

ORIGINAL PAPER

Effects of phthalate esters on the sensitization phase of contact hypersensitivity induced by fluorescein isothiocyanate

Y. Imai, A. Kondo¹, H. Iizuka¹, T. Maruyama and K. Kurohane

Department of Microbiology and COE Program in the 21st Century, University of Shizuoka School of Pharmaceutical Sciences, Shizuoka, Japan

Clinical and Experimental Allergy

Summary

Background Many different types of phthalate ester are used as plasticizers and are thus found in the air. There have been several studies that suggest an association between allergies and phthalate esters. We previously found that di-butyl phthalate (DBP) has an adjuvant effect in a mouse contact hypersensitivity model, in which fluorescein isothiocyanate (FITC) is involved as an immunogenic hapten.

Objective We examined whether other phthalate esters enhance the process of sensitization to FITC by facilitating the trafficking of FITC-presenting dendritic cells or macrophages from skin sites to draining lymph nodes.

Methods Mice were epicutaneously sensitized with FITC dissolved in acetone containing a phthalate ester. Sensitization was evaluated as ear swelling after a challenge with FITC.

Draining lymph node cells obtained 24 h after skin sensitization were examined for FITC fluorescence by means of flow cytometry. FITC-positive cells were characterized with anti-CD11c and anti-CD11b by three-colour flow cytometry.

Results When mice were sensitized with FITC in acetone containing DBP or di-*n*-propyl phthalate (DPP), strong enhancement of the ear-swelling response was observed. Di-methyl phthalate (DMP) and di-ethyl phthalate (DEP) were less effective but produced some enhancement. Consistent enhancement was not observed with di-(2-ethylhexyl) phthalate or di-isononyl phthalate. Upon sensitization in the presence of DBP or DPP, the number of FITC-positive dendritic cells (total CD11c⁺ as well as CD11c⁺/CD11b⁺) was increased in draining lymph nodes. As to the other four phthalate esters, there was no significant increase in the FITC-positive cell number in the draining lymph nodes.

Conclusion During the process of sensitization to FITC, DBP, and DPP exert strong adjuvant effects that are associated with enhancement of trafficking of antigen-presenting dendritic cells from the skin to draining lymph nodes.

Keywords contact sensitivity, DC trafficking, di-butyl phthalate, draining lymph nodes, FITC
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Correspondence:

Dr Yasuyuki Imai, Department of Microbiology, University of Shizuoka School of Pharmaceutical Sciences, 52-1 Yada, Suruga-ku, Shizuoka-shi, Shizuoka 422-8526, Japan.
E-mail: imai@u-shizuoka-ken.ac.jp

Introduction

Phthalate esters are found in the living environment because they are widely used plasticizers for plastics such as polyvinyl chloride (PVC). For example, they are used for vinyl flooring, wall coverings, synthetic leather, and soft PVC toys. There are many types of phthalate esters differing in the alkyl chain type. For topical use, phthalate esters with short alkyl chains are widely used as ingredients of cosmetics and mosquito repellents, for example [1, 2]. Many studies have been conducted to assess the

potential risks of exposure to phthalate esters from the environmental [3, 4] and toxicological [5, 6] perspectives. From the immunological standpoint, there have been some reports describing contact hypersensitivity cases induced by di-butyl phthalate (DBP) [7, 8]. A recent report also suggested an association between allergic symptoms in children and certain types of phthalate esters in house dust [9]. In addition, some phthalate esters have been shown to enhance antibody production when subcutaneously injected with antigen in mouse models [10, 11]. On the other hand, negative results, i.e. a lack of skin sensitization activity of phthalate esters in human volunteer studies [12, 13], have also been reported.

¹These authors contribute equally.

For a mouse contact hypersensitivity model involving fluorescein isothiocyanate (FITC) as a hapten, a mixture of acetone and DBP has been used empirically as a standard solvent for FITC [14]. We previously demonstrated that DBP had an adjuvant effect during the sensitization phase with FITC when several different types of solvent were compared [15]. We also demonstrated that DBP facilitates the trafficking of FITC-presenting cells to the draining lymph nodes from skin sites where the hapten had been applied. The facilitated trafficking of FITC-presenting cells was shown to be one of the factors that influences the extent of sensitization [15].

