

The alkylphenols 4-nonylphenol, 4-tert-octylphenol and 4-tert-butylphenol aggravate atopic dermatitis-like skin lesions in NC/Nga mice

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ABSTRACT: Phthalate esters in plastics act as adjuvants for immunoglobulin production, which aggravates allergic disease. However, the effects of alkylphenols (used as plasticizers and surfactants) on atopic dermatitis have not been studied in detail. Therefore, the goal of the present study was to investigate the effects of the alkylphenols 4-nonylphenol (NP), 4-tert-octylphenol (OP) and 4-tert-butylphenol (BP) in a murine model of atopic dermatitis. NC/Nga mice were intraperitoneally administered NP, OP or BP and were subcutaneously injected with mite allergen in one ear to induce atopic dermatitis-like skin lesions (ADSLs). The condition of the skin was observed, and the levels of immunoglobulin in serum and inflammatory cytokines in lesions were determined. NP exacerbated mite allergen-induced ADSLs according to dose. OP and BP also significantly exacerbated skin lesions but not as a function of dose. Alkylphenols tended to increase the levels of IgE and antigen-specific IgG₁ in serum. Further, the treatment of the alkylphenols increased the expression in lesions of inflammatory cytokines, interleukin-4 and monocyte chemoattractant protein-3. Thymic stromal lymphopoietin levels increased according to ADSL severity. In contrast, the levels of the T-helper 1 cytokines (interleukin-18 and interferon- γ) decreased. NP, OP or BP may enhance T-helper 2-type immune responses in NC/Nga mice, which aggravates mite allergen-induced ADSLs. Therefore, the uptake of very low levels of alkylphenols may contribute to the increase in the incidence of atopic dermatitis. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: 4-nonylphenol; 4-tert-octylphenol; 4-tert-butylphenol; atopic dermatitis; T-helper 2 cytokine; immunoglobulin

Introduction

Alkylphenols are used in industry as raw materials for detergents and surfactants and as antioxidants in manufacturing plastic and rubber polymers. The family of alkylphenols includes 4-nonylphenol (NP), 4-tert-octylphenol (OP) and 4-tert-butylphenol (BP). NP is a degradation product of nonylphenol ethoxylate and is decomposed by microorganisms in aquatic environments. Nonylphenol ethoxylate, an industrial detergent, accounts for approximately 80% of the total alkylphenol ethoxylate production and is the most commonly used alkylphenol. OP and BP are used as industrial materials, but to a lesser extent than NP. NP was detected in vegetables, fruits, cereal products and meat samples obtained from commercialized products (Sasaki *et al.*, 1999). It is possible that this contamination originated from the polyvinyl chloride (PVC) wrapping film covering the products, or the plastic food container, because the residual NP was found in instruments, containers and packaging made of PVC (Inoue *et al.*, 2001; Kawamura *et al.*, 2000; Sun *et al.*, 2001). Moreover, the migration of alkylphenols from plastic bottles to water or milk surrogate was demonstrated (Li *et al.*, 2010; Loyo-Rosales *et al.*, 2010).

NP migrates in to the cooked rice samples wrapped in PVC film after reheating using a microwave oven (Inoue *et al.*, 2001). Therefore, alkylphenols are suspected to be endocrine-disrupting chemicals, which can cause reproductive and developmental problems in mammals (David *et al.*, 2009; Waring and Harris, 2005), and the kind and amount of plasticizers in the products has been subject to controls (Consumer Product Safety Commission, 2008; European Union, 2005). However, the

small amounts of nonylphenol and bisphenol A detected in food products (Gyllenhammar *et al.*, 2012; Lu *et al.*, 2013) may have the unintended effects of residual plasticizers on humans. Intraperitoneal (i.p.) administration of di-(2-ethylhexyl) phthalate (DEHP) or diisononyl phthalate, which are strongly suspected to be endocrine disruptors (Hoyer, 2001; Latini *et al.*, 2004; Lee and Koo, 2007; Poon *et al.*, 1997), aggravate atopic dermatitis (AD)-like injury in mice (Koike *et al.*, 2010; Takano *et al.*, 2006). In both experiments, the injection volume was less than the no observed adverse effect level (NOAEL) determined by developmental and reproductive toxicity tests. In contrast, the few published studies on the effects of low-dose exposure to alkylphenols on allergic diseases do not establish an etiological relationship.

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Alkylphenols bind to the estrogen receptor and act as female sex hormones (Bolger *et al.*, 1998; Danzo, 1997; Soto *et al.*, 1995; White *et al.*, 1994); however, the nature of the genetic and molecular mechanisms by which endocrine-disrupting chemicals aggravate allergic reactions is not clear. Endocrine disruptors, including NP, bisphenol A and OP, stimulate the generation of reactive oxygen species (Aydogan *et al.*, 2008; Bindhumol *et al.*, 2003; Okai *et al.*, 2004). Oxidative stress is associated with allergic disease as it mediates the activation of transcription factors required for T-helper (Th) 2 cytokine expression (Wu *et al.*, 2004; Jung *et al.*, 2008). Further, exposure to OP enhances interleukin (IL)-4 production in T cells via activation of the nuclear factor of activated T cells transcription factor (Lee *et al.*, 2004). *In vitro* studies demonstrated that nonylphenols enhance human mast cell degranulation (Narita *et al.*, 2007). Increased IL-4 production and degranulation of mast cells are key factors leading to the onset and development of AD in humans (Leung *et al.*, 1987) and in a mouse model (Matsuda *et al.*, 1997). Hence, alkylphenols act as aggravating factors of AD. However, to the best of our knowledge, there is no direct evidence regarding whether alkylphenolic compounds modulate the symptoms of AD. Therefore, in the present study, we investigated the effects of alkylphenols (NP, OP and BP) on a model mouse (NC/Nga) of AD.

