Adjuvant effect of an alternative plasticizer, diisopropyl adipate, on a contact hypersensitivity mouse model: link with sensory ion channel TRPA1 activation

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Summary

Due to health concerns about phthalate esters, the use of alternative plasticizers is being considered. Phthalate esters enhance skin sensitization to fluorescein isothiocyanate (FITC) in mouse models. We have demonstrated that phthalate esters stimulate transient receptor potential ankyrin 1 (TRPA1) cation channels expressed on sensory neurons. We also found a correlation between TRPA1 activation and the enhancing effect on FITC-induced contact hypersensitivity (CHS) when testing various types of phthalate esters. Here we investigated the effects of an alternative plasticizer, diisopropyl adipate (DIA). Activation of TRPA1 by DIA was demonstrated by calcium mobilization using Chinese hamster ovary cells expressing TRPA1 in vitro. The effect of DIA was inhibited by a TRPA1-specific antagonist, HC-030031. The presence of DIA or dibutyl phthalate (DBP; positive control) during skin sensitization of BALB/c mice to FITC augmented the CHS response, as revealed by the level of ear-swelling. The enhancing effect of DIA was inhibited by in vivo pretreatment with HC-030031. FITC-presenting CD11c\(^+\) dendritic cell (DC)-trafficking to draining lymph nodes was facilitated both by DIA and by DBP. DBP and DIA were similarly active in the enhancement of interferon-\(\gamma\) production by draining lymph nodes, but the effect on interleukin-4 production was weaker with DIA. Overall, DIA activated TRPA1 and enhanced FITC-induced CHS, as DBP did. The adjuvant effects of adipate esters may need to be considered because they are used as ingredients in cosmetics and drug formulations topically applied to the skin.

Keywords: adipate ester; adjuvant; contact hypersensitivity; transient receptor potential...
Plasticizers are widely used for plastics such as polyvinyl chloride. As plasticizers, phthalate esters are widely used for consumer products such as synthetic leather, vinyl flooring, wall coverings, paints, adhesive agents and cosmetics. Many studies have been conducted for assessment of the risk of exposure to phthalate esters in the air and food, and their accumulation in the body.\textsuperscript{1,2} Epidemiological studies revealed an association between phthalate esters and allergies in children.\textsuperscript{3,4} A case of contact hypersensitivity in association with topical contact with dibutyl phthalate (DBP), a short-chain phthalate ester, has also been reported.\textsuperscript{5} Due to health concerns about phthalate esters, the use of other types of acid esters is being considered. Adipate esters are candidate plasticizers.

Diisopropyl adipate (DIA) is used as an emollient ingredient for cosmetics to protect the human skin from dryness.\textsuperscript{6} DIA is also used in tacrolimus ointments as a skin penetration enhancer.\textsuperscript{7} As to drug formulations, adipate esters are used as plasticizers for biodegradable flexible films and for enhancement of skin penetration of hydrophilic drugs.\textsuperscript{8,9} Regarding safety assessment, DIA has been concluded to be safe as a cosmetic ingredient.\textsuperscript{10,11}

We have been studying fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS) mouse models. In such a model, DBP is empirically added to solvents for FITC.\textsuperscript{12} We found that several phthalate esters, such as DBP, enhance skin sensitization to FITC and the trafficking of dendritic cells (DC) that present FITC.\textsuperscript{13,14} We found the accumulation of cells bearing macrophage C-type lectin mMGL (CD301) in draining lymph nodes after epicutaneous application of DBP.\textsuperscript{13} CD301 was subsequently shown to be expressed on DC as well.\textsuperscript{15} We then performed skin organ culture experiments in which DC migration from skin explants was observed. When DBP was applied on the skin of mice and then skin explants were cultured \textit{in vitro}, the
CD301+ cells disappeared from the dermis, presumably due to migration from the explants. In contrast, when DBP was applied on skin explants after dissection, the CD301+ cells remained in the dermis.16 These results suggested the possibility that DBP was detected by a physiological system involving the whole animal, such as the nervous system.