In this study, we examined whether or not several different phthalate esters have adjuvant effects on skin sensitization with FITC. We also examined whether or not the adjuvant effects are correlated with the effect on the trafficking of FITC-presenting dendritic cells or macrophages from the skin to draining lymph nodes.

Materials and methods

Mice

Specific pathogen-free female CD-1 (ICR) and BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan), and were used between 5 and 8 weeks of age. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka.

Reagents

Acetone, di-methyl phthalate (DMP), di-ethyl phthalate (DEP), DBP, di-(2-ethylhexyl) phthalate (DEHP), and diisononyl phthalate (DINP) were purchased from Wako Pure Chemicals (Osaka, Japan); di-*n*-propyl phthalate (DPP) from Kanto Chemicals (Tokyo, Japan); FITC from DOJINDO Laboratories (Kumamoto, Japan); sodium pentobarbital from Dainippon Pharmaceutical Co. (Osaka, Japan); Dulbecco's minimum essential medium (DMEM) from Nissui Pharmaceuticals (Tokyo, Japan); and bovine serum albumin (BSA) (fraction V) from Sigma (St Louis, MO, USA). R-phycoerythrin (PE)-conjugated hamster anti-mouse CD11c mAb (clone HL3; IgG1), PE-conjugated hamster IgG1 isotype control (clone G235-2356), and PerCP-Cy5.5-conjugated streptavidin were purchased from BD Biosciences (San Jose, CA, USA), and biotin-conjugated rat anti-mouse CD11b mAb (clone M1/70; IgG2b), and biotin-conjugated rat IgG2b isotype control (clone G2b-1-2) from eBioscience (San Diego, CA, USA).

Sensitization and elicitation of a contact hypersensitivity reaction

The method used for contact sensitization to FITC was that used in earlier studies [14, 15] with modifications. Mouse

forelimbs were shaved using small animal clippers 1 day before sensitization. Mice were anaesthetized by intraperitoneal injection of pentobarbital, and then 160 µL of a solution of FITC (0.5%, w/v) dissolved in a solvent was epicutaneously applied to the shaved forelimbs (day 0). The solvents used for sensitization were as follows: acetone, acetone (A)/DMP (A/DMP) (1 : 1, v/v), A/DEP (1 : 1), A/DPP (1 : 1), A/DBP (1 : 1), A/DEHP (1 : 1), and A/DINP (1 : 1). On day 7, the same amount of the FITC solution in the respective solvent was applied again. On day 14, the baseline ear thickness (0 h) in each animal was measured using a dial thickness gauge (Mitutoyo, Kanagawa, Japan). Mice were challenged by applying 20 µL of an FITC solution (0.5% in A/DBP) on the right auricle, and then the ear thickness was measured after 4–48 h. The left auricle was treated with 20 µL of A/DBP alone as a control.

Ear swelling at X h is defined as follows: [(ear thickness of the right ear at X h) – (ear thickness of the right ear at 0 h)] – [(ear thickness of the left ear at X h) – (ear thickness of the left ear at 0 h)].

Flow cytometry

Brachial lymph nodes were obtained from mice that had been epicutaneously treated with FITC on forelimb skin 24 h earlier. Lymph nodes from three mice were pooled for each condition. Single-cell suspensions of lymph nodes were prepared in DMEM by gentle teasing using needles. After washing in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.1% NaN₃ (B-PBS), cells were resuspended in the same buffer. A total of 5×10^5 cells were examined with a flow cytometre (EPICS XL, Beckman Coulter, Fullerton, CA, USA) using gates for forward and side light scatter to collect signals of cell-associated fluorescence. To characterize cells exhibiting FITC fluorescence, 1×10^6 lymph node cells were incubated with 1 µg of biotin-conjugated rat anti-CD11b or biotin-conjugated rat IgG2b (isotype control), for 30 min at 4 °C in 100 µL of B-PBS. After washing in the B-PBS twice, the cells were incubated with 0.2 µg of PE-conjugated hamster anti-CD11c or PE-conjugated hamster IgG1 (isotype control) together with 0.12 µg of PerCP-Cy5.5-conjugated streptavidin for 30 min at 4 °C in 100 µL of B-PBS. Cells were washed in B-PBS twice and then examined with a flow cytometre with appropriate filter sets as follows: FITC (FL-1), CD11c (FL-2), and CD11b (FL-4).