Materials and Methods

Animals

The Research Ethics Committee of the Oita University of Nursing and Health Sciences, Oita, Japan approved this study. The Committee's guidelines follow those of the United States National Institutes of Health for the use of experimental animals (Resources IoLA, 1996). Eighty-four male NC/Nga mice [body weight (BW), 22–25 g] aged 7 weeks were purchased from Charles River Japan (Osaka, Japan) and maintained under conventional conditions for 1 week before starting the experiment. Six animals were housed per cage under a 12/12 h light/dark cycle and were provided *ad libitum* access to a commercial diet (CE-2; Japan CLEA Co., Tokyo, Japan) and water. The animal facility was maintained at 23–25 °C and 50–70% humidity.

Chemicals

NP, technical grade, OP, purity 99.3%, and BP, purity 99.3% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mite crude extract (*Dermatophagoides pteronyssinus*, Der p) was obtained from Cosmo Bio LSL (Tokyo, Japan) and was used as an allergen for inducing AD.

Experimental Design

Mice were divided into 14 experimental groups as follows: (1) saline + vehicle; (2) Der p + vehicle; (3) Der p + NP 1.75; (4) Der p + NP 17.5; (5) Der p + NP 175; (6) Der p + NP 1750; (7) Der p + OP 2.625; (8) Der p + OP 26.25; (9) Der p + OP 262.5; (10) Der p + OP 2625; (11) Der p + BP 10.5; (12) Der p + BP 105; (13) Der p + BP 1050; and (14) Der p + BP 10 500 (as shown in Table 1). The saline + vehicle group was not treated with an induction of AD, nor application of alkylphenols. The animals in this group were treated with 10 µl of saline, and the other groups were treated with 5 µg of Der p dissolved in saline. The mice were anesthetized with 4% halothane (Takeda Pharmaceutical

Table 1. Experimental groups

Experimental groups (*n* = 84)

1	Saline + vehicle
2	Der p + vehicle
3	Der p + NP 1.75
4	Der p + NP 17.5
5	Der p + NP 175
6	Der p + NP 1750
7	Der p + OP 2.625
8	Der p + OP 26.25
9	Der p + OP 262.5
10	Der p + OP 2625
11	Der p + BP 10.5
12	Der p + BP 105
13	Der p + BP 1050
14	Der p + BP 10 500

BP, 4-*tert*-butylphenol; Der p, *Dermatophagoides pteronyssinus*; NP, 4-nonylphenol; OP, 4-*tert*-octylphenol.

Company, Osaka, Japan) and then injected subcutaneously with saline or Der p on the ventral side of their right ears on days 0, 2, 5, 7, 9, 12, 14 and 16. The alkylphenols in 0.1 ml olive oil were administered i.p. on days –6, 1, 8, and 15 to 12 groups as follows: NP (1.75, 17.5, 175 or 1750 µg per animal), OP (2.625, 26.25, 262.5 or 2625 µg per animal) or BP (10.5, 105, 1050 or 10 500 µg per animal). These compounds were not administered to the saline + vehicle group and Der p + vehicle group. These two groups received 0.1 ml olive oil i.p.

Evaluation of Skin Diseases

One day before starting the subcutaneous injection of antigen, and 24 h after each subcutaneous injection, we measured ear thickness using a gauge (G-1A; Ozaki Mfg. Co., Ltd, Tokyo, Japan) and evaluated the clinical scores for the ear skin using a previously published method (Takano *et al.*, 2006). Briefly, the total clinical severity score was defined as the sum of the individual scores for skin dryness, eruption, edema, crust and erosion as follows: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms.

Measurement of Total IgE and *Dermatophagoides Pteronyssinus*-Specific IgG₁

At the end of the experiment, the mice were anesthetized, and blood samples were collected by cardiac puncture. Sera were assayed for total IgE and Der p-specific IgG₁ using published protocols (Sadakane *et al.*, 2002; Yanagisawa *et al.*, 2006).

Quantification of Cytokine and Chemokine Levels in Ear Tissue Homogenates

The right ears of the mice that were killed were removed on day 17 and were homogenized and centrifuged as previously described (Takano *et al.*, 1997, 2002). The levels of cytokines and chemokines in ear tissue supernatants were assayed using ELISAs according to the manufacturer's instructions. The levels of IL-6, IL-13, eotaxin, keratinocyte chemoattractant (KC), macrophage inflammatory protein-1 alpha (MIP-1α) and thymic

stromal lymphopoietin (TSLP) were measured using Quantikine ELISA Kits from R&D Systems, Inc. (Minneapolis, MN, USA). IL-4, IL-5 and interferon gamma (IFN- γ) levels were measured using ELISA kits from Endogen, Inc. (Rockford, IL, USA), and the monocyte chemotactic protein (MCP)-3 level was measured using an Instant ELISA Kit from BMS Inc. (Vienna, Austria). The IL-18 level was measured using an ELISA kit from MBL (Nagoya, Japan). The detection limits of IL-6, IL-13, eotaxin, KC, MIP-1 α , TSLP, IL-4, IL-5, IFN- γ , MCP-3 and IL-18 were 1.6, 1.5, 3.0, 2.0, 1.5, 2.63, 5.0, 5.0, 5.3, 2.6 and 25 pg ml⁻¹, respectively.

Statistical Analysis

Data represent the mean $\pm$ SE. Statistical analyses were performed using KyPlot (KyensLab, Tokyo, Japan). Based on the outcome of

Bartlett's test, either parametric or nonparametric analysis was performed. If the sample data had equal variances ($P > 0.05$), Dunnett's multiple comparison test was performed; if not, then Steel's test was used. A P -value of < 0.05 was considered significant.

