

## RESEARCH ARTICLE

## Effects of airway exposure to di-(2-ethylhexyl) phthalate on allergic rhinitis

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## Abstract

Recent epidemiological studies have suggested a positive link between atopy morbidity and exposure to phthalate esters, which are environmental chemicals mainly involved in house dust. Nevertheless, experimental studies applying several allergic *in vivo* models (in addition to epidemiological studies) are needed to prove the precise correlation between phthalates and facilitation of the allergic response/pathophysiology. Among the phthalate esters, di-(2-ethylhexyl) phthalate (DEHP) has been widely used in flexible polyvinyl chloride products, including vinyl flooring and wall covering, and has been widely suggested to have immunomodulating potential. In the present study, we examined the effects of airway exposure to DEHP on allergen (ovalbumin: OVA)-induced rhinitis in mice. The repeated administration of OVA via an intranasal route induced nasal inflammation characterized by the infiltration of granulocytes (neutrophils and eosinophils) into the nasal cavity. In this experimental setting, DEHP did not exaggerate OVA-related inflammatory pathology. However, local (nasal) IL-13 levels were significantly higher in mice treated with allergen plus DEHP than with allergen alone. Taken together, phthalate esters including DEHP have the potential to exacerbate the allergic milieu in the nasal system, as well as dermal and respiratory systems.

## Keywords

Allergic rhinitis, cytokine, di-(2-ethylhexyl) phthalate, neutrophils, ovalbumin

## History

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## Introduction

The prevalence of allergic disorders has rapidly increased, especially in urban areas, over the past several decades<sup>1</sup>. Environmental factors, in addition to genetic ones such as atopic predisposition, should be considered as a reason for this increase. Among these factors, environmental pollutants including chemical compounds have been implied to play, at least in part, a key role<sup>2</sup>.

Phthalic acid ester plasticizers are widely used in flexible polyvinyl chloride products, including vinyl flooring, wall covering, food containers and infant toys, and are mainly detected in house dust. A causal relationship between phthalate exposure and the induction and/or exacerbation of allergic diseases including asthma, dermatitis and eczema has been epidemiologically implicated, particularly in children<sup>3,4</sup>. We also reported that di-(2-ethylhexyl) phthalate (DEHP), widely used [1.8 million metric tons/year] among phthalates,

potentiates atopic dermatitis-like skin lesions in mice<sup>5</sup>. Additionally, Larsen et al. demonstrated that DEHP facilitates allergic asthma in mice<sup>6,7</sup>. More recently, we showed that DEHP augments allergen-provoked peritonitis in mice<sup>8</sup>. Furthermore, phthalates have reportedly been connected to autoimmune disorders such as systemic lupus erythematosus<sup>9</sup>, which suggests that these chemicals may act as “immuno-disruptors”. However, experimental evidence regarding the correlation between phthalates, allergic responses and the underlying immunopathology has been insufficiently elucidated.

Allergic rhinitis is also a very common allergic condition, affecting approximately 10%–50% of the world's population<sup>10</sup>. Although it has not been directly correlated with a fatal outcome, the burden of this condition on the national economy is relatively high<sup>11,12</sup>. Searching for and identifying the precipitating factors of this disease may, thus, have a significant impact on reducing medical costs. The aim of the present study was, therefore, to investigate the effects of DEHP on allergen-related rhinitis (inflammatory leukocyte infiltration) and the local (nasal) hypersecretion/expression of allergy-related molecules such as cytokines and chemokines in mice.

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## Materials and methods

### Materials

Di-(2-ethylhexyl)phthalate (DEHP) was purchased from Wako Pure Chemicals (Osaka, Japan). Diff-Quik stain solution was purchased from International Reagents (Kobe, Japan). Decalcifying solution (EDTA method), periodic acid Schiff (PAS), and hematoxylin and eosin (H&E) stain solutions were bought from Wako Pure Chemicals (Osaka, Japan). Grade VII ovalbumin (OVA) was purchased from Sigma-Aldrich (St. Louis, MO).