Results

Effects of phthalate esters during sensitization with hapten fluorescein isothiocyanate

We previously demonstrated that DBP has an adjuvant effect during epicutaneous sensitization with FITC [15]. We examined whether or not other phthalate esters with alkyl chains of different length exhibit similar effects.

Five-week-old ICR mice were epicutaneously sensitized on forelimb skin with FITC dissolved in acetone containing one of various phthalate esters. Mice were challenged with FITC dissolved in A/DBP by epicutaneous application on one ear, and ear swelling was measured at 24 and 48 h. We used DBP during the challenge because significant ear swelling was not observed if DBP was absent from the FITC solution used for the challenge (data not shown). There was no indication that DBP itself was antigenic, because there was essentially no change in the thickness of the left auricle (treated with A/DBP alone at the time of challenge) before or after the challenge irrespective of whether or not the mice had been exposed to DBP. For example, there was a $7 \pm 7 \mu\text{m}$ difference (mean $\pm$ SD, $n = 20$) for mice that had been sensitized with FITC in A/DBP; a $10 \pm 9 \mu\text{m}$ one ($n = 10$) for those sensitized with FITC in acetone; and a $2 \pm 5 \mu\text{m}$ one ($n = 5$) for those treated with A/DBP alone. Furthermore, we had also established the lack of immunogenicity of DBP in earlier experiments [15].

Although that there was considerable variation between individual mice in the ear-swelling response, the groups sensitized in the presence of DBP, DPP, and DMP exhibited significantly stronger responses at 24 h as compared with the control group that had been sensitized with FITC in acetone (Fig. 1). In the cases of DEHP and DINP, the levels of the responses were not statistically different from that in the control group (acetone alone), but some individual mice occasionally exhibited stronger responses. The ear swelling was generally decreased at 48 h, but the levels of ear swelling were significantly higher in the groups sensitized in the presence of DPP and DEP. In the absence of FITC during the sensitization phase, no ear swelling was observed upon FITC challenge. Consistent enhancement of the ear swelling response to FITC was also reproduced with DBP and DPP in 8-week-old ICR mice (data not shown).

The effects of phthalate esters in the sensitization phase were confirmed using an inbred mouse strain, BALB/c (8-week old). The enhancement of the ear-swelling response at both 24 and 48 h after elicitation was evident under the conditions with DBP, DPP, or DEP (Fig. 2). The ear-swelling response was stronger at 24 h. In the cases of DEHP and DINP, the mean values were not significantly higher than that for the acetone control. However, some mice exhibited stronger responses than the level under the control conditions (acetone alone). The variation in the ear-swelling response between individual mice was smaller for BALB/c than for ICR mice. In both strains, DBP and DPP produced strong and consistent enhancement.

Time course of ear-swelling response

There is a concern that the 24-h ear-swelling response could represent trailing off from an earlier ear-swelling