Results

Clinical Scores

We determined the clinical scores 24 h after antigen (Der p) injection. From day 6, all groups treated with Der p had significantly higher clinical scores (Fig. 1), including those for dryness, redness, erosion, crust formation, edema and ear thickening than the vehicle treatment group. The clinical scores after exposure to NP at doses of 1.75–1750 μ g increased in a dose-dependent manner after day 13. In particular, exposure to

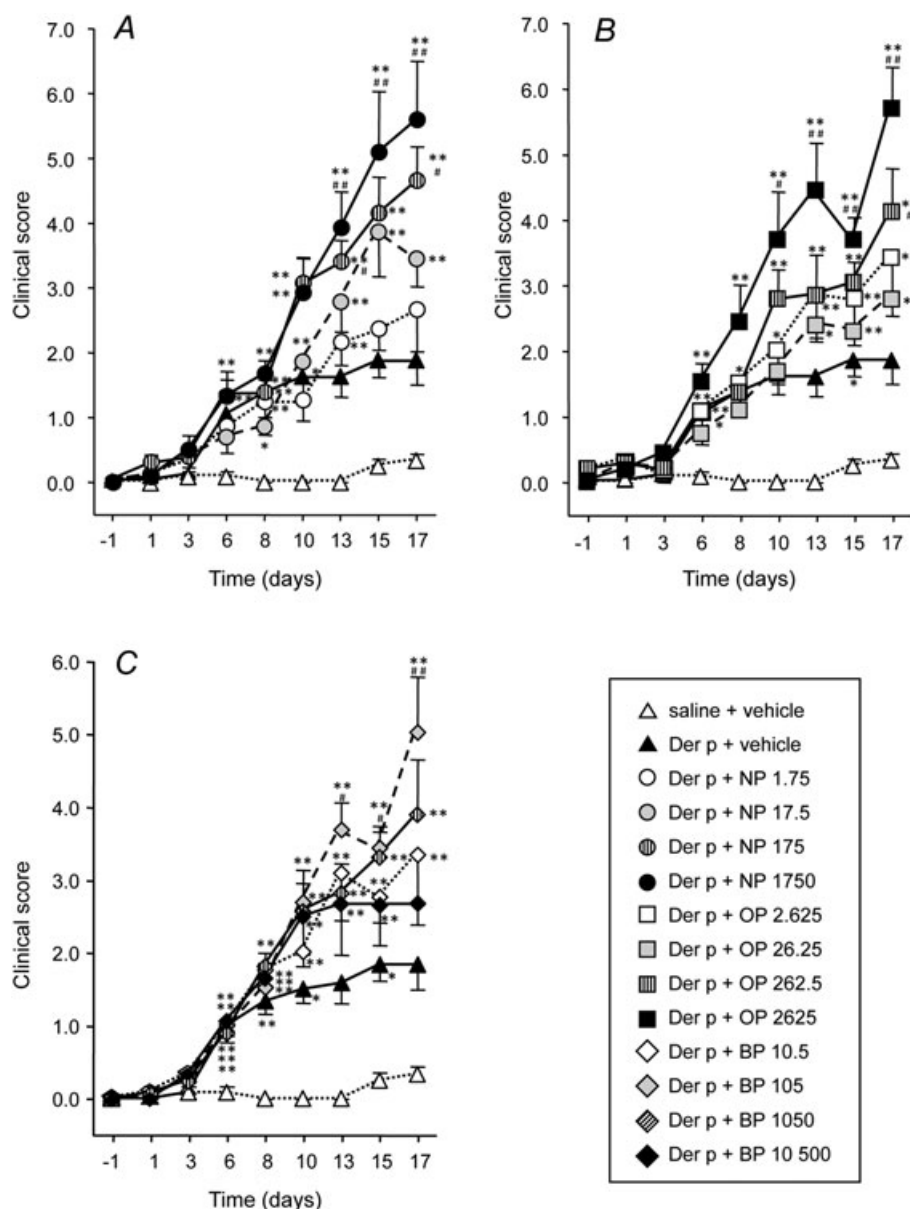


Figure 1. Effect of alkylphenols on atopic dermatitis-like skin lesions induced by Der p on mouse ear (mean $\pm$ SE, $n = 5$ –6 per group). (A) NP, (B) OP, and (C) BP. * $P < 0.05$ and ** $P < 0.01$ versus the saline + vehicle group. # $P < 0.05$ and ## $P < 0.01$ versus the Der p + vehicle group. BP, 4-*tert*-butylphenol; Der p, *Dermatophagoides pteronyssinus*; NP, 4-nonylphenol; OP, 4-*tert*-octylphenol.

1750 μg NP resulted in the most prominent aggravation, followed by exposure to 175 μg NP, compared with Der p + vehicle. The low-dose exposure groups (1.75 and 17.5 μg NP) demonstrated a greater tendency for aggravation of allergic symptoms than the Der p + vehicle group. From day 10, the mice exposed to the highest dose of OP (2625 μg) had greater clinical scores than those treated with the Der p + vehicle. On day 17, the mice exposed to the second highest dose of 262.5 μg also had higher clinical scores than those treated with Der p + vehicle. The other lower-dose exposures also tended to increase the score, although the scores were not as high as Der p + OP 2625 and Der p + OP 262.5 on day 17. The clinical scores after exposure to BP at doses of 10.5–10 500 μg were higher compared with those for Der p + vehicle; however, this difference was not dose dependent. In particular, exposure to 105 μg BP resulted in prominent aggravation on day 13 and day 17 compared with Der p + vehicle. Figure 2 shows the right ears of mice from various mouse groups. We observed no change in the saline + vehicle group (Fig. 2A); however, we noted dryness, redness and edema in the Der p + vehicle group mice (Fig. 2B). Mice exposed to Der p and treated with either NP, OP or BP showed more severe symptoms such as blood crust formation and erosion (Fig. 2C–N).

