48 hr after the challenge (Nakae *et al.*, 2002; Schulz *et al.*, 2008). Normalization of the Th1/Th2 balance might attenuate DTH. Th17 cells, which produce IL-17, have recently been identified as pro-inflammatory T helper cells distinct from both Th1 and Th2 cells. These effector cells constitute 1-2% of peripheral CD4 $^{+}$ T cells and specifically express orphan nuclear receptor ROR γ t. This nuclear receptor, which is a key transcriptional factor that regulates the differentiation of Th17, induces transcription of the gene encoding IL-17 (Ivanov *et al.*, 2006). IL-17 enhanced local tissue inflammation through the up-reg-

ulation of pro-inflammatory and neutrophil-mobilizing cytokines and chemokines, thereby enabling activated T cells to penetrate the extracellular matrix (Qian *et al.*, 2007). On the other hand, regulatory T (Treg) cells, which constitute 5-10% of peripheral CD4 $^{+}$ T cells (1-2% of peripheral lymphocytes), play an important role in the maintenance of peripheral immune tolerance and prevention of immune-mediated diseases by suppressing immune responses (Taylor *et al.*, 2001). Failure of Treg cell function is associated with the development of various autoimmune diseases, including experimental allergic encephalomyelitis (EAE) and CIA (Kelchtermans *et al.*, 2005; Mimran and Cohen, 2005). These functional suppressor cells express CD4, IL-2 receptor α -chain (CD25), and the transcriptional factor Foxp3. Foxp3 is specifically expressed in Treg cells, and this distinguishes Treg cells from CD4 $^{+}$ CD25 $^{+}$ T cells, which lack regulatory function (Fontenot *et al.*, 2003). It was suggested that DEP blocks the induction of oral tolerance in the DTH response induced with hen egg lysozyme (Yoshino *et al.*, 1998). Oral tolerance is classically defined as the suppression of immune response to an antigen that has been administered previously via the oral route. Low-dose antigens favor the induction of Th2 and Treg cell subsets that mediate active suppression of antigen-specific T cells (Miller *et al.*, 1992), whereas high-dose antigens favor induction of clonal anergy or deletion (Whitacre *et al.*, 1991; Melamed and Friedman, 1993). Oral tolerance is suppressed in several animal models of autoimmune disease, including arthritis (Thompson and Staines, 1986) and EAE (Higgins and Weiner, 1988), as well as non-autoimmune diseases, such as asthma (Nakao *et al.*, 1998) and DTH (Miller and Hanson, 1979; Mowat *et al.*, 1982). The effects of DEP on these processes remain to be fully established. In the present study, we examined the effect of DE exposure on the pathology symptoms and pro-inflammatory cytokine profiles of DTH induced with methyl bovine serum albumin (mBSA) in C57BL/6 mice. The results indicate that DE inhalation exacerbates the pathological symptoms of DTH systematically through induction of pro-inflammatory cytokines.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6 weeks of age) were purchased from Sankyo Labo Service (Tokyo, Japan), kept for 1 week at animal facility and used at 7 weeks of age. They were housed in wire-mesh cages at a temperature of $22 \pm 2^{\circ}\text{C}$ and relative humidity of $55 \pm 10\%$ under a 12 hr light-dark cycle, and were allowed free access to

tap water and experimental normal diet, Labo MR Stock (Nosan Co., Kanagawa, Japan). Mice divided into a normal group, a DTH-disease control group, a DE exposed normal group and a DE exposed DTH-disease group. Each group consisted of 5 mice. They were treated and handled according to the Guide Principles for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society and with the approval of Tokyo University of Science's Institutional Animal Care and Use Committee.

Exposure of mice to DE

Mice were exposed to DE in an inhalation chamber at the Nano-particles Health Science Research Center, Tokyo University of Science (Chiba, Japan) according to the method described elsewhere Umezawa *et al.*, (2008). A 2179-cc diesel engine (Isuzu Automobile, Inc., Tokyo, Japan) was operated at 1,580 rpm with commercial diesel oil. The exhaust was introduced into a stainless steel dilution tunnel and mixed with clean air that was passed through a high-efficiency particulate air filter and a charcoal filter. The diameter distributions of DEP in the exhaust was analyzed using a scanning mobility particle sizer (SMPS, model 3936, TSI Inc., St. Paul, MN, USA). The SMPS apparatus comprised a condensation particle counter (CPC, model 3785) and a differential mobility analyzer (DMA, model 3081). Concentrations of DEP in DE gas were adjusted to approximately 0.1 and 1.78 mg/m³.