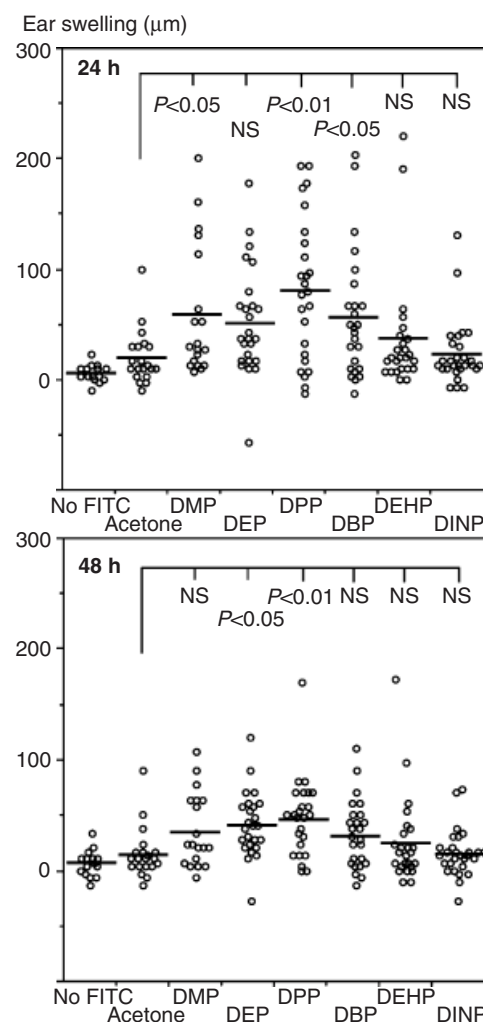


Fig. 1. Effects of various phthalate esters during sensitization with fluorescein isothiocyanate (FITC). ICR mice (5-week old) were epicutaneously treated with 0.5% FITC dissolved in acetone, or in a 1 : 1 mixture of acetone and a phthalate ester (di-methyl phthalate (DMP), di-ethyl phthalate (DEP), di-*n*-propyl phthalate (DPP), di-butyl phthalate (DBP), di-(2-ethylhexyl) phthalate (DEHP), or di-isononyl phthalate (DINP)) on forelimb skin on days 0 and 7. Some mice were treated with acetone (A)/DBP without FITC (no FITC). On day 14, mice were challenged with 0.5% FITC in A/DBP on the auricles as described under Materials and methods. Ear swelling at 24 and 48 h was determined. The values for individual mouse are plotted, and the means for each group are shown by bars. Statistical significance (compared with the acetone control) was analysed by means of one-way ANOVA, followed by Dunnett's test. NS: not significant.

response due to the contribution of circulating antibodies against FITC. We compared the ear swelling at 4, 8, and 24 h after elicitation using 8-week-old BALB/c mice. The extent of ear swelling gradually increased throughout the early period after elicitation (Fig. 3). This trend was seen for both the A/DBP and A/DPP conditions that were used in the sensitization phase. The sensitization with FITC in acetone did not produce ear swelling at the early stage either.

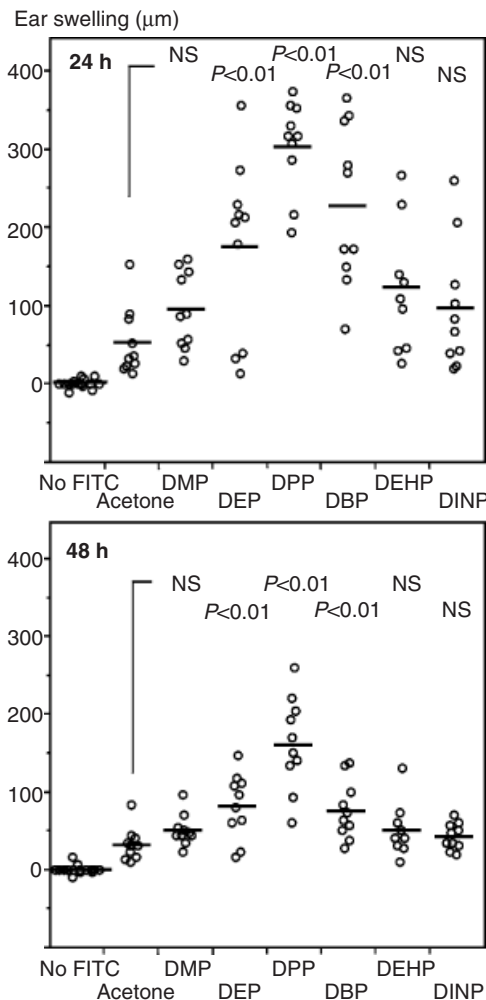


Fig. 2. Effects of various phthalate esters during sensitization with fluorescein isothiocyanate (FITC) using an inbred mouse strain. BALB/c mice (8-week old) were epicutaneously treated with 0.5% FITC on days 0 and 7, as described in Fig. 1. On day 14, the mice were challenged, and the ear swelling at 24 and 48 h was determined. The values for individual mice are plotted, and the means for each group are shown by bars. Statistical significance (compared with the acetone control) was analysed by means of one-way ANOVA, followed by Dunnett's test.

