

Organic Chemicals in Diesel Exhaust Particles Enhance Picryl Chloride-Induced Atopic Dermatitis in NC/Nga Mice

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Key Words

Atopic dermatitis · Diesel exhaust particles · Mast cells · NC/Nga · Picryl chloride

Abstract

Background: Diesel exhaust particles (DEP) have been reported to worsen allergic airway inflammation in mice. Recently, the organic chemical components of DEP (DEP-OC) were found to be important contributors to the aggravation of allergic airway inflammation in mice. The purpose of this study was to examine the effects of DEP-OC on atopic dermatitis (AD)-like skin lesions induced by picryl chloride (PiCl) in NC/Nga mice. **Methods:** DEP were extracted with benzene/ethanol, and the soluble organic fraction formed the DEP-OC. NC/Nga male mice received simultaneous application of DEP-OC and/or PiCl on their ears once a week for 9 or 3 weeks. We evaluated skin lesions by noting scaling, eruption, excoriation, erosion, hemorrhage, pathologic changes, production of cytokines, and IgE level in the serum. **Results:** PiCl application alone produced progressively severe AD-like skin lesions. The application of PiCl plus DEP-OC resulted in a marked worsening of skin lesions in the early stages of AD. Moreover, mast cell counts significantly increased in the

subcutaneous tissue. Administration of PiCl combined with DEP-OC resulted in a greater increase in the local expression of interleukin-4, keratinocyte chemoattractant, and neutrophils in subcutaneous tissue compared with PiCl treatment alone. In contrast, the combination treatment produced lower levels of IFN- γ compared with PiCl treatment alone. **Conclusions:** DEP-OC application to the skin aggravated PiCl-induced AD. This aggravation may be due to activation of the Th2-associated immune responses by the organic chemicals in DEP.

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Introduction

Atopic dermatitis (AD) is a highly pruritic chronic inflammatory skin disease characterized by elevated serum IgE levels and skin lesions infiltrated by eosinophils, CD4⁺ T helper type 2 (Th2) cells [1–3], and Th2 cells that produce interleukin (IL)-4, IL-5, and IL-13 [4, 5]. It is a multifactorial disorder influenced by both genetic and environmental factors, which complicates the development of treatment. The prevalence and severity of AD are increasing in industrialized countries worldwide [6], and

one possible reason is the increase in air pollution, which may be caused by industrial processes, gaseous chemicals from building materials, and automobile diesel exhaust.

Epidemiological studies have shown a correlation between the incidence of allergic diseases and the distance to major traffic routes, suggesting that traffic-related air pollution plays a role in the increase in such conditions, including AD [7–9]. Further, diesel exhaust particles (DEP) emitted from diesel engine vehicles have been shown to exacerbate allergic bronchial asthma and allergic rhinitis [10, 11]. Several studies in mice have shown that intranasal, intraperitoneal, or intratracheal administration of DEP to mice causes increases in antigen-specific serum IgE antibody production and induces local infiltration of inflammatory cells in the lung [12–16]. It has also been suggested that these effects are associated with the production of various cytokines, including IL-4, IL-5, IL-8, IL-10, and IL-13 [12–16]. Similarly, Yanagisawa et al. [17] reported that administration of organic chemicals extracted from DEP (DEP-OC) exaggerated ovalbumin allergic airway inflammation through the enhancement of Th2 responses in mice.

The NC/Nga strain of mice, which originates from Japanese fancy mice, was established as an inbred strain by Dr. Kondo in 1957 [18]; moreover, it is a commonly used model for human AD [19–21]. Some previous studies reported the effect of environmental factors on AD using this mouse model [22–24]. The aim of the present study was to evaluate the effects of crude DEP-OC on AD in this mouse strain.

Materials and Methods

Animals

Ninety-two male NC/Nga mice (6 weeks old), born and reared under specific pathogen-free conditions, were purchased from SLC (Hamamatsu, Japan). The cages were kept in a 12/12-hour light/dark cycle, at 23–25°C, and 55–70% humidity. Mice were provided ad libitum access to a commercial diet (CE-2; Japan Clea Co., Tokyo, Japan) and water. The study adhered to the US National Institutes of Health guidelines for the use of experimental animals [25]. The animals were treated humanely and with regard to alleviation of suffering. They were handled according to the animal care guidelines of the National Institute for Environmental Studies. The animal care methods were also approved by the Animal Care and Use Committee of the Oita University of Nursing and Health Sciences.