Levels of Serum Immunoglobulins

To evaluate adjuvant activity caused by exposure to NP, OP and BP, we measured total IgE and allergen-specific IgG₁ levels in sera. There was a trend toward higher levels of total IgE production in the groups exposed to NP than in the Der p group (Fig. 3A). The trends for OP (Fig. 3B) and BP (Fig. 3C) exposure

were similar. Exposure to 17.5 and 175 μg NP resulted in a higher level of Der p-specific IgG₁ than that of the Der p group (Fig. 3D). However, the lowest level of exposure for NP did not cause the adjuvant effect. OP exposure tended to increase the IgG₁ antibody level (Fig. 3E). High-dose BP exposure induced higher levels of IgG₁ than vehicle exposure with Der p (Fig. 3F). In particular, the allergen-specific IgG₁ levels after exposure to 1050 μg BP were significantly higher compared with those of the Der p + vehicle group. The levels of total IgE and Der p-specific IgG₁ in the 13 groups treated with Der p were significantly higher compared with those in the saline + vehicle group or tended to be higher than those in the saline + vehicle group.

Cytokine and Chemokine Levels in Ear Tissue

We then measured the production of cytokines (Th1 and Th2) and chemokines in ear tissue. The levels of all cytokines and chemokines, except IFN- γ , were significantly higher or tended to be higher in all groups treated with Der p than in the saline + vehicle group (Tables 2 and 3). The level of IFN- γ in the Der p + vehicle group was significantly lower than that in the saline + vehicle group (Table 2). The IL-4 and TSLP levels tended to increase in the groups exposed to Der p and treated with either NP or OP. The levels of IL-4, IL-5, IL-6 and TSLP were considerably higher in the groups exposed to Der p and treated with BP than in the groups treated with Der p + vehicle (Table 2). In particular, a significant difference was observed in the IL-4 level for Der p + BP 1050 and for Der p + BP 10 500, the IL-5 level for Der p + BP 1050, and the IL-6 level for Der p + BP 10 500. In mice treated with NP, the highest levels of TSLP production

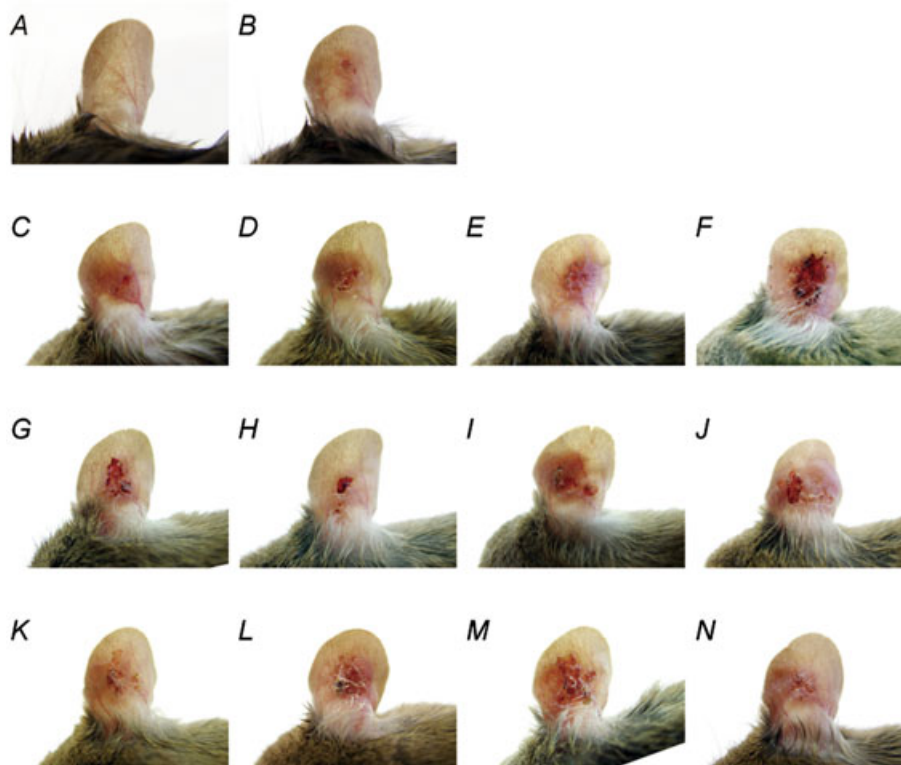


Figure 2. Macroscopic features of the right ear on day 17. Groups: (A) saline + vehicle; (B) Der p + vehicle; (C) Der p + NP 1.75; (D) Der p + NP 17.5; (E) Der p + NP 175; (F) Der p + NP 1750; (G) Der p + OP 2625; (H) Der p + OP 26.25; (I) Der p + OP 262.5; (J) Der p + OP 2625; (K) Der p + BP 10.5; (L) Der p + BP 105; (M) Der p + BP 1050; and (N) Der p + BP 10 500. BP, 4-*tert*-butylphenol; Der p, *Dermatophagoides pteronyssinus*; NP, 4-nonylphenol; OP, 4-*tert*-octylphenol.