The ability of DBP to stimulate sensory neurons was directly demonstrated by the activation of nerve cells isolated from mouse dorsal root ganglia.17 We found that more than 90% of neurons that were stimulated with DBP also responded to allyl isothiocyanate (AITC), an agonist of transient receptor potential ankyrin 1 (TRPA1). Transient receptor potential (TRP) channels constitute a molecular family of calcium-permeable cation channels expressed on sensory neurons.18 We expressed TRPA1 on Chinese hamster ovary (CHO) cells, and found that DBP induced calcium mobilization, which was inhibited by HC-030031, a specific antagonist of TRPA1.17,19 We also found that DBP activates TRPV117 but not TRPM8,20 suggesting its selectivity for channel subtypes. The involvement of TRPA1 in skin sensitization was confirmed by the fact that known TRPA1 agonists (cinnamaldehyde, carvacrol and menthol) enhanced skin sensitization to FITC as well as DC-trafficking. Furthermore, FITC-induced CHS (in the presence of DBP) was inhibited by HC-030031.19 We also compared several types of phthalate esters with alkyl chains of different lengths. We found that phthalate esters with short alkyl chains (less than C6) stimulated TRPA1 as well as enhanced FITC-induced CHS and DC-trafficking.17,19 These results suggested that TRPA1 activation is involved in FITC-induced CHS, and screening for TRPA1 activation may be useful for revealing the adjuvant effects of chemicals.

In this study, we examined the effect of DIA on TRPA1 activation as well as on
FITC-induced mouse CHS. The results indicated that TRPA1 activation in vitro is a good predictor of the adjuvant effect in the case of DIA, which is an alternative plasticizer to phthalate esters.

MATERIALS AND METHODS

**Chemicals and Reagents** Acetone, AITC, DBP, DIA, kanamycin sulfate, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and a 0.5% (w/v) methylcellulose 400 sterile solution were purchased from Wako Pure Chemicals (Osaka, Japan), FITC, Fluo 4-AM and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) from Dojindo Laboratories (Kumamoto, Japan), dimethylsulfoxide (DMSO) from Nacalai Tesque (Kyoto, Japan), and ionomycin, blasticidin, zeocin and tetracycline from Life Technologies (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were purchased from Nissui Pharmaceuticals (Tokyo, Japan), Ham's F-12 medium, Hanks' balanced salt solution (HBSS), bovine serum albumin (BSA; fraction V), probenecid and poly-L-lysine from Sigma (St. Louis, MO), fetal bovine serum (FBS) from Hyclone (South Logan, UT), pentobarbital from Kyoritsu Seiyaku Corporation (Tokyo, Japan), and 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide (HC-030031) from ENZO Life Sciences (Farmingdale, NY). Phycoerythrin (PE)-conjugated hamster anti-mouse CD11c monoclonal antibodies (mAb) (clone HL3; IgG1) and a PE-conjugated hamster IgG1 isotype control (clone G235-2356) were purchased from BD Biosciences (San Jose, CA), purified rat anti-mouse interferon-γ (IFN-γ) mAb (clone AN-18), biotin-conjugated rat anti-mouse IFN-γ mAb (clone
R4-6A2), and recombinant mouse IFN-γ from BioLegend (San Diego, CA), and purified rat anti-mouse interleukin-4 (IL-4) mAb (clone 11B11), biotin-conjugated rat anti-mouse IL-4 mAb (clone BVD6-24G2), and recombinant mouse IL-4 from eBioscience (San Diego, CA). Horseradish peroxidase (HRP)-avidin D was purchased from Vector Laboratories (Burlingame, CA), and HRP-avidin from Invitrogen (Frederick, MD).

**Animals**  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 to 24°C with 50 to 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 humanely in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka and those of Japan Ministry of Education, Culture, Sports, Science and Technology, with due consideration to the alleviation of distress and discomfort. The plans of animal studies were reviewed and approved (approval number: 136031) by the Institutional Animal Care and Use Committee of the University of Shizuoka.

**Cells**  Chinese hamster ovary (CHO) cells stably expressing TRPA1 (TRPA1-CHO cells) and control T-REx CHO cells were used as described previously. Cells were maintained in Ham's F-12/DMEM (1:1) containing 10% FBS, 60 μg/mL kanamycin, 5 μg/mL blasticidin and 200 μg/mL zeocin at 37°C under a humidified atmosphere of 5% CO₂/95% air.