Experimental design for DE exposure and immunization with mBSA (Yoshimoto *et al.*, 2000)

As shown in Fig. 1, mice were divided into 4 groups with or without DE exposure and with or without immunization with mBSA to induce DTH. Normal mice and DTH mice were exposed to DE (0.1 or 1.78 mg DEP/m³; 8 hr/day) from Day1 to Day 14, or housed with clean air as controls. On Day 7, DTH mice and DE-exposed DTH mice were injected s.c. with 200 µl of 1.25 mg/ml mBSA (Sigma) emulsified with CFA (Chondrex, Redmond, WA, USA) in the dorsal region as initial sensitization. On Day 14, mice were injected s.c. into one footpad with 20 µl of 10 mg/ml mBSA dissolved in PBS. An equal volume of PBS was injected into another footpad as a control. At 24 hr after the challenge, footpad thickness was measured with a digital caliper. DE exposures were conducted at doses of 0.1 and 1.78 mg DEP/m³ under the same experimental protocol as shown in Fig. 1. In this report, only data obtained at a dose of 1.78 mg DEP/m³ are presented in this study, since DEP at a dose of 0.1 mg/m³ appeared to have little effect.

Determination of footpad swelling and splenomegaly

The magnitude of the DTH response was determined as follows: [footpad swelling (mm)] = [footpad thickness of mBSA-injected footpad (mm)] - [footpad thickness of PBS-injected footpad (mm)], [footpad swelling (%)] = ([footpad swelling (mm)]/[footpad thickness of PBS-injected footpad (mm)]) x 100. Splenomegaly was deter-

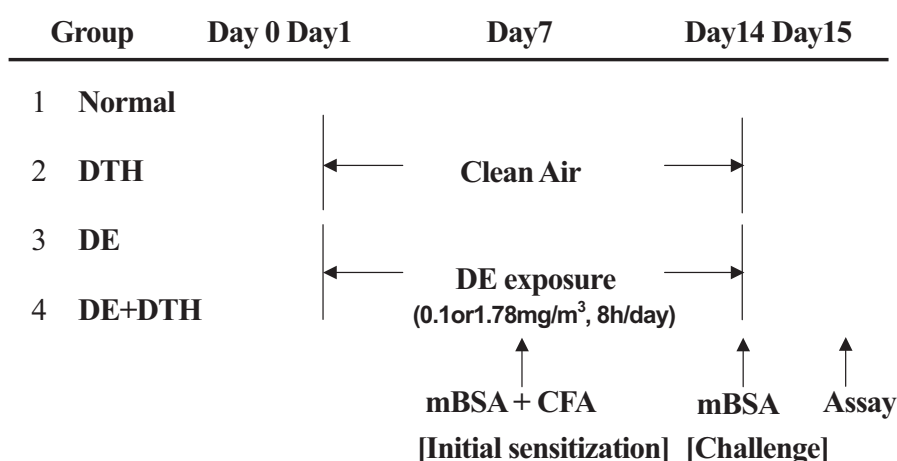


Fig. 1. Experimental design for exposure to DE and immunization with mBSA. Mice of groups 3 and 4 were exposed to DE (1.78 mg DEP/m³; 8 hr/day) for 14 days. Mice of groups 1 and 2 were housed in clean air as controls from Day1 to Day 14. On Day 7, mice of groups 2 and 4 were injected s.c. with 200 µl of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization and on Day14, they were further injected s.c. with 20 µl of 2.5 mg/ml mBSA into one foot-pad as the challenge. At 24 hr after the challenge, footpad thickness and spleen weight were measured.

mined as follows: [splenomegaly (%)] = [spleen weight (g)] / [body weight (g)] x 100.