Effects of phthalate esters on trafficking of fluorescein isothiocyanate-presenting cells

Contact sensitization of skin is initiated by trafficking of antigen-presenting cells from the skin to draining lymph nodes. To determine whether or not some phthalate esters can facilitate skin-to-lymph node trafficking of antigen-presenting cells, FITC-positive cells in draining lymph nodes were compared at 24 h after epicutaneous sensitization with FITC. Because FITC is a fluorescent hapten, cells exhibiting FITC fluorescence in draining lymph nodes can be detected with a flow cytometre [15]. The autofluorescence level was determined by analysing lymph node cells from untreated mice under the same conditions (Fig. 4a). When FITC in an acetone solution was applied on mouse

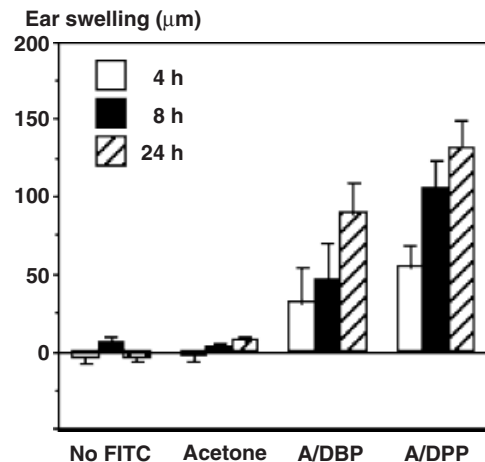
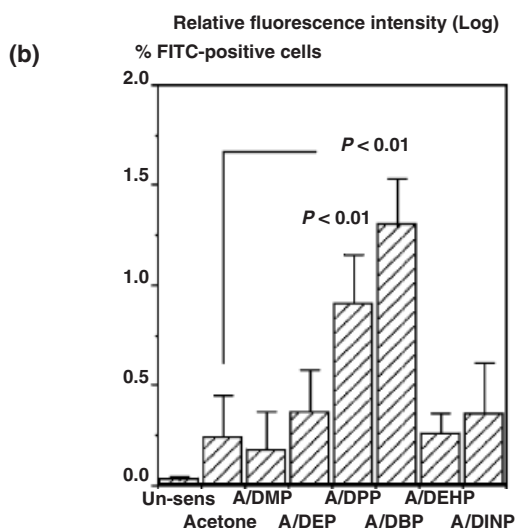
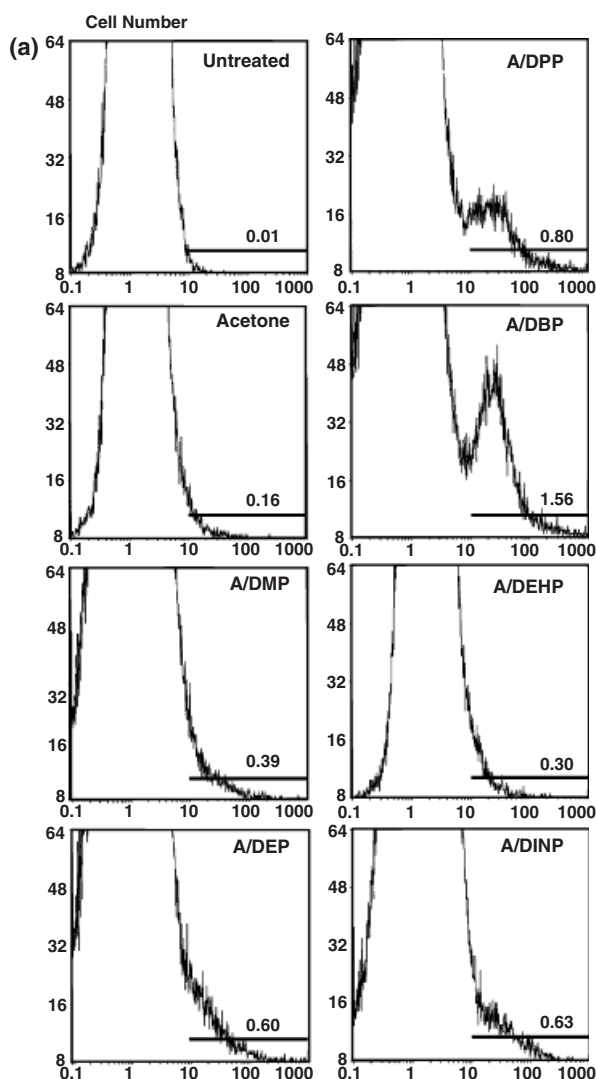


