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Di-(2-ethylhexyl) phthalate enhances skin sensitization to isocyanate haptens in mice

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ABSTRACT

Toxicological and epidemiological studies have suggested the involvement of di-(2-ethylhexyl) phthalate (DEHP) in increases in allergies. The effects of DEHP have been implicated in the deflection to T helper 2 (Th2)-biased immune responses. However, we could not observe an adjuvant effect of DEHP in a Th2-dominant fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS) mouse model when applied epicutaneously together with a hapten. In the present study, we focused whether there is a hapten to which DEHP exhibits an adjuvant effect through the epicutaneous route. BALB/c mice were epicutaneously sensitized with 2-methoxy-4-nitrophenyl isocyanate (MNICN) or phenethyl isocyanate (PEICN) in the presence or absence of phthalate esters of various kinds including DEHP. After challenge with a respective hapten, the ear-swelling response was measured to determine the level of hapten sensitization. Sensitization to MNICN and PEICN was enhanced when DEHP was present in the vehicle for the hapten preparation. The enhancement of sensitization to MNICN was accompanied by an elevated production of interferon- γ and reduced production of interleukin-4 (IL-4), suggesting polarization to a T helper 1 (Th1)-dominant response. The results suggested that the combination of a hapten and a phthalate ester significantly affects the outcome of CHS.

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1. Introduction

Phthalate esters are artificial chemical products commonly used as plasticizers. Some of them have been reported to be risk factors for allergies (Bornehag et al., 2004; Chowdhury and Statham, 2002; Kolarik et al., 2008). The predominant phthalate ester in indoor dust has been considered to be di-(2-ethylhexyl) phthalate (DEHP), and the predominant ones in indoor air are diethyl phthalate (DEP) and dibutyl phthalate (DBP) (Kolarik et al., 2008). The concentrations of DEHP in dust samples have been typically estimated to be 0.01–10 mg/g dust, and those of DEP and DBP to be 0.05–5 $\mu\text{g}/\text{m}^3$ in indoor air (Kolarik et al., 2008). In epidemiological studies, a positive association between asthma in children and the DEHP concentrations in dust samples from the children's home has been found (Bornehag et al., 2004; Kolarik et al., 2008).

Regarding animal studies, there have been several reports suggesting an adjuvant effect of DEHP on immune response against ovalbumin (OVA) (Larsen et al., 2001) and mite antigens (Takano et al., 2006; Yanagisawa et al., 2008). DEHP is considered to deflect an immune response to a T helper 2 (Th2)-type one, which exhibits features of immunoglobulin E (IgE) (Lee et al., 2004) and IgG1

(Larsen et al., 2001) synthesis (antibody response), and interleukin-4 (IL-4) production (cytokine production) (Lee et al., 2004). In these studies (Larsen et al., 2001; Lee et al., 2004; Takano et al., 2006; Yanagisawa et al., 2008), direct injection (subcutaneously or intraperitoneally) of DEHP was performed. In addition, inhalation of OVA together with DEHP in aerosols was reported to enhance IgG1 production to OVA (Larsen et al., 2007). On the other hand, a recent study suggested that topical skin application of DEHP did not have an adjuvant effect on the production of IgE or IgG1 specific to OVA (Dearman et al., 2008). Despite the possible adjuvant effect of DEHP, discordance between the results of animal and epidemiological studies has been pointed out (Nielsen et al., 2007).

We have been studying a fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS) mouse model. We previously found that several phthalate esters, including DEP, di-*n*-propyl phthalate (DPP), DBP, dipentyl phthalate (DPNP), dihexyl phthalate (DHXP), and diheptyl phthalate (DHPP), enhanced sensitization to FITC in BALB/c mice on epicutaneous application (Imai et al., 2006; Shiba et al., 2009). On the other hand, DEHP and diisononyl phthalate (DINP) did not exhibit such an effect (Imai et al., 2006). We also observed that phthalate esters exhibiting adjuvant activity in the FITC-induced CHS mouse model were typically able to activate the transient receptor potential (TRP) A1 receptor and TRPV1 receptor in *in vitro* experiments, whereas DEHP did not cause activation of TRPA1 or TRPV1 (Shiba et al., 2009). Our results suggested

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that DEHP is a relatively inert chemical as an adjuvant for skin sensitization.