**Measurement of TRPA1 activation in vitro**  The ligand-induced increase in the intracellular calcium level was measured in TRPA1-CHO cells using a fluorescent calcium indicator, Fluo 4-AM, as described previously. TRPA1-CHO cells were
cultured in the poly-t-lysine-coated wells of a 96-well plate for 24 h at 37°C in the presence of 1 µg/mL tetracycline to induce TRPA1. T-REx CHO cells, which do not express TRPA1, were cultured under the same conditions and used as a negative control. Cells were loaded with 3 µM Fluo 4-AM in HBSS containing 20 mM HEPES (pH 7.4), 1 mM CaCl2, 0.1% BSA and 2.5 mM probenecid for 1 h at 37°C. Calcium levels were automatically measured over time with a fluorometric imaging plate reader, FLEXstation II (Molecular Devices, Sunnyvale, CA). To record the maximal response level, 5 µM ionomycin was added at the end of each measurement. As a competitive inhibitor of TRPA1, HC-030031 was used. Stock solutions of samples and the inhibitor were prepared in DMSO. The final concentration of DMSO did not exceed 0.1%.

**FITC-induced CHS** FITC-induced contact hypersensitivity experiments were performed as described previously. In brief, BALB/c mice (anesthetized by intraperitoneal injection of pentobarbital, 60 mg/Kg) were sensitized on days 0 and 7 with 160 µL of a 0.5% (w/v) FITC solution by epicutaneous application to shaved forelimbs. FITC was dissolved in one of the following solvents: acetone alone, 2% (v/v) DBP in acetone, and 20% (v/v) DIA in acetone. On day 14, the ear thickness baseline (0 h) was determined using a dial thickness gauge (Mitutoyo; Kanagawa, Japan). Mice were challenged by applying 20 µL of a 0.5% FITC solution in 50% (v/v) DBP in acetone on the right auricle, while 20 µL of 50% DBP in acetone without FITC was applied on the left auricle as a control. Ear thickness was measured after 24, 48 and 72 h. Ear swelling at X h is defined as follows: [ear thickness of the right – ear thickness of the left] at X h – [ear thickness of the right – ear thickness of the left] at 0 h. To differentiate the effect on the sensitization from that on the elicitation, we used the same solvent (50% DBP in acetone) during elicitation. In experiments involving a selective
TRPA1 antagonist, mice received 200 μL of the HC-030031 suspension or vehicle alone by intraperitoneal injection at 1 h before sensitization (4 mg/mouse) on days 0 and 7. HC-030031 was suspended at 20 mg/mL in a 0.5% sterile methylcellulose solution.

**DC-trafficking**  Trafficking of FITC-presenting DC from the skin to draining lymph nodes was examined as described previously. In brief, BALB/c mice were epicutaneously treated with 160 μL of a 0.5% FITC solution in one of the following solvents on shaved forelimb skin: acetone alone, 2% or 50% DBP in acetone, and 20% DIA in acetone. After 24 h, brachial lymph nodes were obtained and pooled for each condition. Single-cell suspensions of lymph nodes were prepared and the cells were stained with the PE-conjugated anti-CD11c mAb or isotype control mAb as described previously. In total, 5 × 10^5 cells were analyzed with a flow cytometer (FACS Canto II; BD Biosciences, San Jose, CA, USA) using gates for forward and side scatter to collect signals of cell-associated fluorescence. An isotype control was used to monitor non-specific binding of antibodies. Data were analyzed with FACSDiva software.

**Cytokine production by draining lymph nodes**  Cytokine production by lymph nodes upon skin sensitization to FITC was examined as described previously. In brief, BALB/c mice were sensitized on days 0 and 7 with 160 μL of a 0.5% FITC solution on shaved forelimbs. The solvents were acetone alone, 50% DBP in acetone, and 20% DIA in acetone. Twenty-four hours after the second sensitization, brachial lymph nodes were collected and single-cell suspensions were prepared for each mouse. The lymph node cells were cultured for 72 h in RPMI 1640 supplemented with 10 mM HEPES (pH 7.2), 10% FBS and 30 μg/mL kanamycin at 37°C under a humidified atmosphere of 5% CO₂/95% air. Culture supernatants were collected at 48 and 72 h, and a pooled sample was made for each mouse. The concentrations of IL-4 and IFN-γ in
each sample were determined by means of a sandwich enzyme-linked immunosorbent assay (ELISA).\textsuperscript{24} Cytokines in the supernatants were captured with immobilized purified anti-IL-4 or anti-IFN-\(\gamma\) antibodies. The captured IL-4 and IFN-\(\gamma\) were detected using biotin-conjugated anti-IL-4 and anti-IFN-\(\gamma\) antibodies, respectively, and then with HRP-avidin (for IL-4) or HRP-avidin D (for IFN-\(\gamma\)). Recombinant IL-4 and IFN-\(\gamma\) were used to generate standard curves. As enzyme substrates, ABTS and H\textsubscript{2}O\textsubscript{2} were added, and then absorbance readings were made at 405 nm. The cytokine concentration of each sample was determined based on a dose–response curve generated with standard recombinant cytokines.