DEP-OC Preparation

DEP-OC was prepared at the Kanazawa University as described previously [26]. Briefly, a diesel engine car (made in Japan, 2,500 cm³, direct injection, 1996 model) was run under idling con-

ditions with a commercial light oil (JIS No. 2). DEPs were collected on glass fiber filters (Pallflex T60A20 55 mm i.d.; Pall Co., Port Washington, N.Y., USA) using a low-volume air sampler. The filters were exchanged every 5 min. The collected DEP were ultrasonically extracted 3 times with benzene/ethanol (3:1) for 20 min. The organic phase was evaporated, and the remaining residue was then reconstituted in ethanol.

Experimental Design

We performed two experiments. For the first, 44 mice were divided into 4 groups: (1) control (n = 10), (2) DEP-OC (n = 10), (3) PiCl (n = 12), and (4) DEP-OC plus PiCl (n = 12). AD was induced according to previously described protocols [19]. For each mouse in the PiCl and DEP-OC plus PiCl groups, 5% (w/v) PiCl (Nacalai Tesque, Kyoto, Japan) was dissolved in acetone/ethanol solution (1:3) and a total volume of 150 µl was then applied to the abdominal skin and each footpad for early sensitization. Five days later (day 0), for the DEP-OC and DEP-OC plus PiCl groups, 2 mg of DEP-OC were dissolved in 50 µl of dimethyl sulfoxide for each mouse, and 25 µl of this solution were gently applied to the dorsal side of each ear using a micropipette with a fine plastic tip. The control and PiCl groups received the same volume of dimethyl sulfoxide in the same manner. Three hours after these treatments, 50 µl of 0.8% (w/v) PiCl dissolved in olive oil containing 50% acetone were applied on the same dorsal areas of the ears of PiCl and DEP-OC plus PiCl mice. The control and DEP-OC groups received 50 µl of olive oil containing 50% acetone using the same protocol. These applications were performed once a week for 9 weeks. Two days after the last application, the mice were sacrificed by exsanguination under deep diethylether anesthesia.

For the second experiment, we repeated the above procedure with only 3 applications (3 weeks) instead of 9, using 48 mice (n = 12 in each group). One day after the last application (day 15), the mice were sacrificed. All of the mice in both experiments were anesthetized by inhalation of halothane during the application treatment.

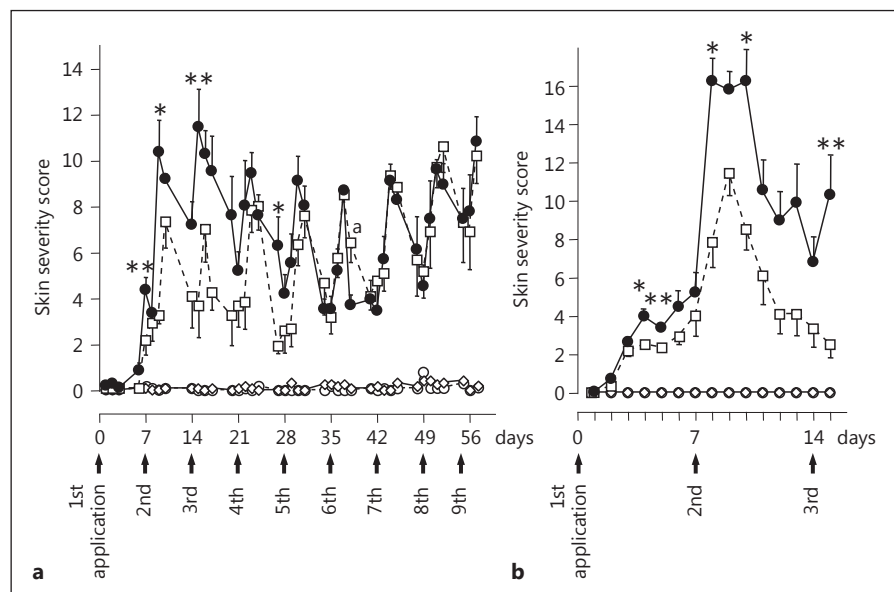
Evaluation of Skin Lesions

Any skin symptoms on the ears, including scaling, eruption, excoriation, erosion, and hemorrhaging as AD indicators [27], were recorded 5 days per week in the first experiment and 7 days per week in the second experiment. Based on these findings, the severity of dermatitis was scored with blind tests as 0, no symptoms; 1–4, very slight symptoms; 5–8, slight symptoms; 9–12, moderate symptoms; 13–16, moderate/extreme symptoms, and 17–20, extreme symptoms. We ran the test twice and averaged the scores.