Fig. 3. Time course of ear-swelling response after challenge. BALB/c mice (8-week old) were epicutaneously sensitized with 0.5% fluorescein isothiocyanate (FITC) dissolved in acetone alone, acetone (A)/di-butyl phthalate (DBP), or A/di-*n*-propyl phthalate (DPP). Some mice were treated with A/DBP without FITC (no FITC). The ear-swelling response was determined 4 (open columns), 8 (closed columns), and 24 h (hatched columns) after challenge. Data are expressed as means $\pm$ SEM for five mice.

forelimb skin, cells exhibiting fluorescence above the background level were detected, although the difference was very subtle. When mice were treated with FITC in A/DBP or A/DPP, a much higher number of FITC-positive cells was observed (Fig. 4a). In the case of A/DMP, A/DEP, A/DEHP, or A/DINP, the increase in the number of FITC-positive cells was less prominent. The background threshold was arbitrarily set at a relative fluorescence intensity of 10 (Fig. 4a), and the proportion of FITC-positive cells in the draining lymph nodes was determined in three independent experiments (Fig. 4b). The proportion of FITC-positive cells was significantly higher under the A/DBP and A/DPP conditions as compared with acetone alone. Other phthalate esters (DMP, DEP, DEHP, and DINP) did not have a significant effect.

Dendritic cell marker on fluorescein isothiocyanate-positive cells in draining lymph nodes

To determine whether or not hapten FITC is transported by antigen-presenting cells such as dendritic cells and macrophages, we analysed the expression of cell surface markers (CD11c and CD11b) by means of three-colour flow cytometry. Cells exhibiting FITC fluorescence above the background level (a relative fluorescence intensity of 10) were gated, and then the expression of CD11c and CD11b was analysed. Appropriate isotype control antibodies were used to detect the background level. Representative results with lymph node cells 24 h after FITC sensitization are shown in Figs 5a–c (A/DBP conditions) and Fig. 5d (A/DPP conditions).



When lymph node cells were stained with anti-CD11c and anti-CD11b (Fig. 5a), 30% of cells appeared in the gate double positive for CD11c (x-axis) and CD11b (y-axis). To

estimate background, we used staining with appropriate isotype control antibodies; anti-CD11b (Fig. 5b) or anti-CD11c (Fig. 5c) was replaced by a respective isotype control. The background level due to random binding of antibodies was estimated to be 10.9%. Thus, 19.1% were calculated to be CD11c⁺/CD11b⁺ cells in this particular example. On the other hand, only a small population of cells (3.29%) was in the gate for CD11c⁻/CD11b⁺ cells (Fig. 5a). This is not distinguishable from background (4.26%) when isotype control was used in place of anti-CD11b (Fig. 5b). Thus, the contribution of FITC-presenting macrophages seems to be negligible. As for total dendritic cells, 55.6% of FITC-positive cells (above the background shown in Fig. 5c) were calculated to be CD11c positive. A similar calculation was carried out for other samples. As a representative result for A/DPP conditions, a dot plot of a sample with anti-CD11c and anti-CD11b double staining is shown in Fig. 5d.

We examined whether DBP or DPP facilitates the trafficking of FITC-positive cells expressing dendritic cell markers. The results of three independent experiments are summarized in Figs 5e and f. The number of FITC-positive CD11c⁺/CD11b⁺ (Langerhans cells (LC) or myeloid dendritic cells (DCs)) cells was significantly increased under the conditions with DBP or DPP, as compared with acetone alone (Fig. 5e). As for total dendritic cells (CD11c⁺), significant enhancement was also observed under the conditions with DBP or DPP (Fig. 5f).