Despite our results suggested the lack of adjuvant effect of DEHP on FITC-induced CHS, earlier studied by others prompted us to examine whether there might be haptens with which DEHP exhibits an adjuvant effect during skin sensitization. We tested the effect of DEHP on skin sensitization to 2-methoxy-4-nitrophenyl isocyanate (MNICN) and phenethyl isocyanate (PEICN). We also investigated whether DEHP has an effect on cytokine production from draining lymph nodes upon sensitization. The studies on the combined effects of antigenic chemicals and adjuvant chemicals will contribute to the risk assessment of chemicals as to hypersensitivity.

2. Materials and methods

2.1. Mice

Female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan) at 7 weeks of age and held for 1 week before use. They were housed at 22–24 °C with 50–60% humidity under artificial lighting conditions with a 12-h light/dark cycle. They had access to food (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

2.2. Reagents

Acetone, DEP, DBP, DHPP, DEHP, and olive oil were purchased from Wako Pure Chemicals (Osaka, Japan); DPP and DPNP from Kanto Chemicals (Tokyo, Japan); and MNICN and PEICN from Sigma–Aldrich (Tokyo, Japan). Purities of DEP, DBP, DHPP, DPP, DPNP and PEICN were more than 98%, and those of DEHP and MNICN were more than 97%. Purified anti-mouse IL-4 monoclonal antibodies (mAb) (clone 11B11, rat IgG1), biotin-anti-mouse IL-4 mAb (clone BVD6-24G2, rat IgG1), purified anti-mouse IFN- γ mAb (clone XMG1.2, rat IgG1), biotin-anti-mouse IFN- γ mAb (clone R4-6A2, rat IgG1), recombinant mouse IL-4, and recombinant mouse IFN- γ were purchased from eBioscience (San Diego, CA), and horseradish peroxidase (HRP)–avidin from Zymed (South San Francisco, CA).

2.3. CHS

The methods used for contact sensitization as well as for elicitation were based on those for FITC-induced CHS (Imai et al., 2006; Shiba et al., 2009) with modifications. Mice were epicutaneously sensitized on shaved forelimbs with 160 μ l of 0.5% MNICN or 0.5% PEICN on days 0, 1, 7 and 8. The dose of MNICN was selected to be same as that of FITC used in our previous experiments (Imai et al., 2006). The dose of PEICN was selected to be a suboptimal one in pilot experiments. The solvents used in the MNICN model were acetone, or a 1:1 (v/v) mixture of acetone and a phthalate ester, while those used in the PEICN model were a 3:1 (v/v) mixture of acetone and olive oil, or a 1:1 (v/v) mixture of acetone and a phthalate ester, as specified in the figure legends. In the case of PEICN, olive oil was included in the control condition to alleviate skin irritation by PEICN. On day 14, mice were challenged by applying 20 μ l of an MNICN solution (0.5% in acetone/DBP) or a PEICN solution (0.5% in acetone/DBP) on the right auricle. The left auricle was treated with the solvent alone as a control. Ear swelling at 24 or 48 h after challenge was calculated as previously described (Imai et al., 2006; Shiba et al., 2009). Ear swelling at Xh is defined as follows: [(thickness of the right ear at Xh) – (thickness of the right ear at 0h)] – [(thickness of the left ear at Xh) – (thickness of the left ear at 0h)]. Numbers of mice used in the experiment involving MNICN sensitization were 9–14 mice per group, and those involving PEICN-sensitization were eight mice per group.

2.4. Cytokine production

Cytokine production by the draining lymph node cells was assessed as described previously (Maruyama et al., 2007a). In brief, mice were euthanized 24 h after the fourth sensitization (Exp. 1 involving DBP) with MNICN. In the Exp. 2 involving DEHP, mice were sensitized on days 0 and 7, and then they were euthanized 24 h after the second sensitization. Brachial lymph nodes were collected and pooled for each experimental group (three mice each), and single-cell suspensions were prepared. In either case, the draining lymph nodes were collected 24 h after the last sensitization considering that both circulating MNICN-specific T cells and MNICN-presenting DC that had migrated from the skin were present within lymph nodes. The lymph node cells (5×10^5 cells/well) were cultured for 72 h in the wells of a flat-bottomed 96-well culture plate (Falcon 3072) in RPMI 1640 (Wako) supplemented with 10 mM HEPES (pH 7.4; Nacalai Tesque, Kyoto, Japan), 10% fetal bovine serum (Hyclone, South Logan, UT), and 30 μ g/ml kanamycin (Wako) at 37 °C under a humidified atmosphere of 5% CO₂/95% air. No polyclonal T cell stimulator such as anti-CD3