### Animals

A total of 84 female BALB/c mice (weighing about 22 g on average and aged 5 weeks) were bought from Charles River Laboratories Japan Inc. (Yokohama, Japan). After 1 week of screening-out sick mice, mice with abnormal body weights, and mice stressed from different environmental breeding, the remaining mice were fed a commercial diet CE-2 (CLEA Japan, Inc., Tokyo, Japan) and given water *ad libitum*. Mice were housed in plastic cages lined with soft wood chips. The cages were placed in a conventional room, which was air conditioned at 23 °C and 55%–70% humidity with a light/dark (12 h/12 h) cycle. The study adhered to the U.S. National Institutes of Health guidelines for the use of experimental animals. The animal care method was approved by the Animal Care and Use Committee at Oita University of Nursing and Health Sciences in Oita, Japan.

### Study protocol

Mice were divided into six groups ( $n = 14$ ) according to the treatment with normal saline (control) alone, OVA (50 µg) alone, and OVA plus four doses of DEHP: OVA + DEHP (0.0004 µg:DEHP<sub>1</sub>), OVA + DEHP (0.008 µg:DEHP<sub>2</sub>), OVA + DEHP (0.16 µg:DEHP<sub>3</sub>) and OVA + DEHP (3.2 µg:DEHP<sub>4</sub>). OVA (50 µg/16 µl) was dissolved in normal saline for instillation (Otsuka Co., Kyoto, Japan). DEHP was dissolved in dimethyl sulfoxide (DMSO: 0.01%, at the beginning of the study, we have confirmed that both local and systemic effects of DMSO instillation alone at this concentration were negligible). The final concentration of DEHP diluted by normal saline was 0.0004, 0.008, 0.16 or 3.2 µg/16 µl. Mice were administered with a total 16 µl (8 µl/one side) of saline, OVA and OVA + DEHP solution into both anterior nares four times at 2-week intervals and then four times at 2-day intervals. Five hours after the last intranasal administration, the mice in all groups were sacrificed by exsanguination under deep anesthesia through an i.p. injection of pentobarbital.

### Sample preparation and analysis of cell contents in nasal cavity lavage fluids

The cell content in the nasal cavity lavage fluids (NCLF) of eight mice was examined. After the head of each mouse was removed, the connected part of the larynx and pharynx was cannulated. The nasal cavity was lavaged with two instillations of 0.5 ml of sterile saline at 37 °C, instilled by a syringe. The lavaged fluid was collected from the nostrils. The average

volume retrieved was 98% of the amount instilled (1 ml). The fluids from two lavages were combined and then cooled to 4 °C. The fluids were centrifuged at 1500 rpm for 10 min. Total cell counts were determined in fresh fluid specimens using a hemocytometer. Differential cell counts were assessed on cytologic preparations. The slides were prepared using a Cytospin (Sakura Co., Ltd., Tokyo, Japan) and stained with Diff-Quik. A total of 200 cells were counted under oil immersion microscopy. Aliquots of the supernatants were stored at –80 °C until analysis for inflammatory cytokines and chemokines. Nasal tissues lavaged with saline were removed and stored at –80 °C until analysis for analyses of cytokines and chemokines.

### Pathological evaluation

Six of the 14 mice from each group were used for the pathologic examination. After mice were sacrificed, the lower jaw, skin and musculature were removed, and the head was immersed in 10% neutral phosphate-buffered formalin. After fixation, the heads were decalcified in a decalcifying solution containing 0.5 M ethylenediaminetetraacetic acid disodium salt (pH, 7.5) for 3 weeks at 37 °C. The decalcified heads were rinsed in tap water. A tissue block was removed from the anterior nasal cavity by making two transverse cuts perpendicular to the hard palate immediately posterior to the upper incisors according to the procedure described in a previous report<sup>13</sup>. The tissue block was imbedded in paraffin and then 4 µm thick sections were cut from the anterior surface. Sections were stained with H&E to identify eosinophils and were stained with PAS to identify goblet cells in the bronchial epithelium. Pathological observations were mainly performed in the epithelium and subepithelium of the respiratory region and olfactory region in the nasal cavity using a Nikon ECLIPSE light microscope (Nikon Co., Tokyo, Japan).