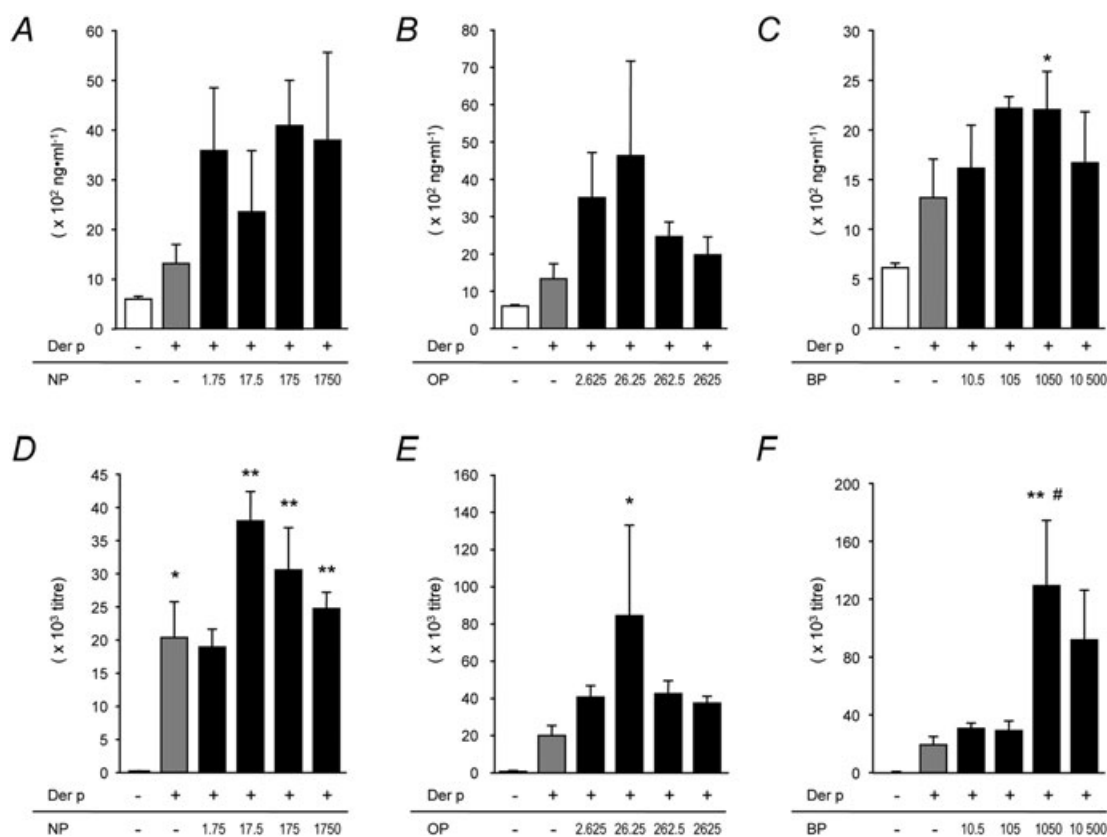


Figure 3. Effect of alkylphenols on serum levels of immunoglobulins in NC/Nga mice. (A–C) The levels of total IgE induced by exposure to NP, OP and BP. (D–F) Antibody titers of Der p-specific IgG₁ induced by exposure to NP, OP and BP. The data represent the mean $\pm$ SE values, $n = 5–6$ per group. * $P < 0.05$ and ** $P < 0.01$ versus saline + vehicle group. # $P < 0.05$ versus Der p + vehicle group. BP, 4-*tert*-butylphenol; Der p, *Dermatophagoides pteronyssinus*; NP, 4-nonylphenol; OP, 4-*tert*-octylphenol.

Table 2. Effects of alkylphenols on T-helper 2 cytokine levels in ear homogenates

(pg mg⁻¹ total ear protein)

	<i>n</i>	IL-4	IL-5	IL-6	TSLP	IL-13
Saline + vehicle	6	11.1 $\pm$ 0.5	16.3 $\pm$ 1.1	44.3 $\pm$ 3.9	52.5 $\pm$ 8.7	6.7 $\pm$ 0.9
Der p + vehicle	6	16.6 $\pm$ 2.6	17.0 $\pm$ 2.7	68.1 $\pm$ 4.9	188.7 $\pm$ 60.8	33.3 $\pm$ 3.9 *
Der p + NP 1.75	5	22.2 $\pm$ 4.1	12.8 $\pm$ 0.8	61.3 $\pm$ 7.0	169.5 $\pm$ 42.1	28.9 $\pm$ 2.1 *
Der p + NP 17.5	6	25.2 $\pm$ 3.4	22.7 $\pm$ 3.1	55.1 $\pm$ 2.6	312.1 $\pm$ 66.7 *	26.1 $\pm$ 1.7 *
Der p + NP 175	6	29.7 $\pm$ 3.5 **	16.2 $\pm$ 2.5	62.7 $\pm$ 6.5	290.3 $\pm$ 30.7 *	28.1 $\pm$ 1.6 *
Der p + NP 1750	6	26.1 $\pm$ 4.8	15.7 $\pm$ 1.3	92.2 $\pm$ 38.4	410.4 $\pm$ 72.0 **	29.1 $\pm$ 1.8 *
Der p + OP 2.625	6	23.3 $\pm$ 4.1	15.7 $\pm$ 1.7	77.0 $\pm$ 7.4 **	262.2 $\pm$ 80.0	34.6 $\pm$ 1.9 *
Der p + OP 26.25	5	38.2 $\pm$ 11.9	23.3 $\pm$ 2.9	70.4 $\pm$ 3.5 **	246.7 $\pm$ 20.2	35.1 $\pm$ 2.5 *
Der p + OP 262.5	6	26.5 $\pm$ 5.1	17.1 $\pm$ 1.9	59.4 $\pm$ 3.5	282.8 $\pm$ 75.1	30.5 $\pm$ 2.6 *
Der p + OP 2625	6	39.8 $\pm$ 9.7 *	15.8 $\pm$ 1.4	62.6 $\pm$ 3.5	427.0 $\pm$ 85.5 **	26.5 $\pm$ 2.0 *
Der p + BP 10.5	6	32.6 $\pm$ 6.5 *	32.3 $\pm$ 6.1	60.6 $\pm$ 6.4	234.9 $\pm$ 68.6	32.8 $\pm$ 2.1 *
Der p + BP 105	6	26.3 $\pm$ 2.6	26.6 $\pm$ 3.9	81.3 $\pm$ 7.4 *	319.6 $\pm$ 61.4 **	28.5 $\pm$ 4.2 *
Der p + BP 1050	6	40.7 $\pm$ 6.8 **	33.7 $\pm$ 7.4 **	93.5 $\pm$ 10.8 **	273.4 $\pm$ 43.0	36.6 $\pm$ 4.4 *
Der p + BP 10 500	6	40.8 $\pm$ 5.4 **	31.7 $\pm$ 2.9	114.6 $\pm$ 10.8 **	229.1 $\pm$ 58.6	32.2 $\pm$ 2.4 *

IL, interleukin; TSLP, thymic stromal lymphopoietin.

Data represent the mean $\pm$ SE, $n = 5–6$ per group.