Reagents

Anti-mouse IFN- γ mAb (XMG1.2), biotin-conjugated anti-mouse IFN- γ mAb (R4-6A2), purified anti-mouse IL-4 mAb (11B11), biotin-conjugated anti-mouse IL-10 mAb (JES5-2A5), purified anti-mouse TNF- α mAb (1F3F3D4), biotin-conjugated anti-mouse TNF- α mAb (MP6-XT22), purified anti-mouse IL-6 mAb (MP5-20F3) and biotin-conjugated anti-mouse IL-6 mAb (MP5-32C11) were purchased from eBioscience (San Diego, CA, USA). Purified anti-mouse IL-10 mAb (JES-16E3), purified anti-mouse IL-17 mAb (TC11-18H10.1), biotin-conjugated anti-mouse IL-17 mAb (TC11-8H4) and recombinant mouse IL-17 were purchased from BioLegend (San Diego, CA, USA). Recombinant mouse IFN- γ , TNF- α IL-6 and IL-10 were purchased from eBioscience. Phycoerythrin (PE)-conjugated anti-mouse CD3 mAb (17A2), which recognizes T cell receptor-associated complex present on all mature T cells; PE-Cy5-conjugated anti-mouse CD4 mAb (H129.19), which recognizes protein on helper T cell; FITC-conjugated anti-mouse CD8a mAb (53-6.7), which recognizes protein on cytotoxic T cells; and FITC-conjugated anti-mouse CD19 mAb (1D3), which recognizes protein on B cells, were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). PE-Cy5-conjugated anti-mouse CD38 mAb (90), which recognizes protein on plasma cells; FITC-conjugated anti-mouse CD25 mAb (PC61.5), which recognizes protein on activated T cells; and PE-conjugated anti-mouse Foxp3 mAb (FJK-16a), which recognize a transcriptional factor specifically expressed in Treg cells, were purchased from eBioscience.

Phenotype of lymphocytes

Immunophenotyping of splenic lymphocytes was carried out using a FACS-LSR flow cytometer (Becton Dickinson, San Jose, CA, USA). Spleens were harvested from normal mice and DTH mice with or without DE exposure on Day 15. Each spleen was minced with scissors, and a single-cell suspension was prepared. Splenocytes were purified by means of hemolysis, counted under a microscope, and suspended in RPMI1640-based buffer (containing 102 mM NaCl, 5 mM KCl, 0.4 mM CaCl₂, 0.4 mM MgSO₄, 23.8 mM NaHCO₃, 5.6 mM Na₂HPO₄, 11.1 mM glucose and 10 mM HEPES-NaOH; pH 7.4) at a concentration of 2×10^6 cells/ml for use as samples. Cells were stained with fluorochrome-conjugated antibodies for 30 min, and washed. Analysis of 10,000 lymphocyte events per tube was performed using Cell Quest

software (Becton Dickinson). The percentages of T cells (CD3⁺), helper T cells (CD3⁺CD4⁺), cytotoxic T cells (CD3⁺CD8⁺), B cells (CD19⁺) and plasma cells (CD3⁺CD19⁺CD38⁺) in splenocytes were calculated.

Analysis of Treg cells

Splenocytes were prepared from normal and DTH mice with or without DE exposure on Day 15. Cells (5×10^6) were stained with PE-Cy5-conjugated anti-CD4 and FITC-conjugated anti-CD25 antibodies for 30 min at room temperature, washed with RPMI1640-based buffer, fixed with 4% para-formaldehyde for 10 min on ice, and then treated with 0.1% Triton X-100 for 5 min at 4°C. After having been washed with RPMI1640-based buffer and blocked with 1% BSA/PBS(-) for 30 min at room temperature, the splenocytes were stained with PE-conjugated anti-Foxp3 antibody for 1 hr at room temperature, and washed with RPMI1640-based buffer for assay. Analysis of 10,000 lymphocyte events per tube was performed using Cell Quest software (Becton Dickinson).