### Quantization of cytokines and chemokines in NCLF and nasal tissues

Cytokine protein levels in the NCLF were determined using enzyme-linked immunosorbent assays (ELISAs). The nasal tissues (0.1 g) were sonicated in 1 ml of normal saline for 2 min with an ultrasonic disrupter, UD-201 type with a micro tip (Tomy, Tokyo, Japan), under a cooling condition. The solutions containing nasal tissues were centrifuged at 14 000 rpm for 10 min. Cytokine and chemokine protein levels in the supernatants were determined by means of ELISA as well as NCLF. Interleukin (IL)-6, IL-13, eotaxin and keratinocyte chemoattractant (KC) were measured using each of ELISA kit from R&D Systems Inc. (Minneapolis, MN). IL-5 and IL-12 were measured using each of ELISA kit from Endogen (Cambridge, MA). MCP-3 was measured using an ELISA kit from Bender Med Systems (Burlingame, CA).

### Statistical analysis

Statistical analyses of pathologic evaluations in the cellularity and cytokine and chemokine proteins in the NCLF were conducted using the Tukey Test for Pairwise Comparisons (KyPlot Ver.5, Kyens Lab Inc, Tokyo, Japan).

Differences among groups were determined as significant at a level of  $p < 0.05$ .

## Results

### Effects of DEHP on NCFL cellularity

To estimate the effects of DEHP on OVA-related tissue inflammation, we first investigated the cellular profiles of NCLF (Figure 1). The total number of total cells and neutrophils in the lavage fluid was greater in the OVA + DEHP groups than in the control or OVA group ( $p < 0.05$  for control versus OVA + DEHP<sub>4</sub>) in a dose-dependent manner with overall trend. Also, the number of eosinophils was significantly greater in the OVA + DEHP<sub>2</sub> group than in the control group ( $p < 0.05$ ).

### Effects of DEHP on histological findings in nasal tissues

To estimate the effects of DEHP exposure on nasal histology, we evaluated nasal specimens stained with H&E 5 h after intranasal instillation (Figure 2a). A negligible degree of eosinophilic infiltration was seen in nasal specimens in the control-treated group. The infiltration of eosinophils was moderate in the OVA-treated groups. In the presence of OVA, the degree of nasal inflammation was slightly worsened by DEHP exposure. Next, nasal sections were stained with PAS to evaluate epithelial injury and the hypersecretion of mucus (Figure 2b). Although OVA induced modest mucus hyperplasia in the nasal epithelium, DEHP exposure did not have a marked influence on these histopathological changes.

### Effects of DEHP on cytokine and chemokine levels in NCLF supernatants

To elucidate the effects of DEHP on the level of inflammation- and allergy-related molecules in the nasal cavity, we measured the protein levels of cytokines and chemokines (Table 1) in NCLF supernatants. The levels of KC ( $p < 0.01$ ) were significantly greater in the OVA + DEHP<sub>2</sub> ( $p < 0.05$ ) group or OVA + DEHP<sub>3</sub> ( $p < 0.01$ ) group than in the control group. The levels of KC ( $p < 0.01$ ) were significantly greater in the OVA + DEHP<sub>4</sub> group than in the control group. The level of Eotaxin was significantly greater in the OVA + DEHP<sub>4</sub> than in the control ( $p < 0.01$ ) group. Of note, the level of IL-13 was significantly greater in the OVA + DEHP groups than in the control or the OVA group ( $p < 0.001$ ). On the other hand, these cytokine and chemokine levels in nasal tissue homogenates were not different between experimental groups (data not shown).