Measurement of the Total IgE Antibody Level

Sera were collected from the mice by puncture of the retro-orbital venous plexus before the first immunization and every 2 weeks during the experiments. At each sampling, we mixed the sera of all 12 mice in the respective group and stored it at –80°C for later measurement. Total IgE antibody concentration was measured using an ELISA kit from Shibayagi Co. Ltd. (Gunma, Japan) and obtained from the standard curves prepared using varying concentrations of standard IgE solution. The detection limit of this assay was less than 1 ng/ml.

Fig. 1. Skin severity scores for the ears of NC/Nga mice. **a** First experiment with 9 weekly applications of PiCl/DEP-OC. **b** Second experiment with 3 weekly applications of PiCl/DEP-OC. **a**, **b** ○ = Control; ◇ = DEP-OC; □ = PiCl; ● = DEP-OC plus PiCl. Means ± SE of 12 mice. * $p < 0.05$, ** $p < 0.01$ vs. the PiCl group; ^a $p < 0.01$ vs. the DEP-OC plus PiCl group.



Histopathological Analysis

Six mice from each group in the second experiment were used for histopathological observation. The ears were fixed in 10% neutral phosphate-buffered formaldehyde. Three pieces of approximately 2-mm-wide sections from each ear were embedded in paraffin, and 2 sections 3 μ m thick were prepared from each paraffin block. One 3- μ m section was stained with hematoxylin and eosin (HE) and the other with toluidine blue. We arbitrarily selected 3 points on each piece, which indicated the apical, central, and basal parts. Inflammatory cells in the subcutaneous tissue were counted in these 9 arbitrarily selected areas using an Olympus BH-2 light microscope (Olympus Co., Tokyo, Japan). The area consisting of perpendicular lines extended from the edges of the cartilage of the external ear by a length of 250 μ m to the epidermal layer.

Cytokine and Chemokine Quantification in Ear Tissue

In the second experiment, 6 animals from each group were used to measure cytokine levels in the ear tissue. The ear tissue was homogenized and centrifuged as described in previous studies [15, 28]. The cytokine and chemokine levels in the supernatants were assayed by ELISA according to the manufacturer's instructions. IL-4 levels were assessed using an ELISA kit from Amersham Pharmacia Biotech, Inc. (Little Chalfont, UK); keratinocyte chemoattractant (KC) levels were measured using an ELISA kit from R&D Systems Inc. (Minneapolis, Minn., USA), and interferon (IFN)- γ levels using an ELISA kit from Pierce Endogen (Rockford, Ill., USA). The detection limits of IL-4, KC, and IFN- γ were 5.0, 2.0, and 10.0 pg/ml, respectively.

Statistical Analysis

All data are reported as means $\pm$ SE. Based on the outcome of Bartlett's test, either parametric or nonparametric analysis was performed. If the sample data had equal variances ($p > 0.05$), one-way ANOVA with Fisher's protected least-significant difference was performed; if not, Kruskal-Wallis one-way ANOVA for inde-

pendent groups was performed. With overall significance established, a Steel-Dwass' multiple comparison procedure was used to determine differences. A value of $p < 0.05$ was considered statistically significant in all analyses.

Results

Skin Lesions

Figure 1a shows the changes in the skin severity score for each group in the first experiment (9 weekly applications). The control group and DEP-OC group showed no AD-like symptoms, while PiCl and DEP-OC plus PiCl mice developed skin lesions (including erosion and hemorrhage). In the PiCl group, these skin lesions appeared 7 days after the first application of PiCl. Skin lesions worsened 3 days after the second PiCl application and then resolved spontaneously. Thereafter, lesion scores were elevated for 2–3 days after the PiCl application, and the deterioration and remission patterns were repeated until the end of the experiment. In the DEP-OC plus PiCl group, the first symptoms appeared 6 days after the first DEP-OC and PiCl applications. The skin scores for this group were significantly higher 7, 9, and 15 days after the first application compared to the PiCl group ($p < 0.01$, $p < 0.05$, and $p < 0.01$, respectively), but the skin scores for both groups were similar after day 31.