Assays of INF- γ , TNF- α , IL-6, IL-17 and IL-10

Splenocytes were suspended in RPMI1640 medium containing 10% FBS at a concentration of 7×10^6 cells/ml and cultured with mBSA (20 mg/ml) in a 6-well plate. After incubation at 37°C under an atmosphere of 5% CO₂ for 96 hr, the culture supernatant was harvested for assays of IFN- γ , IL-10, TNF- α , IL-6 and IL-17. The concentrations of these cytokines were measured by ELISA as described below. A 96-well plate was coated with purified anti-mouse IFN- γ (XMG1.2) (1:500), IL-10 (JES-16E3) (1:250), TNF- α (1F3F3D4) (1:500), IL-6 (MP5-20F3) (1:500), or IL-17 (TC11-18H10.1) (1:250) mAb. Nonspecific binding was blocked with 1% bovine serum albumin. Culture supernatant was added for 2 hr at room temperature. The plate was washed and anti-mouse biotin-conjugated IFN- γ (XMG1.2) (1:500), IL-10 (JES5-2A5) (1:500), TNF- α (1F3F3D4) (1:1000), IL-6 (MP5-20F3) (1:500), or IL-17 (TC11-8H4) (1:500) mAb was added. The plate was then washed, and avidin-horseradish peroxidase (Sigma, Saint Louis, MO, USA) was added. The plate was washed again, and 3,3',5,5'-tetramethylbenzidine was added and left for a few min. The reaction was stopped by adding 2.5 M H₂SO₄, and the absorbance at 450 nm was measured with an Immuno Reader NJ-2000 (Nihon Inter Med, Tokyo, Japan). These antibodies were purchased from eBioscience. The assay sensitivities were 16-1,000 pg/ml (IFN- γ system), 2-125 pg/ml (TNF- α system), 8-2,000 pg/ml (IL-6 system), 63-4,000 pg/ml (IL-17 system) and 63-4,000 pg/ml (IL-10 system). Values are the average $\pm$ S.E.M. of three independent assays.

Statistics

Each value is given as the mean $\pm$ S.D. Comparison between two values was performed by using the unpaired Student's t-test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni's post hoc analysis. Calculations were done with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA, USA). The criterion of significance was $P < 0.05$.

RESULTS

The particle diameter distribution of the DEP in the DE gas was shown in Fig. 2. The mean diameter of DEP was 104 nm. Though chemicals and gaseous components included in DE were not examined in this study, they would be almost similar to those which were already reported by our research group (Ono *et al.*, 2008). DE exposures were conducted at doses of 0.1 and 1.78 mg DEP/m³ according to the protocol as shown in Fig. 1. Since DE exposure at the dose of 0.1 mg DEP/m³ had little effect, only data obtained at the dose of 1.78 mg/m³ are presented in this section.

Footpad swelling and splenomegaly in normal and DTH mice with or without DE exposure were investigated in order to determine the effect of DE on mBSA-induced DTH. Significant footpad swelling was observed in DTH mice, compared with normal control mice. The footpad swelling in DTH mice was further augmented by DE exposure (Fig. 3A). No significant splenomegaly was observed in any of the groups (Fig. 3B).

Pro-inflammatory cytokines involved in the pathology of DTH were assayed. The levels of IFN- γ and TNF- α in DTH mice were both significantly increased in comparison with those in normal control mice. DE exposure significantly increased the level of TNF- α , but not that of IFN- γ in normal control mice (Figs. 4A and B). However, these values in DE-exposed DTH mice were significantly higher than those in DTH mice. The level of IL-6, which induces activation of B cells and antibody production, was also significantly increased in DTH mice compared with that in normal control mice, and further enhanced by DE exposure (Fig. 4C). Moreover, we examined the effect of DE exposure on the production of IL-17 by Th17 cells. The level of IL-17 in DTH mice was much higher than that in normal control mice, and the level was further significantly augmented by DE exposure (Fig. 5A). It is likely that augmented production of these pro-inflammatory cytokines is involved in the exacerbation of DTH pathology induced by DE exposure. Next, anti-inflammatory cytokine IL-10 was assayed. As shown in Fig. 5B,

the level of IL-10 was higher in DTH mice than in normal control mice, but there was no difference in IL-10 production between DTH mice and DE-exposed DTH mice.

To elucidate the involvement of Treg in the DE-induced exacerbation of DTH, changes in the population of Treg cells in CD4⁺ T cells were examined. CD4⁺CD25⁺Foxp3⁺ T cells were considered as Treg cells in this study. The percentage of CD25⁺Foxp3⁺ T cells in CD4⁺ T cells of DTH mice was slightly, but not significantly, higher than that of normal control mice, and DE exposure had no effect on the value in DTH mice (Fig. 6A). The percentages of helper T cells (CD3⁺CD4⁺), cytotoxic T cells (CD3⁺CD8⁺), B cells (CD19⁺) and plasma cells (CD3⁺CD19⁺CD38⁺) in splenocytes in all groups were analyzed. No significant differences were found (Fig. 6B).