## Discussion

We previously demonstrated that DEHP aggravates atopic dermatitis in NC/Nga mice<sup>5</sup>, which identified a positive biological link between DEHP exposure and the facilitation of allergic pathophysiology. Larsen et al. also demonstrated that DEHP worsens allergic asthma in BALB/c mice<sup>6,7</sup>. More recently, we showed that DEHP facilitated allergen-provoked peritonitis (characterized by neutrophilic inflammation) in ICR mice<sup>8</sup>. However, more research is required to certainly implicate this environmental chemical as an atopic/allergic amplifier. In the present study, we added these previous reports that airway (intranasal) exposure to DEHP (0.0004–3.2  $\mu\text{g}/\text{body}$ ) did not significantly deteriorate allergen-related

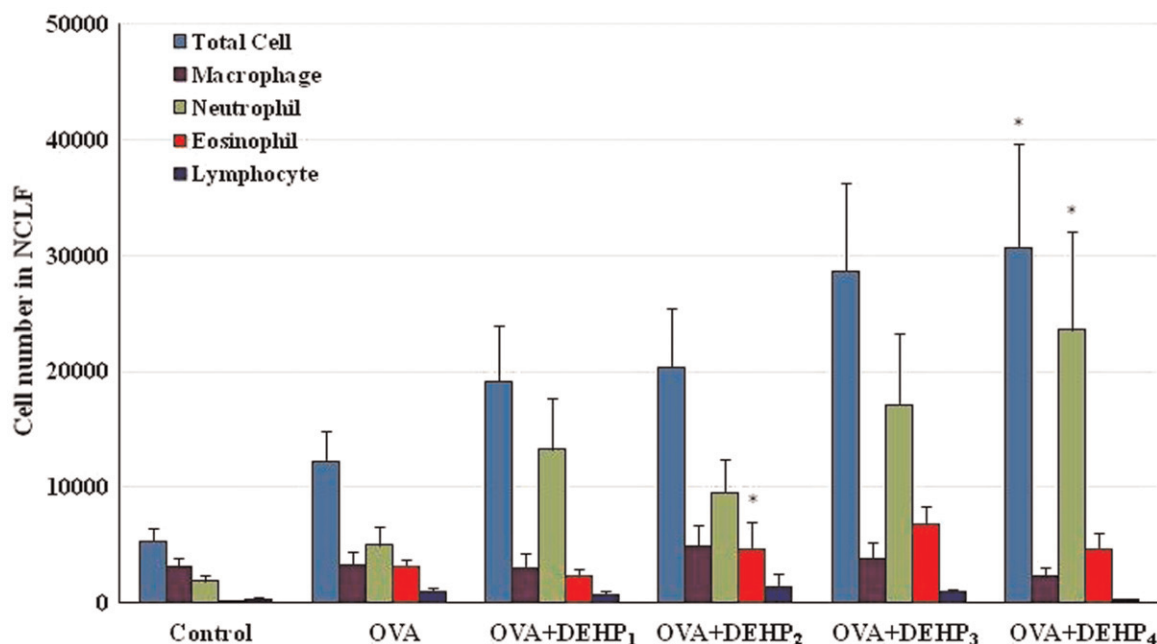


Figure 1. Effects of DEHP on OVA-related nasal inflammation on the cellular profiles of NCLF. Mice were divided into six groups ( $n = 8$  in each group) according to the treatment with normal saline (control) alone, OVA (50  $\mu\text{g}$ ) alone, and OVA plus four doses of DEHP: OVA + DEHP (0.0004  $\mu\text{g}$ :DEHP<sub>1</sub>), OVA + DEHP (0.008  $\mu\text{g}$ :DEHP<sub>2</sub>), OVA + DEHP (0.16  $\mu\text{g}$ :DEHP<sub>3</sub>), and OVA + DEHP (3.2  $\mu\text{g}$ :DEHP<sub>4</sub>) and administered with a total 16  $\mu\text{l}$  (8  $\mu\text{l}/\text{one side}$ ) of each solution into both anterior nares four times at 2-week intervals and then four times at 2-day intervals. Five hours after the last intranasal administration, the mice in all groups were sacrificed and analyzed. Total cell counts were determined in fresh fluid specimens using a hemocytometer. Differential cell counts were assessed in cytologic preparations stained with Diff-Quik. Results are means  $\pm$  SE. \* $p < 0.05$  versus control group.