The changes in the skin severity score for the second experiment (3 weekly applications) were similar to the early phase of the first experiment (fig. 1b). Scores for the

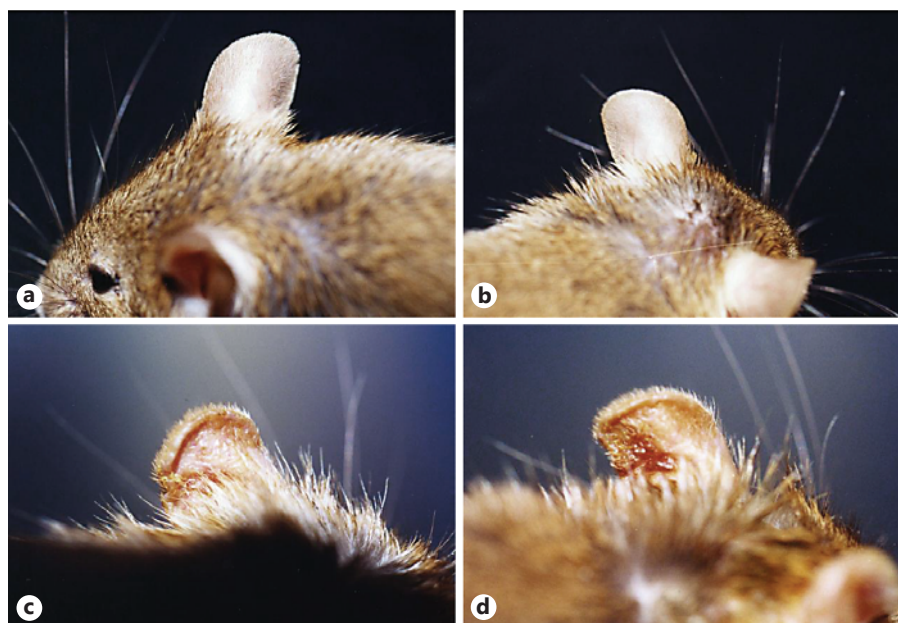


Fig. 2. Macroscopic features of the mouse ears on day 16. **a** Control. **b** DEP-OC. **c** PiCl. **d** DEP-OC plus PiCl.

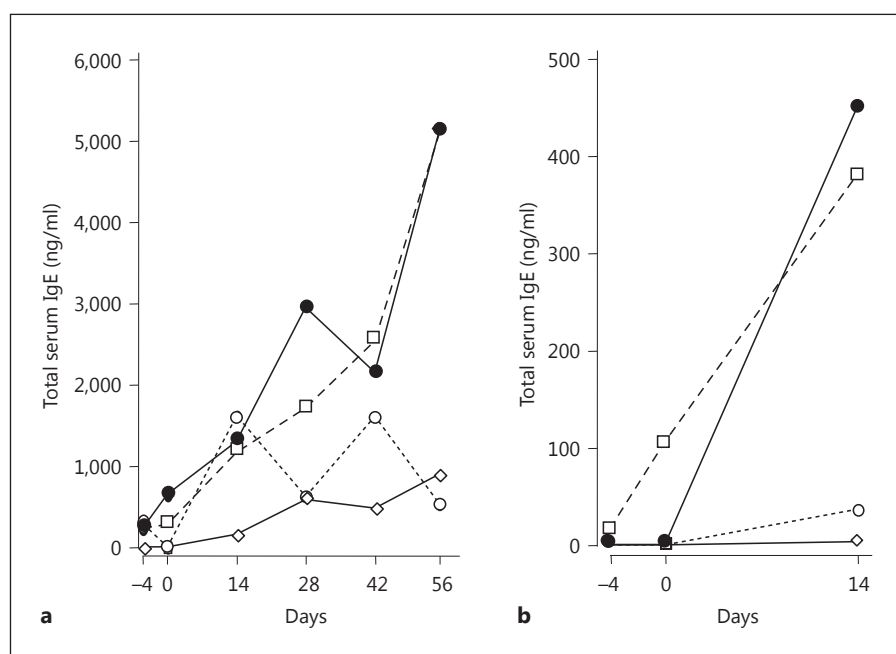


Fig. 3. Total IgE levels in the serum of the NC/Nga mice. ○ = Control; ◇ = DEP-OC; □ = PiCl; ● = DEP-OC + PiCl. **a** First experiment with 9 weekly applications of PiCl/DEP-OC. **b** Second experiment with 3 weekly applications.

DEP-OC plus PiCl group were significantly higher than for the PiCl group on days 4 and 5 ($p < 0.05$ and $p < 0.01$, respectively). On day 8, DEP-OC plus PiCl scores markedly increased ($p < 0.05$) and remained elevated until day 15. On days 10 ($p < 0.05$) and 15 ($p < 0.01$), the severity scores were significantly different between the DEP-OC plus PiCl and PiCl groups.