DISCUSSION

In this study, we examined the effect of DE exposure in a mouse model of DTH. The significant footpad swelling observed in DTH mice compared with normal control

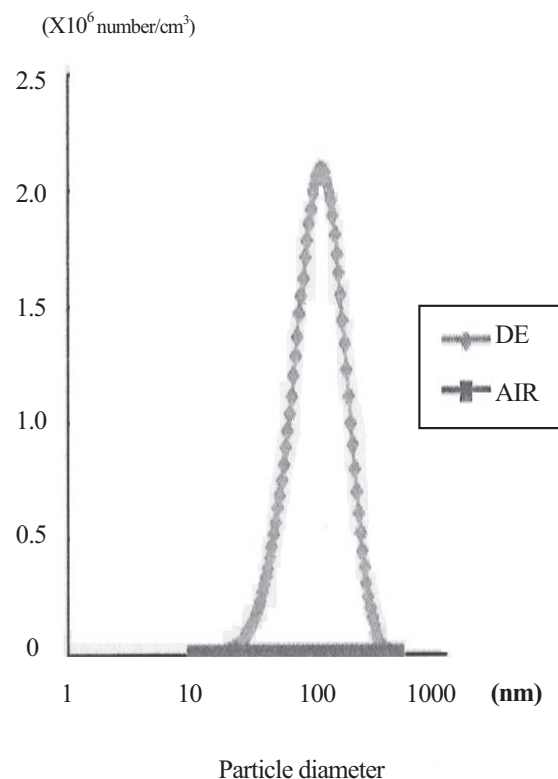


Fig. 2. Particle diameter distribution of diesel exhaust particles in diesel exhaust gas.

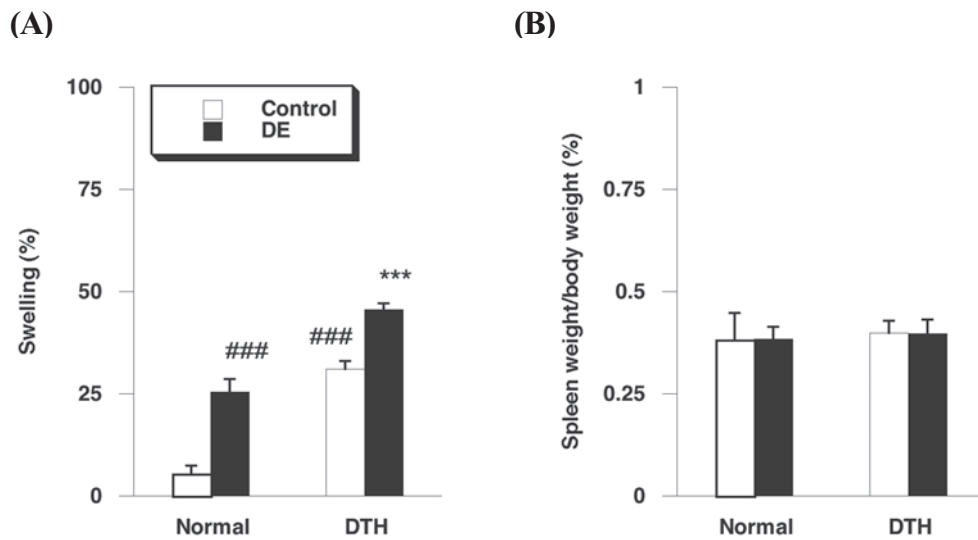


Fig. 3. Effects of DE exposure on footpad swelling and splenomegaly in DTH mice. Mice were exposed to DE (1.78 mg DEP/m³; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μ l of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μ l of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On Day 15, footpad thickness and spleen weight were measured to determine footpad swelling and splenomegaly as described in “Materials and Methods”. (A) Footpad swelling and (B) Splenomegaly. Each value represents the mean $\pm$ S.D. for five mice. ###Statistically significant difference ($p < 0.001$) compared to the normal control group. ***Statistically significant difference ($p < 0.001$) compared to the DTH-disease control group.