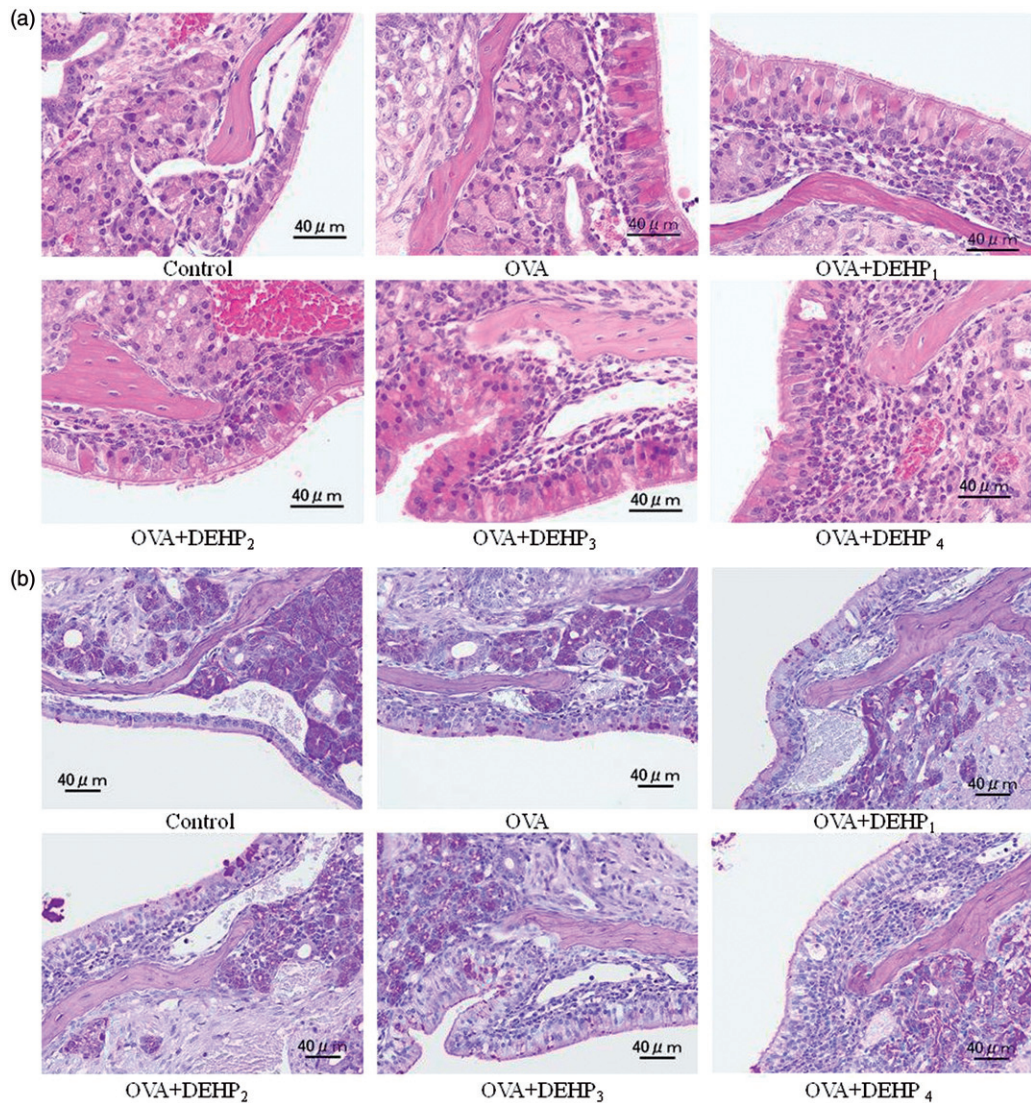


Figure 2. (a) Effects of DEHP on histological findings of hematoxylin and eosin (H&E)-stained nasal tissues. Mice were divided into six groups ( $n = 6$  in each group) according to the treatment with normal saline (control) alone, OVA ( $50 \mu\text{g}$ ) alone, and OVA plus four doses of DEHP:OVA + DEHP ( $0.0004 \mu\text{g}$ :DEHP<sub>1</sub>), OVA + DEHP ( $0.008 \mu\text{g}$ : DEHP<sub>2</sub>), OVA + DEHP ( $0.16 \mu\text{g}$ :DEHP<sub>3</sub>), and OVA + DEHP ( $3.2 \mu\text{g}$ :DEHP<sub>4</sub>) and administered with a total  $16 \mu\text{l}$  of each solution into both anterior nares four times at 2-week intervals and then four times at 2-day intervals. Nasal tissues were removed 5 h after the last intranasal administration and analyzed. Representative photographs of H&E-stained samples are shown. (b). Effects of DEHP on histological findings of PAS-stained nasal tissues. Mice were divided into six groups ( $n = 6$  in each group) according to the treatment with normal saline (control) alone, OVA ( $50 \mu\text{g}$ ) alone, and OVA plus four doses of DEHP:OVA + DEHP ( $0.0004 \mu\text{g}$ :DEHP<sub>1</sub>), OVA + DEHP ( $0.008 \mu\text{g}$ :DEHP<sub>2</sub>), OVA + DEHP ( $0.16 \mu\text{g}$ :DEHP<sub>3</sub>), and OVA + DEHP ( $3.2 \mu\text{g}$ :DEHP<sub>4</sub>) and administered with a total  $16 \mu\text{l}$  of each solution into both anterior nares four times at 2-week intervals and then four times at 2-day intervals. Nasal tissues were removed 5 h after the last intranasal administration and analyzed. Representative photographs of PAS-stained samples are shown.

Table 1. Cytokines and chemokines in NCLF.

| Group                   | Animals ( $n$ ) | Cytokines and chemokines (pg protein/ml) |                              |                            |                            |