* $P < 0.05$ and ** $P < 0.01$ versus saline + vehicle group. # $P < 0.05$ and ## $P < 0.01$ versus Der p + vehicle group.

were as follows: Der p + NP 1750 > Der p + NP 175 > Der p + NP 175 > Der p + NP 1.75; this order resembled the skin score, except the second and third grade were exchanged. For OP treatment,

the highest expression of TSLP levels were as follows: Der p + OP 2625 > Der p + OP 262.5 > Der p + OP 2.625 > Der p + OP 26.25. The same trend was observed for the skin scores for OP treatment.

A similar relationship was observed between TSLP production and skin scores for BP. The levels of IL-13 in the alkylphenol-treated groups were not higher compared with those in the Der p + vehicle group.

Exposure to NP, OP or BP along with the Der p allergen decreased the levels of the Th1 cytokine, IFN- γ (Table 3). A significant difference was observed in the Der p + NP 1750 and the Der p + OP 2625 groups compared with the Der p + vehicle group. IL-18 production tended to be lower in almost all groups (Der p + NP, Der p + OP and Der p + BP) than in the Der p + vehicle group (Table 3). However, this effect was not observed in the groups co-treated with 1050 μ g or 10 500 μ g BP. In particular, exposure to NP (1.75–1750 μ g) markedly decreased Der p-induced IL-18 production. Exposure to OP (2.625 and 262.5 μ g) and BP (10.5 μ g) decreased the expression of this cytokine compared with the Der p + vehicle.

In the presence of the allergen, exposure to NP, OP and BP tended to increase MCP-3 production (Table 3). Exposure to 262.5 μ g OP, 1050 μ g BP or 10 500 μ g BP significantly increased the expression of this chemokine compared to that in the Der p + vehicle group. Further, Der p + 1050 μ g BP caused greater production of eotaxin than that obtained with Der p + vehicle (Table 3). The protein levels of MIP-1 α tended to be higher in the other groups treated with alkylphenols than in the Der p + vehicle group, but this difference was not statistically significant. The level of KC was not higher in the alkylphenol-treated groups than in the Der p group (Table 3).

Discussion

The present study was undertaken to clarify the effects of NP, OP and BP in a mouse model of AD. Exposure to NP aggravated AD-like skin lesions (ADSLs) induced by mite allergen in a dose-dependent manner. Exposure to OP or BP caused significant exacerbation of skin lesions, which was not dose dependent. We found that the Th1/Th2 balance shifted toward Th2-dominant conditions in the inflamed ear.

The dose of 10 mg kg⁻¹ BW day⁻¹ has been identified as the NOAEL of NP for general chronic oral toxicity in rats (Nagao *et al.*, 2001). The maximum dose used in this experiment 1750 μ g per animal, calculated according to BW and was the same dose as the NOAEL for NP. Similarly, the maximum dose for OP (2625 μ g per animal) or BP (10 500 μ g per animal) was based on the NOAEL for OP or BP. The NOAEL for OP was defined as 15 mg kg⁻¹ BW day⁻¹ based on abnormal symptoms, salivation and decreasing albumin–globulin ratio in rats (Ministry of Health & Welfare, 1994). The effect of OP was also dose-dependent for induction of skin lesions at the highest (2625 μ g per animal) and second highest dose (262.5 μ g per animal). Thus, our results indicate that NP and OP aggravate AD at extremely low doses of NOAEL or less. In contrast, dose-dependent effects were not observed for BP. The skin symptoms of AD were most prominent at 105 μ g per animal. This dose is 100 times lower than the NOAEL for BP (60 mg kg⁻¹ BW day⁻¹), which is determined by oral toxicity tests that examine decreasing total serum protein levels in rats (Ministry of Health & Welfare, 1994). Thus, our results indicate that BP can aggravate ADSLs at doses lower than the NOAEL.

However, because NOAEL values are determined using oral toxicity tests in rats, interspecies differences in sensitivity to alkylphenols need to be considered. Furthermore, alkylphenol was generally taken into the body orally, for example, drinking water or eating foods, including some alkylphenols migrated

from plastic bottles and containers. The amount of orally ingested alkylphenols in the body may be reduced through intestinal absorption and metabolism, thereby resulting in lower levels than the actual dose. Other NOAELs for alkylphenols based on oral administration studies in rats were proposed as 100 mg kg⁻¹ BW day⁻¹ for mid- and long-term toxicity of NP (Hüls, 1989), 150 mg kg⁻¹ BW day⁻¹ for developmental and reproductive toxicity of OP (Tyl *et al.*, 1999), and 200 mg kg⁻¹ BW day⁻¹ for developmental and reproductive toxicity by repeated dose toxicity study of BP (Ministry of Health & Welfare, 1996), respectively. The present study indicates that alkylphenols can aggravate mite-induced ADSLs at lower doses than previously reported, even though a NOAEL for oral administration cannot be compared with i.p. administration. However, few studies on the effect of alkylphenols on AD have been reported. Therefore, we first investigated their effects on AD *in vivo*, using i.p. injection. In future studies, it may be necessary to evaluate the effects of oral administration of a low dose of alkylphenols in mice.

The mean values of plasma levels of total IgE and antigen-specific IgG₁ were elevated in most alkylphenol-treated mice compared with those of mice treated with Der p + vehicle. Although these differences were not statistically significant, they may be attributable to the natural individual variation between animals. Treatment with NP or OP resulted in a significant increase in IgE levels in the sera of antigen-primed mice (Lee *et al.*, 2003, 2004). Our present results and those of previous studies show that alkylphenols have an adjuvant effect on sensitized animals. Further, IgE plays a role in allergic inflammation that is induced partly by mast cell degranulation (Metcalf *et al.*, 1997). The cell surface IgE receptors bind to IgE and are cross-linked to the antigen, causing the activation of mast cells and the release of proinflammatory molecules. The degranulation of eosinophils results from the formation of cross-links between the antigen and IgG₁, which binds to cell surface Fc-gamma receptors (Kaneko *et al.*, 1995). The proinflammatory molecules that are released cause damage to subcutaneous tissue in allergic dermatitis or respiratory tract tissue in allergic airway inflammation (Hart, 2001; Katunuma and Kido, 1988; Steinhoff *et al.*, 1999). The increased levels of IgE and IgG₁ may have contributed to worsening of the clinical status in our present study.