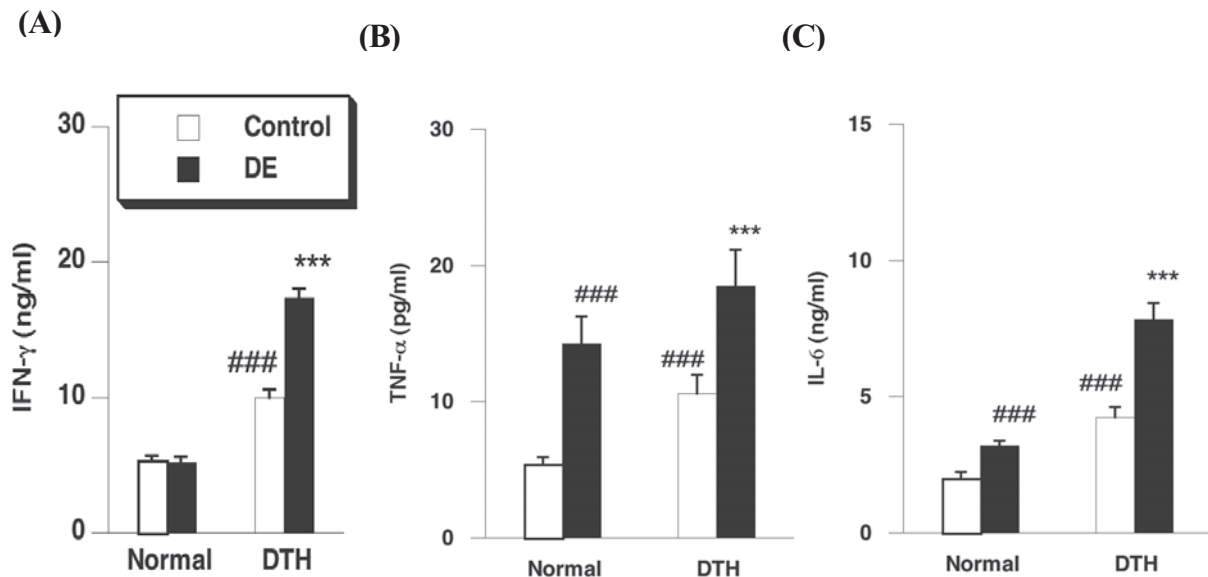


Fig. 4. Effect of DE exposure on production of IFN- γ , TNF- α and IL-6 by splenocytes. Mice were exposed to DE (1.78 mg DEP/m³; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μ l of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μ l of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On day 15, the spleen was harvested. Splenocytes (7×10^6 cells/ml) were cultured with mBSA (10 mg/ml) for 96 hr in a CO₂ incubator. The supernatants were subjected to assays of IFN- γ , TNF- α and IL-6 by ELISA as described in “Materials and Methods”. (A) IFN- γ , (B) TNF- α , and (C) IL-6. Each value represents the mean $\pm$ S.D. for four or five mice. ###Statistically significant difference ($P < 0.001$) compared to the normal control group. ***Statistically significant difference ($p < 0.001$) compared to the DTH-disease control group.