**Statistics** To detect significant differences between multiple groups, one-way ANOVA followed by Tukey's test was employed. Statistical analyses were performed using Graphpad Prism 5 (Version 5.02; Graphpad Software, San Diego, CA).

RESULTS

**Activation of TRPA1 by DIA** We previously demonstrated a correlation between TRPA1 activation and its adjuvant effect on FITC-induced CHS using various types of phthalate esters.\textsuperscript{17,19} Thus, we first examined the ability of an alternative plasticizer, DIA, to activate TRPA1. As a positive control, AITC (a TRPA1 agonist) was shown to induce dose-dependent calcium mobilization in TRPA1-CHO cells, whereas there was no increase in cytoplasmic calcium in T-REx CHO cells, which lack TRPA1 expression (Fig. 1A). DIA concentration-dependently induced calcium mobilization in TRPA1-CHO but not in T-REx cells (Fig. 1B). The DIA-induced calcium influx in TRPA1-CHO cells was inhibited by HC-030031, a TRPA1-specific antagonist (Fig. 1C).

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The concentration–response curves for calcium responses plotted against DIA concentrations shifted toward higher concentrations of DIA when increasing amounts of HC-030031 were used, while the maximal responses remained the same. These results are interpreted as representing competitive antagonism. The 50% effective concentration (EC$_{50}$) values for TRPA1 activation by AITC and DIA were calculated to be 0.22 µM and 37 µM, respectively.

<insert Fig. 1>

**Adjuvant effect of DIA** We then examined the adjuvant effect of DIA using an FITC-induced CHS mouse model. Mice were epicutaneously sensitized with FITC in the absence or presence of a plasticizer. As a positive control, 2% DBP was employed because not only 50% DBP but also 2% DBP was shown to induce a significant adjuvant effect in previous experiments.$^{19}$ The FITC-specific ear-swelling response was significantly enhanced when mice were sensitized with FITC in the presence of 2% DBP or 20% DIA, as compared with in ones sensitized with FITC in acetone (Fig. 2). The ear-swelling response peaked at 24 h and then decreased with time. The time courses were the same for DBP and DIA. For each time point, the ear-swelling response was significantly higher than that in the negative control group (acetone alone).

<insert Fig. 2>

**Facilitated DC-trafficking with DIA** We have demonstrated that one of the processes leading to enhanced sensitization to FITC with phthalate esters is an effect on
DC-trafficking to draining lymph nodes.\textsuperscript{14,17,19,21} FITC solutions with or without a plasticizer were applied to mouse skin, and draining lymph nodes were collected after 24 h. Cells presenting FITC and expressing a DC-marker (CD11c) were counted by flow cytometry. The presence of 2\% DBP or 20\% DIA in the solvent significantly increased the number of FITC\textsuperscript{+}CD11c\textsuperscript{+} cells in the draining lymph nodes, as compared with under the control (acetone alone) conditions (Fig. 3A and B). In independent experiments, we confirmed that 50\% DBP as well as 20\% DIA increased DC-trafficking (Fig. 3C).

<insert Fig. 3>

Enhanced cytokine production with DIA As another effect of phthalate esters, we have demonstrated enhanced production of IL-4.\textsuperscript{22} The essential roles of IL-4 and its signaling pathway in FITC-induced CHS have been reported.\textsuperscript{25} We then evaluated the cytokine production by draining lymph nodes during the sensitization process. Mice were epicutaneously sensitized with FITC twice on days 0 and 7 in the absence or presence of a plasticizer, 50\% DBP or 20\% DIA. Twenty-four hours after the second sensitization, draining lymph nodes were collected. The lymph node cell suspensions, which contained FITC-presenting DC and FITC-specific T-cells, were cultured. Culture supernatants were harvested at 48 and 72 h, and then examined for the accumulation of IL-4 and IFN-\(\gamma\).