In the present study, alkylphenols increased the levels of IL-4, IL-6, TSLP, MCP-3 and MIP-1 α in the lesions of mice injected with alkylphenols. In contrast, only treatment with BP increased the levels of IL-5. IL-4, IL-5 and IL-6 are Th2 cytokines that regulate humoral immunity (Brandt and Sivaprasad, 2011; Coffman *et al.*, 1988; Mosmann and Coffman, 1989). Development and aggravation of AD are associated with increased production of these cytokines in humans (Jenmalm *et al.*, 2000; Yamada *et al.*, 1996) and in mouse models of AD (Mutou *et al.*, 2007; Segawa *et al.*, 2008). TSLP is highly expressed by keratinocytes, epithelial and dendritic cells during AD where it plays a role in initiating allergic inflammation (Soumelis *et al.*, 2002; Watanabe *et al.*, 2005). MCP-3 and MIP-1 α act as chemoattractants that induce eosinophil chemotaxis to lesions in allergic respiratory diseases (Lukacs *et al.*, 1996; Rojas-Ramos *et al.*, 2003; Santiago *et al.*, 2008), and their levels increase in patients with AD (Kaburagi *et al.*, 2001) and in mouse models of ADSL (Yanagisawa *et al.*, 2010). Our present results suggest that aggravation of skin lesions induced by alkylphenol exposure is associated with increasing levels of these chemokines. Particularly, the level of TSLP in ear tissue and the skin score correlated in the

Table 3. Effects of alkylphenols on cytokine and chemokine levels in ear homogenates

($\mu\text{g mg}^{-1}$ total ear protein)	<i>n</i>	IFN- γ	IL-18	MCP-3	Eotaxin	MIP-1 α	KC
Saline + vehicle	6	3052.1 $\pm$ 312.6	727.3 $\pm$ 22.4	3.2 $\pm$ 3.1	31.8 $\pm$ 12.0	24.4 $\pm$ 1.1	41.9 $\pm$ 3.6
Der p + vehicle	6	1156.3 $\pm$ 99.1 **	2285.9 $\pm$ 154.4 **	9.4 $\pm$ 5.3	394 $\pm$ 8.2	97.5 $\pm$ 18.0 *	25.4 $\pm$ 2.0 *
Der p + NP 1.75	5	902.6 $\pm$ 115.7 **	1381.8 $\pm$ 92.8 **##	18.0 $\pm$ 5.7	26.0 $\pm$ 4.9	126.3 $\pm$ 29.1	20.4 $\pm$ 2.1 *
Der p + NP 17.5	6	710.9 $\pm$ 127.6 **	1686.1 $\pm$ 62.9 **##	14.9 $\pm$ 4.6	16.6 $\pm$ 2.9	131.0 $\pm$ 24.8	16.7 $\pm$ 1.4 **
Der p + NP 175	6	732.5 $\pm$ 88.0 **	1533.9 $\pm$ 137.7 **##	19.3 $\pm$ 3.7 *	38.8 $\pm$ 8.7	186.0 $\pm$ 26.1 **	18.1 $\pm$ 1.8 *
Der p + NP 1750	6	315.4 $\pm$ 60.1 **##	1367.3 $\pm$ 52.4 **##	31.6 $\pm$ 19.6	32.5 $\pm$ 3.2	194.8 $\pm$ 39.0 **	25.9 $\pm$ 6.5
Der p + OP 2.625	6	856.2 $\pm$ 108.5 **	1449.1 $\pm$ 232.1 **##	39.9 $\pm$ 10.7 *	103.5 $\pm$ 56.4	151.6 $\pm$ 44.4	25.2 $\pm$ 2.6 *
Der p + OP 26.25	5	835.9 $\pm$ 91.0 **	1940.7 $\pm$ 123.6 **	39.2 $\pm$ 4.2 *	59.7 $\pm$ 14.3	159.1 $\pm$ 32.4	24.5 $\pm$ 2.0 *
Der p + OP 262.5	6	641.5 $\pm$ 106.0 **	1269.2 $\pm$ 196.5 **	44.8 $\pm$ 13.2 **##	39.9 $\pm$ 11.6	162.7 $\pm$ 62.6	23.9 $\pm$ 1.8 *
Der p + OP 2625	6	477.6 $\pm$ 39.0 ** #	1682.9 $\pm$ 137.9 **	35.8 $\pm$ 6.2	17.7 $\pm$ 1.0	208.8 $\pm$ 28.5 *	23.7 $\pm$ 2.6
Der p + BP 10.5	6	669.7 $\pm$ 96.4 **	1261.0 $\pm$ 230.0 **	41.3 $\pm$ 6.6 *	20.0 $\pm$ 3.6	116.7 $\pm$ 29.3	23.0 $\pm$ 1.4 *
Der p + BP 105	6	651.3 $\pm$ 124.1 **	1769.4 $\pm$ 161.4 **	44.7 $\pm$ 6.8	16.9 $\pm$ 1.7	215.9 $\pm$ 59.5 **	24.4 $\pm$ 2.1 *
Der p + BP 1050	6	828.0 $\pm$ 155.5 **	2169.1 $\pm$ 154.6 **	108.9 $\pm$ 14.5 **##	202.6 $\pm$ 81.3 **	244.3 $\pm$ 49.3 **	26.4 $\pm$ 1.4 *
Der p + BP 10 500	6	959.7 $\pm$ 145.3 **	2 884.3 $\pm$ 316.4 **	82.1 $\pm$ 11.4 **##	110.3 $\pm$ 33.1	134.3 $\pm$ 27.0	29.3 $\pm$ 1.7 *