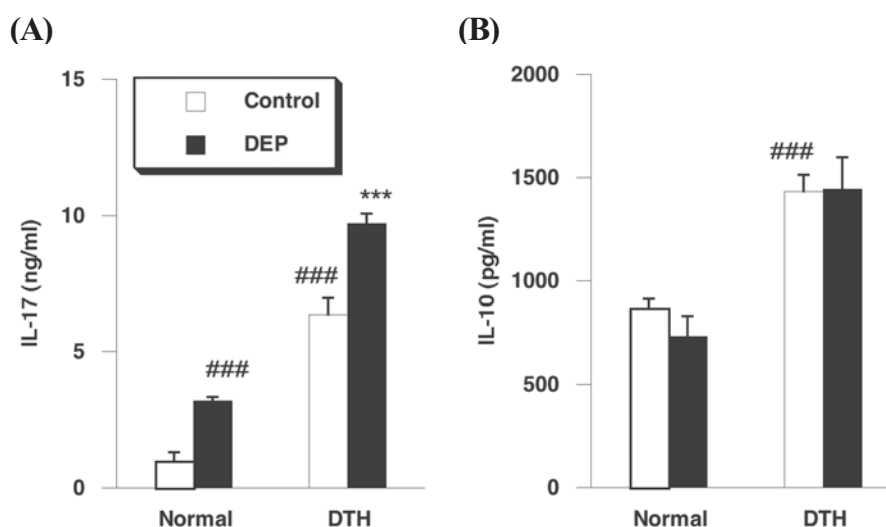


Fig. 5. Effect of DE exposure on production of IL-17 and IL-10 by splenocytes. Mice were exposed to DE (1.78 mg DEP/m³; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μ l of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μ l of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On day 15, the spleen was harvested. Splenocytes (7×10^6 cells/ml) were cultured with mBSA (10 mg/ml) for 96 hr in a CO₂ incubator. The supernatants were subjected to assays of IL-17 and IL-6 by ELISA as described in "Materials and Methods". (A) IL-17 and (C) IL-10. Each value represents the mean $\pm$ S.D. for four or five mice. ###Statistically significant difference ($P < 0.001$) compared to the normal control group. ***Statistically significant difference ($p < 0.001$) compared to the DTH-disease control group.

mice was further increased by DE (Fig. 3A). Interestingly, footpad swelling was also observed in DE-exposed normal control mice. These results indicate that DE activates the immune system. The immune system should be activated in DTH-mice, and further augmented by DE exposure, resulting in splenomegaly, although splenomegaly, which would reflect activation of immune cells, unexpectedly was not observed in any of the groups (Fig. 2B). The activation and the augmentation should have been evaluated by the assay of proliferation activity, such as a ³H-thymidine incorporation study, of splenic lymphocytes. Further studies will be required for full elucidation of these details.

The effect of DE exposure on the production of pro-inflammatory cytokines, such as IFN- γ , TNF- α and IL-6, was also examined. These cytokines are involved in the development of DTH. Increased productions of IFN- γ , TNF- α and IL-6 were observed in DTH mice, and were further increased by DE exposure. It was reported that DEP induces mRNAs of IL-6 and chemokines such as monocyte chemoattractant protein-1 (MCP-1) and keratinocyte-derived chemokine (KC) in TNF- α deficient mice (Saber *et al.*, 2006). IL-6 is considered to play a more important role in DEP-induced inflammation than TNF- α , because it controls the initial steps of the T-cell activation

with enhancement of IL-2 responsiveness (Lotz *et al.*, 1988) and Th17 cell differentiation (Bettelli *et al.*, 2006). Naive T helper cells differentiate into Th17 cells and Treg cells under the influence of IL-6 and TGF- β and IL-2 and TGF- β , respectively. Further, IL-6 suppresses the development of Treg cells (Fong and Mosmann, 1989). That is to say, IL-6 regulates the development of Th17 cells and Treg cells. Though IFN- γ plays an important role in the development of DTH, as mentioned above, the pathology of DTH is actually exacerbated in IFN- γ knock-out mice (Irmeler *et al.*, 2007). A similar phenomenon has been found in other Th1-dominant autoimmune diseases, such as CIA, EAE and experimental autoimmune uveitis (Caspi *et al.*, 1994; Ferber *et al.*, 1996; Jones *et al.*, 1997). In other words, IFN- γ is indispensable in the development of DTH and other immune-related diseases, but also has a protective effect against them. This seems paradoxical from the viewpoint of the Th1 paradigm of DTH. However, the apparent paradox has been resolved by the discovery of Th17 cells, a third subset of T helper cells that secrete IL-17. It is reported that IL-17 and IFN- γ are both involved in the development of these cytokine-producing cells during immune responses, and IL-17 plays a more crucial role in DTH than IFN- γ ; IL-17 can induce the expression of a variety of genes encoding many cytokines