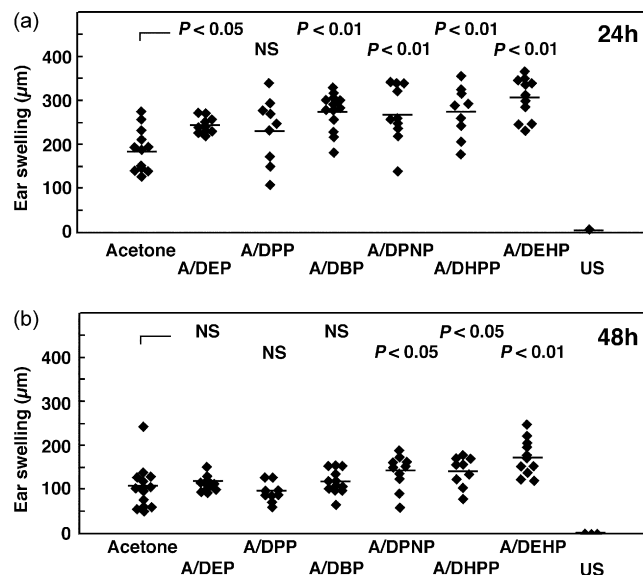


Fig. 1. Effects of various phthalate esters on sensitization to MNICN. BALB/c mice ($n=9-14$) were epicutaneously sensitized with MNICN dissolved in acetone or in a 1:1 mixture of acetone (A) and the phthalate ester specified on the abscissa. US: un-sensitized negative control ($n=5$). Ear swelling at 24 h (a) and 48 h (b) was determined after challenge with MNICN in A/DBP on an auricle. Ear-swelling responses (ordinate) of individual mice (in μ m) are plotted, and the horizontal bars show the mean for each group. Statistical significance (compared with the acetone control) was analyzed by means of one-way ANOVA, followed by Dunnett's test. NS: not significant.

was included. The culture supernatants were collected from the wells, and then the concentrations of IL-4 and IFN- γ were determined by means of a sandwich ELISA as described (Maruyama et al., 2007a). As antibodies to capture cytokines, purified anti-IL-4 mAb and purified anti-IFN- γ mAb were used. The bound cytokines were detected with the respective detection antibodies, i.e. biotin-anti-IL-4 or biotin-anti-IFN- γ , followed by incubation with HRP–avidin. The cytokine concentrations in the supernatants were determined based on a dose–response curve generated with standard recombinant cytokines. In each experimental set, experiments were repeated three times.

2.5. Statistical analysis

To assess significant differences between multiple groups, one-way ANOVA followed by Dunnett's test were employed. For cytokine production, comparison between two groups was performed by means of Student's *t*-test. *P* values of <0.05 were considered significant.

3. Results

3.1. Effects of various phthalate esters on sensitization to MNICN

We examined whether the presence of various phthalate esters might enhance epicutaneous sensitization to MNICN (Fig. 1). DEP, DBP, DPNP, DHPP and DEHP enhanced the sensitization to MNICN. The effect of DPP was not statistically significant in this experiment due to variation. The ear-swelling response at 24 h after sensitization was similarly enhanced by several phthalate esters including DBP and DEHP, whereas that at 48 h was enhanced by phthalate esters with longer alkyl chains (DPNP, DHPP and DEHP) but not with shorter ones (DEP and DBP).

3.2. Effects of various phthalate esters on sensitization to PEICN

Because the adjuvant effect of DEHP was discovered in the MNICN system, we focused on the effect of DEHP on another isocyanate hapten, PEICN, with references of DBP and DPP (Fig. 2). In this case, DBP and DPP did not enhance the sensitization. The ear-swelling response at 24 h was not significantly higher in the