Discussion

In this study, we focused on whether or not several different phthalate esters can enhance sensitization to FITC. We found that not only DBP but also other phthalate esters have the ability to enhance sensitization to FITC. The extent of the effect was dependent on the length of the alkyl chain, and DPP was the strongest among those tested. Thus, the optimal length of the alkyl chain was

Fig. 4. Appearance of fluorescein isothiocyanate (FITC)-positive cells in draining lymph nodes after epicutaneous application of FITC with various phthalate esters. Mice (ICR, 5-week old) were treated with 0.5% FITC dissolved in a solvent on forelimb skin. At 24 h, single-cell suspensions were prepared from pooled (three mice) brachial lymph nodes and analysed for FITC fluorescence. (a) Histogram for FITC fluorescence. The abscissa represents the fluorescence intensity on a log scale. Cells with a fluorescence intensity of 10 or more (horizontal bars) were arbitrarily assigned as FITC-positive. The percentage of FITC-positive cells was shown by a number on each bar. (b) Summary of FITC-positive cell trafficking. The abscissa indicates the solvent conditions for sensitization. Some mice were left untreated (un-sens). The ordinate represents the percentage of FITC-positive cells relative to the total lymph node cell number in each condition. Each bar represents the mean $\pm$ SD of three independent experiments. Statistical significance (compared with the acetone control) was analysed by means of one-way ANOVA, followed by Dunnett's test.

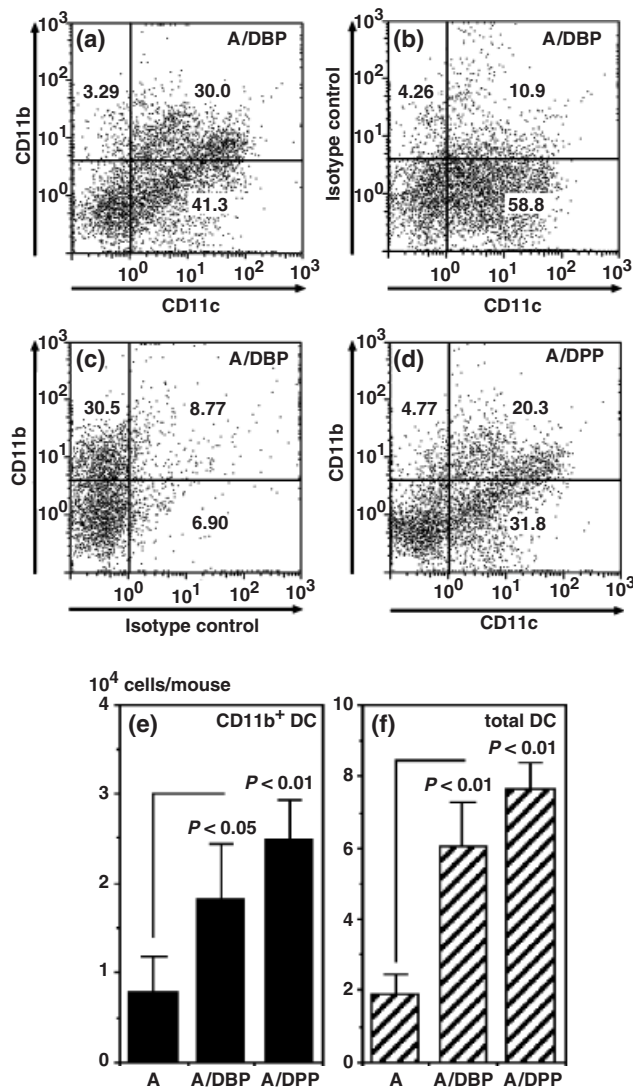


Fig. 5. Effects of various phthalate esters on trafficking of fluorescein isothiocyanate (FITC)-presenting dendritic cells (DCs). (a–d) Representative results for the draining lymph node cells from ICR mice (8-week old) that had been epicutaneously treated with 0.5% FITC dissolved in acetone (A)/di-butyl phthalate (DBP) (a–c) or in A/di-*n*-propyl phthalate (DPP) (d) on forelimb skin 24 h beforehand. Lymph node cells were stained with anti-CD11c and anti-CD11b (a and d). As control experiments, cells were stained with the isotype control plus anti-CD11c (b), or anti-CD11b plus the isotype control (c). FITC-positive cells (> 10 for the fluorescence intensity) are gated and analysed for CD11c (abscissa) and CD11b (ordinate) expression. A quadrant gate was set to discriminate cellular phenotypes. The number in (a–d) gives the percentage of cells in the gate relative to total FITC-positive cells. (e and f) Summary for the trafficking of FITC-presenting CD11b⁺ DCs (e) and total DCs (f). Based on the analysis described in Figs 5a–c, the numbers of FITC-positive CD11c⁺/CD11b⁺ cells (Langerhans cells or myeloid DCs) and CD11c⁺ cells (total DCs) were calculated per mouse for each solvent condition: A, A/DBP, and A/DPP. Each bar represents the mean $\pm$ SD of three independent experiments. Statistical significance (compared with the acetone control) was analysed by means of one-way ANOVA, followed by Dunnett's test.