|-------------------------|-----------------|------------------------------------------|------------------------------|----------------------------|----------------------------|
|                         |                 | IL-6                                     | IL-13                        | Eotaxin                    | KC                         |
| Control                 | 8               | $36.3 \pm 6.95$                          | ND                           | $2.58 \pm 0.82$            | $22.9 \pm 2.60$            |
| OVA                     | 8               | $96.8 \pm 30.2$                          | ND                           | $12.7 \pm 3.19$            | $39.1 \pm 2.46$            |
| OVA + DEHP <sub>1</sub> | 8               | $63.8 \pm 16.3$                          | ND                           | $7.44 \pm 2.73$            | $36.4 \pm 4.39$            |
| OVA + DEHP <sub>2</sub> | 8               | $128 \pm 42.6$                           | $5.15 \pm 0.43^{\text{c,d}}$ | $9.78 \pm 3.19$            | $44.9 \pm 4.12^{\text{a}}$ |
| OVA + DEHP <sub>3</sub> | 8               | $113 \pm 41.6$                           | $5.31 \pm 0.52^{\text{c,d}}$ | $11.9 \pm 2.45$            | $48.4 \pm 8.07^{\text{b}}$ |
| OVA + DEHP <sub>4</sub> | 8               | $90.3 \pm 22.8$                          | $5.09 \pm 0.40^{\text{c,d}}$ | $20.5 \pm 3.30^{\text{b}}$ | $42.7 \pm 4.58$            |

Control:saline; OVA:OVA ( $50 \mu\text{g}$ ); OVA + DEHP<sub>1</sub>:OVA ( $50 \mu\text{g}$ ) + DEHP ( $0.0004 \mu\text{g}$ ); OVA + DEHP<sub>2</sub>:OVA ( $50 \mu\text{g}$ ) + DEHP ( $0.008 \mu\text{g}$ ); OVA + DEHP<sub>3</sub>:OVA ( $50 \mu\text{g}$ ) + DEHP ( $0.16 \mu\text{g}$ ); OVA + DEHP<sub>4</sub>:OVA ( $50 \mu\text{g}$ ) + DEHP ( $3.2 \mu\text{g}$ ).