Representative macroscopic skin features of the mouse ears on day 16 are presented in figure 2. There were no lesions in either the control or DEP-OC groups (fig. 2a, b), whereas the PiCl group exhibited scaling (fig. 2c). The DEP-OC plus PiCl group developed scaling and more severe symptoms such as erosion (fig. 2d).

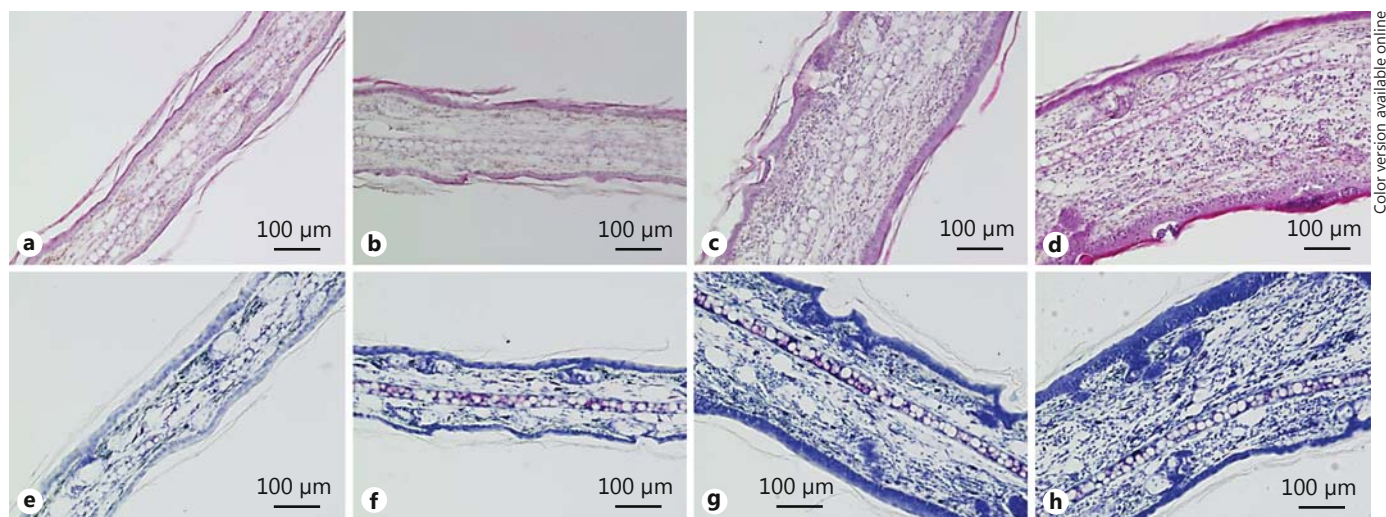


Fig. 4. Histological features of ear sections stained with HE (a–d) and toluidine blue (e–h) in NC/Nga mice. a, e Control. b, f DEP-OC. c, g PiCl. d, h DEP-OC plus PiCl.

Production of Total IgE

In the first experiment, there was little change in total IgE in the DEP-OC group (fig. 3a). The total IgE levels for the DEP-OC plus PiCl and PiCl groups were higher than those of both the control and the DEP-OC groups, except on day 14. However, the difference in total IgE levels between the DEP-OC plus PiCl group and the PiCl group was not significant. In the second experiment, there were no significant differences in the levels of total IgE between the DEP-OC plus PiCl and PiCl groups at any time (fig. 3b).

Inflammation of Subcutaneous Tissue

Histopathological changes were analyzed using the ear tissue sections from the second experiment (3 weekly applications) stained with HE (fig. 4a–d) or toluidine blue (fig. 4e–h). No pathological change was noted in the control and DEP groups (fig. 4a, b, e, f). The PiCl and DEP-OC plus PiCl groups showed thickening of the epidermis layer and subcutaneous tissue layer, and accumulation of inflammatory leukocytes in the subcutaneous tissue compared to the non-PiCl groups (fig. 4c, d, g, h). Figure 5 shows the number of the inflammatory cells in the subcutaneous tissue. The number of neutrophils in the DEP-OC plus PiCl group was higher than that in the PiCl group; however, the difference between these values was not significant (fig. 5a). Moreover, there was no difference in eosinophil counts between the PiCl and DEP-OC plus PiCl groups (fig. 5b). However, the mast cell count

was significantly higher in the DEP-OC plus PiCl group compared to the PiCl group ($p < 0.05$; fig. 5c).