IFN, interferon; IL, interleukin; MCP-3, monocyte chemoattractant protein-3; MIP-1 α , macrophage inflammatory protein-1 alpha; KC, keratinocyte chemoattractant.Data represent the mean $\pm$ SE, *n* = 5–6 per group.* *P* < 0.05 and ** *P* < 0.01 versus saline + vehicle group. # *P* < 0.05 and ## *P* < 0.01 versus Der p + vehicle group.

alkylphenol-treated groups. TSLP may therefore make the most significant contribution to the aggravation of symptoms of AD. Further, our results show that production of Th2 cytokines and chemokines was induced by alkylphenol treatment and suggest that the aggravation of AD in NC/Nga mice treated with alkylphenols was induced by proinflammatory cytokines such as IL-4 and IL-6 and chemokines such as TSLP, MCP-3 and MIP-1 α . The enhanced production of IL-5 induced by treatment with BP also contributed to the aggravation of AD.

Th1-type cytokine levels decreased in Der p-treated animals that were exposed to alkylphenols. In the Der p + vehicle group, the level of IFN- γ , a Th1 cytokine, was lower than that in the saline + vehicle group and was significantly decreased in the presence of antibody and alkylphenols. These data correspond with those of studies using IFN- γ knockout (IFN- $\gamma^{-/-}$) mice. Thus, antigen-sensitized IFN- $\gamma^{-/-}$ mice express markedly higher levels of IL-4 mRNA in skin lesions compared with their wild-type littermates (Spergel *et al.*, 1999). Unsurprisingly, IFN- $\gamma^{-/-}$ mice also express decreased levels of serum IgE, consistent with the inhibition of CD40 ligand expression on Th2 cells by IFN- γ (Roy *et al.*, 1993). CD40 ligand expression is critical for immunoglobulin class switching in B cells (Gascan *et al.*, 1991). Alkylphenol exposure suppressed the levels of IFN- γ in lesions, which may have promoted the Th2 response.

IL-18, which is expressed by activated macrophages, epidermal keratinocytes and dendritic cells, induces IFN- γ and may play a role in immunoregulation by Th1 cells (Dao *et al.*, 1996; Okamura *et al.*, 1995). IL-18 also contributes to ADSLs in mouse models of AD (Terada *et al.*, 2006). In the present study, the production of IL-18 by the Der p treatment groups was higher compared with that of the saline + vehicle group, while the production of IFN- γ decreased. However, exposure to alkylphenols, with the exception of BP, inhibited the production of IL-18. Compared with other alkylphenols, BP exposure appears to have had less of an effect on IL-18 production. Therefore, alkylphenols may suppress the activation of Th1 cells. Evidence indicates that the atopic skin disease induced by allergens is related to the shift in the Th1/Th2 balance toward Th2 (Ayoub *et al.*, 2003; Santa *et al.*, 2003; Xiao *et al.*, 2003). Here, exposure to alkylphenols enhanced Th2-dominant responses and caused the aggravation of AD in NC/Nga mice.

Th2 cells secrete IL-13 and IL-4, which share 20–25% sequence similarity and overlap in some functions. In our present study, the levels of IL-4 and IL-13 in the groups treated with Der p with or without alkylphenols were higher than those in the saline + vehicle group. However, the production of IL-13 in the groups treated with Der p and alkylphenols was the same as that of the Der p + vehicle group, while the production of IL-4 was elevated in the groups treated with Der p and alkylphenols. IL-13 is also produced from super Th1 cells, which arise when differentiated Th1 cells are stimulated by antigen in the presence of IL-18 and IL-2 (Sugimoto *et al.*, 2004). It is unlikely that alkylphenols induce the expression of the IL-13 gene, at least in our model of AD.

Intraperitoneal administration of NP, OP or BP led to the exacerbation of skin lesions and increased the production of immunoglobulins in serum and the expression of Th2 cytokines and chemokines. In particular, our study showed increased levels of TSLP that corresponded approximately to the ear skin scores. These results suggest that NP, OP and BP when present in the environment may aggravate AD.

The number of patients with AD has increased worldwide (Horii *et al.*, 2007; Sugiura *et al.*, 1998; Williams, 1992) and may

be explained by the increase in allergens, including mites, microbes, the hair and dandruff of pets and high-fat diets (Hon *et al.*, 2005; Kulthanan *et al.*, 2011; Ushiyama *et al.*, 2002; Zirngibl *et al.*, 2002). Further, environmental chemicals may enhance allergies. Diesel exhaust particles and diesel exhaust, which are environmental adjuvants, enhance antigen-induced eosinophilic inflammation and IL-5 production in the lung and antigen-specific immunoglobulin in the serum of mice with experimentally induced allergic disease (Ichinose *et al.*, 2004; Miyabara *et al.*, 1998; Sadakane *et al.*, 2002; Takano *et al.*, 1997).

Takano *et al.* (2006) investigated the effect of low-level exposure to DEHP on AD in mice. Exposure to 0.8–20 μ g DEHP, but not to 100 μ g, increased the severity of ADSLs. This result implicates DEHP as an environmental enhancer of AD and implies that low-level exposure, which is a much lower dose than the NOAEL reported previously, can exacerbate AD. Some epidemiological studies reported that the concentration of DEHP in house dust was significantly associated with allergic wheezing and asthma in children (Bornehag *et al.*, 2004; Kolarik *et al.*, 2008). These findings suggest that human allergic diseases may be aggravated by exposure to DEHP; however, few studies have investigated the role of alkylphenols as environmental effectors of allergy. In the present study, we administered the alkylphenols at levels according to the NOAEL values or lower. Our results imply that alkylphenols and phthalates might act as environmental adjuvants.

In conclusion, we have demonstrated that alkylphenols enhanced Th2-type immune responses in NC/Nga mice, which aggravated mite allergen-induced AD-like skin lesions. Therefore, it will be important to determine whether these observations in mice are relevant to human exposure to alkylphenols.

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