\*All values were expressed as mean  $\pm$  SE.

<sup>a</sup> $p < 0.05$  versus Control; <sup>b</sup> $p < 0.01$  versus Control; <sup>c</sup> $p < 0.001$  versus Control; <sup>d</sup> $p < 0.001$  versus OVA.

nasal inflammatory pathology in BALB/c mice in the current research setting, although histological findings were slightly worsened in the OVA + DEHP groups than in the OVA group (Figure 1). These findings slightly different from previous reports on atopic dermatitis, allergic asthma and allergen-related peritonitis may be due to the experimental protocol used, such as the DEHP dosage, exposure design (route and duration) and/or allergic model. Indeed, the exposed DEHP doses used in the present study were lower than those in the previous studies, and this is the first experimental study to examine the effects of DEHP via a local (intranasal) route on allergic indices. Therefore, the current study might represent a more relevant exposure model of phthalates.

Interestingly, the level of IL-13 in NCLF was significantly greater in the OVA plus DEHP at doses above 0.008 µg groups than in the OVA group. IL-13 is a vital cytokine that regulates Th2 type immune and/or inflammatory responses<sup>14,15</sup>. IL-13 reportedly acts on B cells and stimulates both the proliferation/activation of these cells with IgE synthesis<sup>16,17</sup>. IL-13 also appears to be an effector cytokine that strongly contributes to bronchial hyper-reactivity and mucus overproduction/secretion in animal asthma models<sup>18,19</sup>. Furthermore, it can promote its own production through the regulation of several mediators, such as adenosine and histamine, which in turn stimulate related cells such as eosinophils, mast cells, basophils and smooth muscle cells to produce more IL-13<sup>20</sup>. Through the accelerated stimulation/activation of these pathways, IL-13 may also be a major contributor to the chronic phase of allergic reactions<sup>20</sup>. In allergic rhinitis, the IL-13 gene is expressed in the nasal mucosa of patients<sup>21,22</sup>. Miyahara et al. have shown using IL-13-null mice that this cytokine is essential to the late phase immune response, as well as the early response in allergic rhinitis<sup>23</sup>. Taken together, IL-13 can serve as a key player in the aberrant immune response and/or pathophysiology in allergic diseases including rhinitis. Therefore, although DEHP did not markedly (diversely) affect the pathophysiology of allergic rhinitis such as NCLF cellularity in the present study, this chemical may have the potential to disrupt local immune systems in the nasal cavity, implicating its plausible role in the predisposition of immunological abnormalities such as allergies; therefore, further studies are required to clarify the tangible effects of these chemicals on allergic traits, maybe through the use of longer exposure schedules. On the other hand, there was a difference in the levels of IL-13 between tissue samples and NCLF (i.e. the level in nasal tissue homogenates was not different between experimental groups: data not shown). This discrepancy may have arisen from the time lag of sacrifice. Alternatively, DEHP may preferentially act on machinery concerning the release of IL-13 rather than the production and/or targeted leukocytes synthesizing T cells, mast cells and eosinophils. In any case, this issue should be clarified in the future.

Allergic rhinitis is also one of extremely common allergic diseases/conditions. Although it has not been directly correlated to a fatal outcome, the burden of this condition on the national economy is relatively high<sup>11,12</sup>, and additionally reduces the quality of life of patients. Therefore, the current study may provide useful information for future investigations, which may ultimately leads to a reduction in

the number of patients with this disease and consequently related to medical costs.

In conclusion, airway (intranasal) exposure to relatively low doses of DEHP did not significantly exacerbate allergen-related pathology in a murine rhinitis model. Nevertheless, local (nasal) levels of IL-13 were significantly elevated by exposure to allergen plus DEHP than to allergen alone. These results additionally provide experimental evidence that phthalate esters including DEHP may exacerbate the allergic milieu in the nasal environment, as well as dermal and respiratory systems. Yet, further studies are required in order to achieve substantial conclusions.

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## Declaration of interest

We have no declarations of interest.

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