Cytokine and Chemokine Levels

IL-4, KC, and IFN- γ levels were measured from the supernatants of ear tissue from the second experiment (3 weekly applications). The DEP-OC group showed no changes in the expression of cytokines and chemokines (fig. 6). At the end of the experiment, levels of IFN- γ in the PiCl and DEP-OC plus PiCl groups were significantly lower than in the control group (fig. 6a), while IL-4 and KC levels for these groups were significantly higher than in the control group (fig. 6b, c). The DEP-OC plus PiCl group had a lower IFN- γ level compared with the PiCl group, but the difference was not significant. This group also showed a higher level of IL-4 and KC compared with PiCl.

Discussion

This study demonstrated that the application of DEP-OC to the skin of NC/Nga mice accelerated the development of PiCl-induced AD. In the experiment with 9 weekly applications of DEP-OC and/or PiCl, this effect appeared 1 day after the second application and 2 days after the third application. This finding shows that DEP-OC can aggravate skin symptoms at the early stages of AD in NC/Nga mice. To investigate the underlying mech-

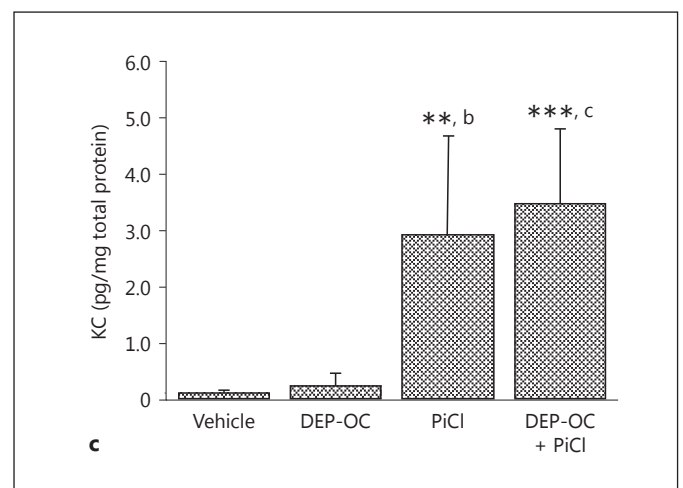
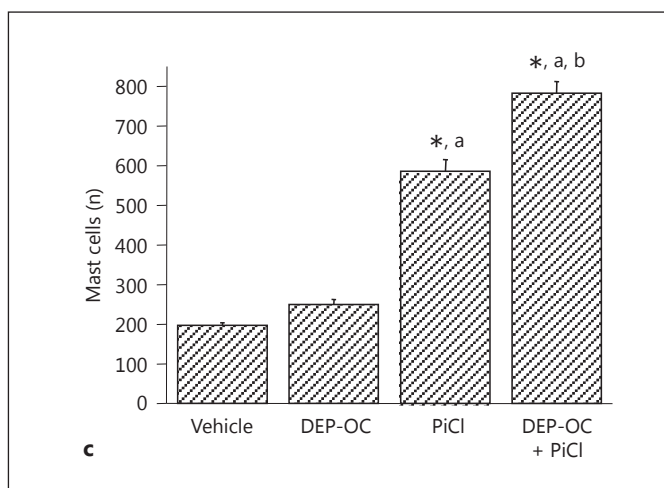
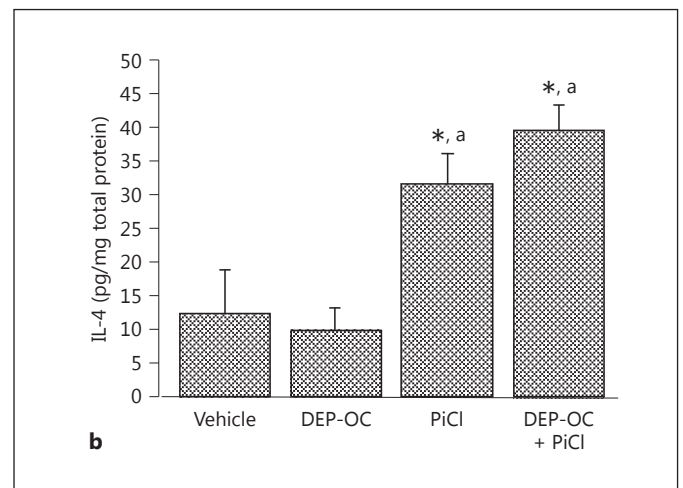
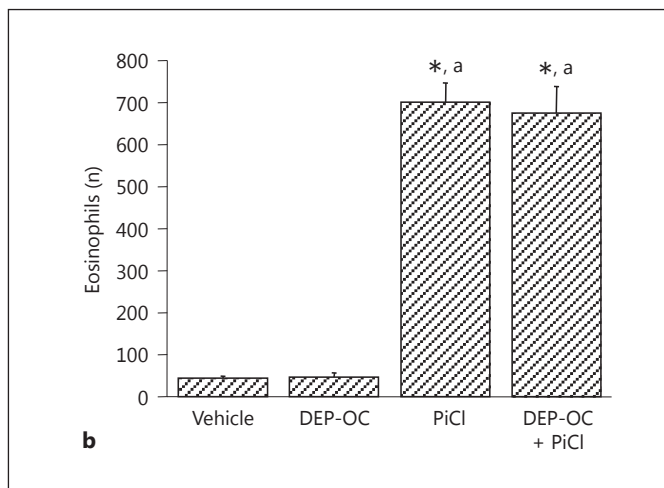
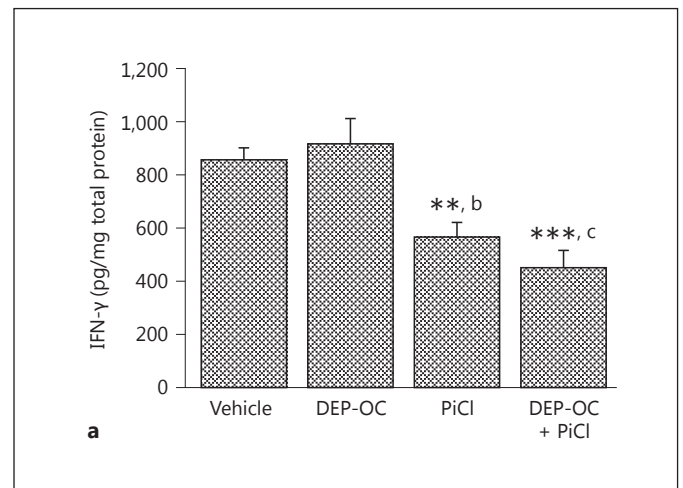
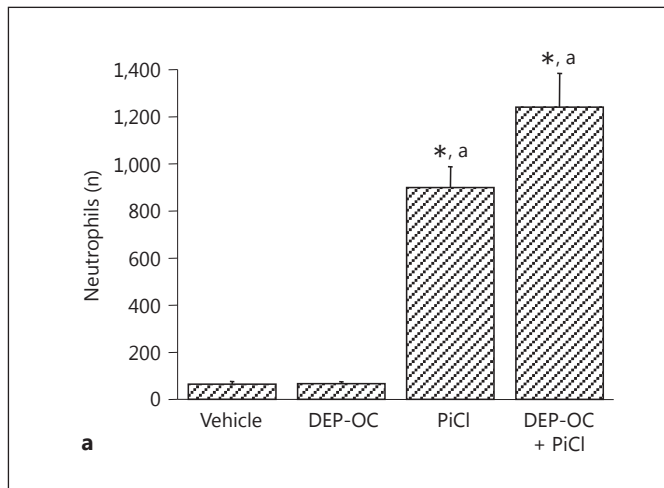