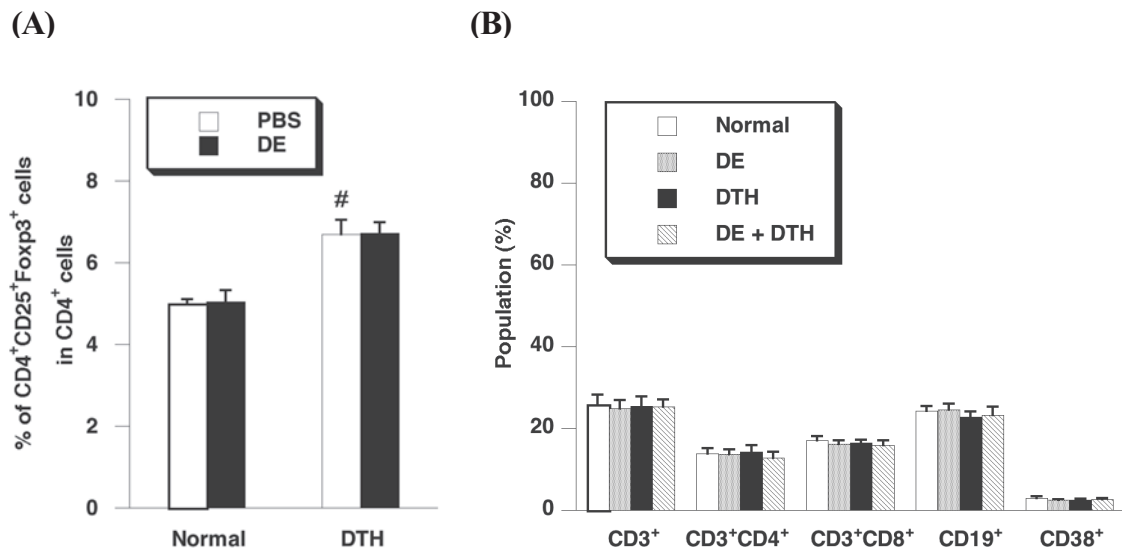


Fig. 6. The percentages of T cells (CD3⁺), helper T cells (CD3⁺CD4⁺), cytotoxic T cells (CD3⁺CD8⁺), B cells (CD19⁺) and plasma cells (CD38⁺) in splenocytes. Mice were exposed to DE (1.78 mg DEP/m³; 8 hr/day) for 14 days. DTH was induced by s.c. injection with 200 μ l of 1.25 mg/ml mBSA in CFA at the dorsal region for initial sensitization on Day 7 and with 20 μ l of 2.5 mg/ml mBSA into one foot-pad on Day 14 as the challenge. On day 15, the spleen was harvested. Immunophenotyping of splenic lymphocytes was carried out by flow cytometry as described in "Materials and Methods". Each value represents the mean $\pm$ S.D. for four or five mice. [#]Statistically significant difference ($p < 0.05$) compared to the normal control group.

involved in the pathology of DTH (Schulz *et al.*, 2008). In fact, Th17 cells and IL-17 production are known to be increased in DTH mice, and blocking IL-17 can ameliorate DTH (Nakae *et al.*, 2002; Irmeler *et al.*, 2007). We found that DEP exposure significantly augmented the production of IL-17 in DTH mice. This result may suggest that increased production of IL-17 is a key mediator of the adverse effect of DE.

Inhibition of oral tolerance has been reported in DEP-administered DTH mice (Yoshino *et al.*, 1998). It can be hypothesized that DEP exposure inhibits Treg cells or activates antigen-specific T cells to exacerbate inflammation in the DTH model. Thus, we measured the percentage of Treg cells in each group; however, no significant difference was observed between DTH mice and DE-exposed DTH mice (Fig. 6A). IL-10, produced by Th2 and Treg cells, is an anti-inflammatory cytokine known to inhibit production of IL-1, IL-4, IL-5, IL-6, IL-8, TNF- α and IFN- γ (Del Prete *et al.*, 1993; Wang *et al.*, 1994). We found no difference in IL-10 production between DTH mice and DE-exposed DTH mice (Fig. 5B). These results may imply that Treg cells are not involved in the DE exposure-induced exacerbation of DTH. Further, we found no significant difference in the percentages of other lymphocytes (CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD19⁺,

CD38⁺) between DTH mice and DE-exposed DTH mice (Fig. 6B).

In conclusion, it has been demonstrated that DEP inhalation may, in part, exacerbate the pathological symptoms of DTH and induction of pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-6, and IL-17.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for the private University Science Research Upgrade Promotion Business Academic Frontier Project.

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