carbon number C3 (DPP), followed by C4 (DBP). Those with shorter alkyl chains, i.e. DMP (C1) and DEP (C2), had some effect. In contrast, phthalate esters with longer alkyl chains, i.e. DEHP (C8) and DINP (C9), were less effective.

Haptens epicutaneously applied on the skin covalently bind to self-proteins, and then the hapten–protein complexes are transported through afferent lymphatic vessels. In the transportation of hapten–protein complexes, the trafficking of skin LC, which present such complexes, to draining lymph nodes is involved [14]. We previously demonstrated that DBP facilitates this process [15]. In the present study, we found that DPP as well as DBP facilitated this process. We analysed the cell surface phenotypes of FITC-positive cells found in the draining lymph nodes. The number of FITC⁺/CD11c⁺/CD11b⁺ cells was significantly increased when FITC was applied in A/DBP or A/DPP as compared with acetone alone. This finding suggests that DBP and DPP facilitated the trafficking of LC and/or myeloid DC from skin sites [16, 17]. We also observed a significant enhancement in the total number of FITC-positive CD11c⁺ cells in the draining lymph nodes under the conditions with A/DBP or A/DPP. We found higher numbers of FITC-positive CD11c⁺/CD11b⁺ and CD11c⁺ cells in the draining lymph nodes under DPP conditions rather than under DBP conditions. This is due to an increase in the total number of cells in draining lymph nodes under the DPP conditions. To examine the possibility that hapten FITC can be taken up by antigen-presenting cells in draining lymph nodes *in situ*, we are currently investigating other combinations of DC markers such as CD8 α and B220 [18–20]. A recent report suggested that fast-migrating dermal DCs (CD207 negative) contributed to contact sensitization using tetramethylrhodamine isothiocyanate (TRITC) as a hapten [21]. Further characterization is needed to see the effects of phthalate esters on the migration of dermal DCs as well. From our present studies, enhanced trafficking of antigen-presenting CD11b⁺ DCs was shown to be correlated with the establishment of sensitization in the presence of DBP or DPP.

In the case of DEP and DMP, there was some enhancement in the ear-swelling experiments. However, these reagents did not facilitate the trafficking of FITC-presenting cells. DEHP and DINP did not produce consistent enhancement in the ear-swelling experiments or enhancement in the trafficking of FITC-presenting cells either. These results suggested that enhanced trafficking of antigen-presenting cells is one of the mechanisms, but that some other mechanisms may also be involved in the adjuvant effects of phthalate esters. We previously found that DBP also facilitates the trafficking of dermal connective tissue macrophages, and the macrophage trafficking appeared to be positively correlated with enhanced sensitization as well [15]. Trafficking of connective tissue macrophages expressing MGL-1 or MGL-2 may possibly explain the effects of DEP and DMP.

The fact that phthalate esters can facilitate the process of sensitization to FITC suggested that the co-existence of different chemicals has some impact on the outcome of contact sensitization. In a human volunteer study in which phthalate esters with carbon numbers C6–C13 (including DEHP and DINP) were investigated by means of a patch test, negative results for skin sensitization were obtained [12]. However, in that study, phthalate esters with shorter alkyl chains were not examined. In another study on human volunteer skin, an uncommon response to DEP was observed [13]. It should be noted that these studies were conducted to examine the effect of a phthalate ester by itself, i.e. in the absence of other chemicals. On the other hand, there has been a case report showing that a patient became allergic to DBP and benzalkonium chloride after using Timodine[®] cream (Reckitt Benckiser, Hull, UK), which contains these two substances [8]. The results may indicate that the effects of phthalate esters should be examined in combination with other chemicals in human systems as well.

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