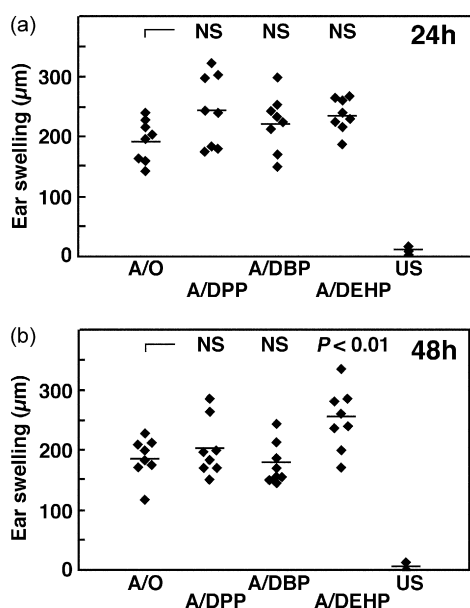


Fig. 2. Effects of various phthalate esters on sensitization to PEICN. BALB/c mice ($n=8$) were sensitized with PEICN dissolved in a 3:1 mixture of acetone and olive oil (A/O) or in a 1:1 mixture of acetone (A) and the phthalate ester specified on the abscissa. US: un-sensitized negative control ($n=4$). Ear swelling at 24 h (a) and 48 h (b) was determined after challenge with PEICN in A/DBP on an auricle. Data were plotted and statistically analyzed as in Fig. 1.

presence of a phthalate ester. However, only the DEHP group exhibited enhanced ear swelling at 48 h after challenge.

3.3. Effects of DBP and DEHP on the production of cytokines by MNICN-sensitized lymph node cells

We analyzed the production of IL-4 and IFN- γ by draining lymph nodes after sensitization with MNICN to examine helper T cell polarization. In our experimental protocol, MNICN-specific T cells were stimulated with MNICN-presenting DC, both of which were obtained from draining lymph nodes. Therefore, no cytokine production was observed from lymph nodes from un-sensitized mice. When mice were sensitized with MNICN in the presence of DBP, enhancement of IFN- γ was not evident but IL-4 production was significantly reduced (Fig. 3, Exp. 1) as compared with acetone control. In the case of DEHP, significant enhancement of IFN- γ production was observed with reduced production of IL-4 (Fig. 3, Exp. 2). In both cases, T helper 1 (Th1)-type polarization was suggested to take place.

4. Discussion

In epidemiological studies, exposure to DEHP has been found to be associated with airway allergies such as asthma (Bornehag et al., 2004; Kolarik et al., 2008), in which Th2-type polarization takes place. In animal studies involving atopic model NC/Nga mice, intraperitoneal injection of DEHP aggravated skin lesions upon challenge with a mite allergen (Takano et al., 2006). Maternal intraperitoneal exposure to DEHP during the lactation period enhanced atopic dermatitis-like skin lesions upon injection of a mite allergen in the offspring (Yanagisawa et al., 2008). The effect of DEHP on NC/Nga mice was accompanied by the appearance of a Th2-type chemokine, eotaxin, at the site of allergen injection.

In contrast, some studies that involved epicutaneous topical exposure of BALB/c mice to DEHP at the site of protein antigen (OVA) injection failed to demonstrate enhanced production of IgE