As we reported previously, the presence of DBP during sensitization to FITC increased the production of IL-4, which accumulated over time. Under the acetone alone conditions, the IL-4 level was below the detection limit. The IL-4 level exceeded
the detection limit under the DIA conditions (Fig. 4A and B). However, it was not judged to be significantly high compared with that under the acetone alone conditions on multiple comparisons. DIA also facilitated IFN-γ production (Fig. 4C and D). The extents of enhancement of IFN-γ production were similar under the DBP and DIA conditions. IFN-γ production under the acetone alone conditions was low.

Pretreatment with a TRPA1 antagonist inhibited the adjuvant effect of DIA in vivo  As for DBP, pretreatment of mice with HC-030031 (a TRPA1 antagonist) was shown to inhibit the enhanced sensitization to FITC in the presence of 2% DBP. We then examined whether or not pretreatment with HC-030031 could also inhibit the sensitization to FITC in the presence of DIA. The ear-swelling response was higher in mice under the 20% DIA conditions as compared with under the acetone alone conditions, as expected. Under the 20% DIA conditions, HC-030031 treatment at 1 h before each sensitization significantly inhibited the ear-swelling response (Fig. 5).

DISCUSSION

In FITC-induced CHS mouse models, DBP has been empirically added to solvents for FITC. We have repeatedly shown that the sensitization activity of FITC is very low without DBP, however, no ear-swelling response occurred when mice were
treated with DBP in the absence of FITC. \(^{13,14}\) Thus DBP does not work as a hapten or non-specific inducer of inflammation but as an adjuvant.

We previously demonstrated there is a good correlation between the ability of TRPA1 activation and the adjuvant effect on FITC-induced CHS using various phthalate esters with different alkyl chain lengths. \(^{17}\) Thus, we started to examine whether or not DIA could activate TRPA1 in vitro. One of the reasons why we chose DIA is that it is widely used as an adipate ester-type plasticizer. Another reason for the choice is that relatively short chain-phthalate esters, such as DBP and di-\(n\)-propyl phthalate (DPP), efficiently activate TRPA1 and exert an adjuvant effect. In contrast, long chain-phthalate esters, such as di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DINP), not only have no effect or only a very weak one on TRPA1 activation, \(^{17}\) but also do not have an adjuvant effect on FITC-induced CHS. \(^{14}\) We demonstrated that DIA induced a calcium response in CHO-K1 cells stably transfected with a TRPA1-expression vector construct, whereas no such response was observed in cells without such a vector construct. A concentration–response curve revealed that \(EC_{50}\) of DIA was 37 \(\mu\)M. Our previous studies indicated that \(EC_{50}\) of DBP was 1.6 \(\mu\)M. \(^{19}\) The activation of TRPA1 by DIA was competitively inhibited by HC-030031, a TRPA1-specific antagonist.

The positive results as to TRPA1 activation by DIA prompted us to investigate the adjuvant effect on an FITC-induced CHS mouse model. As a positive control, DBP was commonly used at 50\% in past experiments, \(^{12,14}\) however, we previously found that 2\% DBP was sufficient to enhance sensitization. \(^{19}\) Because the efficacy of DIA is not known, 20\% (a dose between 2\% and 50\%) was chosen in the present experiments. To differentiate the effects of plasticizers on the sensitization and elicitation processes, all
mice were challenged with FITC under the same conditions, which included 50% DBP. The results indicated that 20% DIA facilitates sensitization to FITC.

The dose of 20% DIA was consistently used throughout the present study. This dose is not extremely out of range for human use. In fact, 1% DIA has been tested as a skin penetration enhancer in an ointment base for human use. To alleviate dry skin, undiluted ester-based oils including DIA have been tested for an emollient effect using human skin samples. In addition, toxicological studies revealed that 5 to 100% DIA caused minimal to mild irritation in skin irritation studies involving rabbits, and such results were also obtained on clinical testing with only moderate cumulative irritation, and no sensitization or photosensitization. Dose–response of DIA for the adjuvant effect should also be examined in a toxicological standpoint. Further studies are needed for the risk assessment of DIA.

As a mechanism underlying the enhanced sensitization, trafficking of DC from skin to draining lymph nodes was analyzed. FITC was painted on the skin with (2% DBP or 20% DIA) or without a plasticizer. FITC-incorporating CD11c⁺ DCs that had reached draining lymph nodes by 24 h after sensitization were counted with a flow cytometer. In independent experiments, 50% DBP was also used as a positive control representing the standard experimental conditions used in the past studies. The presence of 2% as well as 50% DBP significantly increased DC trafficking compared with acetone alone conditions. The presence of 20% DIA facilitated DC-trafficking as well. These results indicated that the presence of DIA augmented the supply of FITC-presenting DC in the draining lymph nodes during skin sensitization.