Fig. 5. Numbers of inflammatory leukocytes in the subcutaneous tissue. **a** Neutrophils. **b** Eosinophils. **c** Mast cells. **a–c** Means $\pm$ SE of 6 mice. * $p < 0.05$ vs. the control group; ^a $p < 0.05$ vs. the DEP-OC group, ^b $p < 0.05$ vs. the PiCl group.

Fig. 6. Levels of cytokines and chemokines from the second experiment with 3 weekly applications. **a** IFN- γ . **b** IL-4. **c** KC. **a–c** Means $\pm$ SE of 6 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the control group; ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ vs. the DEP-OC group.

anism, we performed a shorter experiment with only 3 weekly applications. Histologic results from this second experiment showed infiltration of mast cells and neutrophils in the skin lesions caused by PiCl application. The application of PiCl together with DEP-OC caused significant inflammation in these cells along with increased IL-4 and KC production and decreased IFN- γ production.

AD is characterized by high levels of serum IgE and a shift from a Th1-dominant to a Th2-dominant immune system [29, 30]. Th2-dominant immune responses have been shown for allergic asthma, rhinitis, and allergic airway inflammation [31–33]. Heo et al. [34] demonstrated that co-injection of mice with ovalbumin and DEP enhanced the production of allergen-specific IgE compared with administration of ovalbumin alone. In another study, mRNA of Ym1 and Ym2, proteins involved in allergic peritonitis through the IL-4/STAT 6 signal transduction pathway [35], were upregulated in the lung tissue of mice exposed to DEP [36]. Exposure to DEP was also shown to cause infiltration of inflammatory cells in bronchoalveolar lavage fluid and perivascular areas in mouse lungs [37]. Cultured cells from the popliteal lymph node in DEP-treated mice have been shown to produce IL-4 at significantly higher levels than untreated mice [38]. Thus, these animal studies suggest that DEP aggravates allergic diseases through Th2-mediated immune responses. We also found that serum IgE levels in the DEP-OC plus PiCl group increased at a quicker rate than that observed in the PiCl group, but the periodic changes in IgE levels were not synchronized with skin severity scores. Therefore, we believe that DEP-OC might not accelerate the production of IgE antibodies from B cells stimulated by PiCl.

DEP can be divided into the soluble organic fraction and the residual carbonaceous nuclei. In a previous study, our group found that the benzene-/ethanol-soluble organic chemical components in DEP, but not the residual carbonaceous nuclei, promote allergic airway inflammation through the enhancement of Th2 immune responses [17]. DEP affects inflammatory cells by suppressing IL-12 and IFN- γ production induced by lipopolysaccharide stimulation [39, 40]. Chan et al. [40] demonstrated that DEP-OC interfered in lipopolysaccharide-induced IL-12 production in dendritic cells and IFN- γ production in T cells. Moreover, they found that it inhibited the IL-12p40 production in BC1 cells by simulating the agonist to toll-like receptors 2, 3, and 4. These studies show DEP-OC is able to enhance Th2 response by interfering with Th1 immunity.

The levels of cytokines and chemokines noted at the end of the first experiment did not show any differences between the PiCl group and DEP-OC plus PiCl group. On the other hand, on day 15 in the second experiment, the level of IL-4 in the DEP-OC plus PiCl group was higher than that in the PiCl group, while the level of IFN- γ was lower. Skin severity scores were synchronized to the levels of these cytokines. These results may indicate that DEP-OC application activated the Th2 immune response.

The level of KC was also higher in the DEP-OC plus PiCl group than in the PiCl group. KC is a murine homologue of human IL-8 and acts as a chemoattractant for neutrophils. IL-8 facilitates the generation of chemical mediators in inflammatory cells of atopic patients [41]. Clinical research has shown that AD is associated with higher plasma IL-8 levels than other illnesses, including allergic rhinitis, bronchial asthma, urticaria, and contact dermatitis [42]. Moreover, that study showed that IL-8 levels increase with the severity of the condition. In the present study, the increasing level of KC after DEP-OC plus PiCl treatment coincided with skin severity scores. Therefore, the infiltration and activation of neutrophils caused by KC expression may be involved in skin lesion development.

In an earlier immunohistochemical study, mast cells and CD4+ T cells in AD-like skin lesions in mice showed expression of IL-4 and IL-5, but not IFN- γ [19]. In other studies, infiltration of mast cells and eosinophils in lesional skin of mice increased compared with normal skin [19, 43–45]. In the current study, we observed increases in mast cells and neutrophils, but not eosinophils, in the ears of mice in the 3-week application study, and this may have caused the worsening of the skin lesions. In humans, IL-8 is a potent chemotaxin for mast cells [46]. Neutrophil infiltration has been reported to be induced by KC secretion from mast cells in rodents [47]. In this study, increases in KC produced by application of DEP-OC might have caused chemotaxis for mast cells as well as neutrophils.

PiCl used as a hapten induced the contact dermatitis. Moreover, its repeated application to the skin in NC/Nga mice induced skin disease like AD by affecting cytokine production resulting in a shift from the Th1 to the Th2 response [19]. DEP-OC modulated the early stages during the observation period. Treatment with the organic extract of DEP enhanced the expression of Ia, B7.1, and B7.2, which are all cell surface molecules associated with antigen presentation and antigen-presenting function in rat peripheral blood monocytes, which are immature

cells, but not in alveolar macrophages, which are mature cells [48]. Therefore, DEP-OC might influence antigen presentation in immature cells during the sensitization phase.

The reported level of DEP-OC exposure in actual conditions would be lower than that used in the present study; hence, further studies should focus on the effects of low-level long-term exposure of DEP-OC on AD in humans. Moreover, the effect of DEP-OC exposure through another route, such as intratracheal injection, on AD should also be investigated.

In this study, we showed the aggravating effects of DEP-OC administration during the early stages of PiCl-induced AD in NC/Nga mice. Our findings suggest that contact with diesel exhaust may exacerbate skin problems in patients with AD.

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