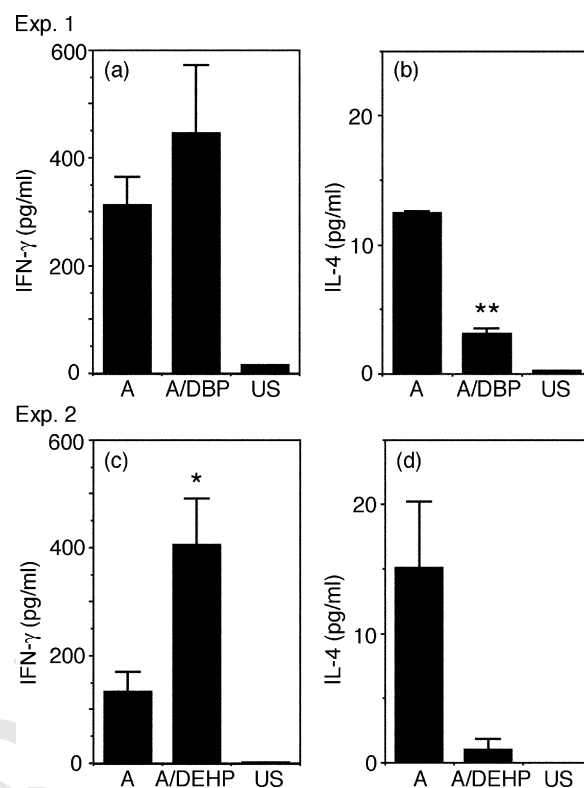


Fig. 3. Effects of phthalate esters on cytokine production by draining lymph node cells. BALB/c mice were sensitized on days 0, 1, 7 and 8 (Exp. 1) or on days 0 and 7 (Exp. 2) with MNICN. Draining lymph nodes were collected 24 h after the final sensitization. The solvent conditions used for sensitization are shown on the abscissa. A lymph node single-cell suspension that contained T cells and DCs was prepared and then cultured for 72 h. IFN- γ (a and c) and IL-4 (b and d) released into the culture supernatants were determined by ELISA. The bars represent the means $\pm$ SEM for three independent experiments. Comparison was made between acetone and A/DBP (a and b), and between acetone and A/DEHP (c and d) by means of Student's *t*-test. * $P < 0.05$, ** $P < 0.001$ compared with the acetone group. US: un-sensitized negative control.

and IgG1, which are known to be associated with Th2-type helper T cell polarization (Dearman et al., 2008). In animal models, it is still controversial whether DEHP is an adjuvant for allergic reactions in which Th2-polarization is a key.

FITC-induced CHS has been widely used as a mouse model in which a Th2-type response is required (Dearman and Kimber, 2000; Takeshita et al., 2004; Tang et al., 1996). In this case, topical application of FITC together with phthalate esters of several types including DBP enhanced the sensitization to FITC. The enhanced sensitization was associated with increased trafficking of FITC-presenting DC to draining lymph nodes (Imai et al., 2006; Shiba et al., 2009). However, DEHP did not enhance the sensitization to FITC or DC-trafficking (Imai et al., 2006). In our experiments, DEHP was dissolved in acetone together with FITC, suggesting that the low water solubility of hydrophobic DEHP is not the reason for the lack of an effect. We also confirmed that DEP, DBP and DPP enhanced IL-4 production by draining lymph nodes (Maruyama et al., 2007b). Lack of the effect of DEHP in OVA-specific IgE and IgG1 production (Dearman et al., 2008) as well as in the FITC-induced CHS suggests that DEHP may not necessarily lead immune responses into Th2-type polarization.

With this background, we examined effects of phthalate esters on the skin sensitization to MNICN. Most phthalate esters that have been observed to enhance the sensitization to FITC also enhanced the sensitization to MNICN. Unexpectedly, DEHP, which does not exhibit an adjuvant effect in the FITC model, had the strongest effect on the sensitization to MNICN. Furthermore, DEHP, but not

DBP, enhanced the sensitization to another hapten with a monoisocyanate group, PEICN. The results indicated that DEHP has an effect during skin sensitization depending on the hapten.

Because a sign of Th2-type polarization was reported as an increased level of eotaxin under the influence of DEHP in the NC/Nga mouse model (Yanagisawa et al., 2008), we examined whether DEHP enhanced Th2-type cytokine production by draining lymph nodes after MNICN sensitization. DEHP enhanced IFN- γ production associated with decreased IL-4 production. Such a Th1-type polarization tendency was also found for DBP, there being a significant reduction in IL-4 production with a slight increase in IFN- γ production. The results suggest that DEHP and DBP induce polarization to Th1. The results also suggest that the direction of helper T cell polarization is not intrinsic to phthalate esters but is influenced by the nature of the hapten simultaneously applied.

Dose of DEHP used in our experimental system was 4.48 g/kg body weight per each epicutaneous exposure. Such a high concentration of exposure may not happen in our daily life. However, exposure to aerosol containing 13 mg/m³ DEHP was reported to enhance the production of IgG1 against OVA in BALB/c mice (Larsen et al., 2007). This exposure level is not too far from that detected in house dust. Isocyanate haptens are well-recognized as substances that cause occupational allergies. Dermal sensitization against toluene diisocyanate is also demonstrated in animal models (Vanoirbeek et al., 2004). Further toxicological studies are required to determine the dose of DEHP that enhance sensitization to isocyanate haptens. It would contribute to know risks in occupational environments where both isocyanate haptens and phthalate esters might present at considerably higher concentrations as compared with those in residential environments.

In conclusion, we found that DEHP has an adjuvant effect in mouse CHS models using haptens with a single isocyanate group. The enhanced sensitization to MNICN was associated with a Th1-biased T cell response. The perspective on the combined effects of antigenic chemicals and adjuvant chemicals will provide a key clue to evaluate risks as to hypersensitivity.

Conflict of interest

None.

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