The facilitated DC trafficking suggests that a vigorous immune response is initiated in the draining lymph nodes after second epicutaneous treatment with FITC in the
presence of a plasticizer. We first examined IL-4 production in lymph nodes because IL-4 signaling is known to be essential in the FITC-induced CHS mouse model.\textsuperscript{25)} Furthermore, we previously demonstrated that 50% DBP enhanced IL-4 production in draining lymph nodes.\textsuperscript{22)} As expected from our previous study,\textsuperscript{22)} sensitization with FITC in the presence of 50% DBP induced a detectable level of IL-4, whereas FITC in acetone did not. In the case of 20% DIA, IL-4 accumulated over time to above the detection limit. However, the level of IL-4 accumulation under the DIA conditions was low.

IL-4 production was enhanced under 50% DBP conditions, while 20% DIA conditions yielded a significantly low amount of IL-4. In contrast, the elevated IFN-\(\gamma\) production was similar under the 50% DBP and 20% DIA conditions. The difference in the affinity to TRPA1 (as revealed by EC\(_{50}\)) could be reflected in this dichotomy. This may suggest some qualitative difference under 20% DIA conditions as opposed to 50% DBP conditions in terms of helper T cell polarization. The initiation of an immune response accompanied by a minimal level of IL-4 production may be sufficient to yield a CHS response. IFN-\(\gamma\) may possibly play a major role in CHS responses under 20% DIA conditions unlike under 50% DBP conditions.

The role of IFN-\(\gamma\) in FITC-induced CHS (in the presence of 50% DBP) may be unclear. In the case of STAT6-deficient mice defective in the IL-4-signaling pathway, FITC-induced CHS was reported to be completely abolished.\textsuperscript{25)} Recombinant IFN-\(\gamma\) was shown to inhibit FITC-induced CHS in wild-type mice, but the effect was partial and weaker than that of anti-IL-4 antibody treatment.\textsuperscript{25)} Early studies demonstrated that the level of IFN-\(\gamma\) was lower in FITC-induced CHS (50% DBP present) than that in dinitrochlorobenzene (DNCB)-induced CHS when draining lymph node T cells were
polyclonally activated with concanavalin A. However, no comparison was made between the conditions in the presence or absence of 50% DBP during sensitization to FITC. It is possible that under the conditions with a plasticizer, a sufficient amount of IL-4 is produced to initiate FITC-induced CHS despite that IFN-γ is simultaneously produced. On the other hand, under the acetone alone conditions, neither IL-4 nor IFN-γ is available due to the paucity of FITC-presenting DC in the draining lymph nodes.

We focused on the cytokine profiles in lymph nodes during the activation process of T helper effector cells. In terms of inflammatory cytokines in the skin, two cytokines need to be mentioned. First, production of thymic stromal lymphopoietin (TSLP), a cytokine that directs T helper type 2 (Th2) responses, produced by skin keratinocytes in response to DBP may be an interesting topic for study. Second, recent studies demonstrated that IL-31, which is produced by skin-homing Th2 effector cells, is involved in the T cell-dependent itch sensation in atopic dermatitis models. An important finding is that a receptor for IL-31 is expressed on subsets of sensory neurons that express TRPA1 or TRPV1. The genetic ablation of TRPA1 or TRPV1 decreased the IL-31-induced itch. This is another example demonstrating an important link between the immune and sensory nervous systems. However, it was not demonstrated that IL-31 could influence the skin sensitization process or that signaling through TRP channels is involved in transmission of the itch sensation. In addition, we have already demonstrated that DBP induced secretion of interleukin-1β from the skin. As for DIA, the role of skin cytokine production will be a subject of further studies. Involvement of TRPA1 activation in the production of skin cytokines is another subject to be studied.

Some immunogenic haptens have been shown to activate TRPA1 in vitro. For example, DNCB was shown to exhibit agonistic activity toward TRPA1, which is
inhibited by TRPA1 antagonists. However, this paper did not address the question of whether sensitization to DNCB was inhibited by \textit{in vivo} treatment with a TRPA1 antagonist. We have not tested the adjuvant effect of DBP using DNCB as an immunogenic hapten. However, we previously demonstrated that DBP did not enhance skin sensitization to dinitrofluorobenzene (DNFB), a hapten chemically related to DNCB. We have not measured the TRPA1 agonistic activity of FITC due to interference to a fluorometric calcium influx assay by the fluorescence of FITC. However, a related non-fluorescent hapten, phenethyl isothiocyanate (PEITC), could be tested. We found that PEITC is an agonist of TRPA1 using our fluorometric assay (unpublished results). We also demonstrated that DBP enhanced skin sensitization to PEITC. These results indicated the question as to what attributes of haptens are responsible for the enhanced sensitization in the presence of phthalate esters or DIA remains to be answered.

We further demonstrated that TRPA1 activation is involved in the elevated sensitization to FITC in the presence of DIA by means of a TRPA1-specific antagonist \textit{in vivo}. Mice exhibited a reduced ear-swelling response if they were given an intraperitoneal injection of an HC-030031 (4 mg/mouse) suspension at 1 h before sensitization with FITC in the presence of 20% DIA. As to the mechanisms, effect of HC-030031 on DC-trafficking and cytokine production should also be examined. Further studies are needed to confirm the link between TRPA1 activation and the initiation of immune responses.

Recent results also demonstrated the involvement of TRPA1 channels in the CHS response in TRPA1-deficient mice. The mechanisms downstream of TRPA1 activation leading to the enhancement of CHS are another subject for further studies.
Activation of TRPA1 may result in the release of neuropeptides from peripheral nerve endings.\textsuperscript{33} We previously demonstrated that treatment with an antagonist of calcitonin gene-related peptide (CGRP) at a skin site of sensitization to FITC (in the presence of DBP) suppressed the CHS response.\textsuperscript{21} Others have shown that CGRP promotes FITC-induced CHS by comparison with mice deficient in the signal transduction of CGRP.\textsuperscript{34} Another candidate is substance P, which could mediate the enhancing effect on CHS as a result of TRPA1 stimulation.\textsuperscript{32} However, it seems more likely that substance P acts during the elicitation phase of a CHS response. In this setting, substance P may act not only downstream of TRPA1 activation but also upstream of peripheral nerve stimulation.

Although it has been concluded that DIA is safe for use in cosmetics,\textsuperscript{10,11} our present results may suggest a potential connection of the topical use of DIA to CHS. In addition, adipate esters are widely used as skin penetration enhancers for therapeutics\textsuperscript{7,8} as well as for drug formulations.\textsuperscript{9} Various types of adipate esters are also practically used. Di(2-ethylhexyl) adipate (DEHA),\textsuperscript{35,36} dibutyl adipate,\textsuperscript{11} and diethyl adipate\textsuperscript{8} are examples. Contamination by a phthalate ester (DEHP) and an adipate ester (DEHA) of packaged food, such as curry paste, was simultaneously checked for and reported in Thailand.\textsuperscript{35} Safety assessment of alternative plasticizers needs to be conducted on a worldwide scale. Although modulation of the skin access route of sensitizers may also be important, as suggested for DBP,\textsuperscript{37} our present simple method will provide a useful means of surveying potential adjuvant chemicals.

In conclusion, TRPA1 activation was shown to be a good predictor of the enhancement of FITC-induced CHS by DIA. As underlying mechanisms for the enhancement of FITC-induced CHS, elevation of DC trafficking and cytokine...
production were shown to be involved. The results suggest that not only phthalate esters but also an alternative plasticizer comprising a di-carboxylic acid ester may have an adjuvant effect through the activation of TRPA1 channels.

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**Conflict of interest**  The authors declare no conflict of interest.
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Figure legends

Fig. 1. TRPA1-mediated calcium mobilization by DIA

(A and B) TRPA1-CHO (filled circles) or control T-REx CHO (open circles) cells were stimulated with various concentrations (abscissa) of AITC (A) or DIA (B). The percentages of the maximal calcium responses are shown on the ordinate. (C) TRPA1 antagonist HC-030031 inhibited DIA-induced calcium influx into TRPA1-CHO cells. The calcium responses to various concentrations of DIA (abscissa) were measured in the absence or presence of various concentrations of HC-030031 (shown in panel C). Each datum is the mean±standard error (n=4). Error bars underneath the symbols are not visible. Reproducibility was confirmed by two independent experiments.

Fig. 2. Skin sensitization to FITC was enhanced in the presence of DIA in a mouse FITC-induced CHS model

BALB/c mice were sensitized with 0.5% FITC dissolved in acetone in the presence of 20% DIA, 2% DBP (positive control), or acetone alone (negative control) on days 0 and 7. On day 14, the mice were challenged on an ear auricle with 0.5% FITC dissolved in 50% DBP in acetone. To differentiate the effects of plasticizers on the sensitization and elicitation processes, all mice were challenged with FITC under the same solvent. The ear-swelling responses were compared under the acetone alone (open circles; n=6), DBP (filled circles; n=6), and DIA (filled triangles; n=7) conditions. The ear-swelling responses at 24, 48 and 72 h after challenge in an individual mouse (each point) and
Biological and Pharmaceutical Bulletin

Means (horizontal bars) are shown. *P < 0.05, **P < 0.01, ***P < 0.001 compared with acetone alone conditions. Reproducibility was confirmed by two independent experiments.

Fig. 3. DIA enhanced DC-trafficking from skin to draining lymph nodes

(A) Representative cytograms showing DC trafficking. Twenty-four hours after skin application of 0.5% FITC in acetone containing 20% DIA or 2% DBP (positive control), or in acetone alone, cells in draining lymph nodes were analyzed for FITC and CD11c by flow cytometry. The numbers in the respective areas are the percentages of cells relative to total lymph node cells. (B) Summary of experiments shown in panel A. The percentages of FITC^+CD11c^+ cells relative to total CD11c^+ cells are shown. (C) Summary of experiments, in which the 50% DBP conditions were used as a control. In the panels (B) and (C), each bar represents the mean±standard error for three experiments (under some conditions, the error bars are too short to see). ***P < 0.001 compared with acetone alone conditions. Reproducibility was confirmed by five sets of independent experiment.

Fig. 4. Cytokine production by draining lymph node cells after skin sensitization with FITC in the presence of DIA.

Mice were sensitized with 0.5% FITC dissolved in acetone in the presence of 20% DIA or 50% DBP (positive control), or in acetone alone on days 0 and 7. Twenty-four hours after the second sensitization, brachial lymph nodes were collected and single cell
suspensions were prepared. The lymph node cells were cultured for 48 h (A, C) or 72 h (B, D), and then IL-4 (A, B) and IFN-γ (C, D) in the culture supernatants were quantitated by means of sandwich ELISA. Bars represent the means±standard error (n=4). *P < 0.05, **P < 0.01, ***P < 0.001, NS (not significant) compared with acetone alone conditions. Reproducibility was confirmed by two independent experiments.

Fig. 5. A TRPA1 antagonist, HC-030031, inhibited an enhanced sensitization to FITC with DIA

Mice were given an intraperitoneal injection of methylcellulose (vehicle) alone or HC-030031 in methylcellulose (4 mg/mouse) on days 0 and 7. Mice were sensitized with 0.5% FITC dissolved in acetone alone or acetone containing 20% DIA. FITC-sensitization was performed 1 h after antagonist injection. On day 14, the mice were challenged on an ear auricle with 0.5% FITC dissolved in 50% DBP in acetone. The ear-swelling responses were compared under methylcellulose/acetone (M F/A; open circles; n=6), methylcellulose/DIA (M F/DIA; filled triangles; n=6), and HC-030031/DIA (HC F/DIA; open triangles; n=7) conditions. The ear-swelling responses at 24 h (A), 48 h (B), and 72 h (C) after challenge in an individual mouse (each point) and means (horizontal bars) are shown. *P < 0.05, **P < 0.01, ***P < 0.001 compared with methylcellulose/DIA (M F/DIA) conditions. Reproducibility was confirmed by two independent experiments.
Fig. 1.

A) AITC

B) DIA

C) DIA + HC-030031
Fig. 2.

A) 24 h

B) 48 h

C) 72 h

Ear swelling (μm)

Acetone  DBP  DIA
Fig. 3.

A) 

B) 

C)
Fig. 4.

A) 48 h

IL-4 (pg/mL)

Acetone  DBP  DIA

*** NS

B) 72 h

IL-4 (pg/mL)

Acetone  DBP  DIA

*** NS

C) 48 h

IFN-γ (pg/mL)

Acetone  DBP  DIA

* **

D) 72 h

IFN-γ (pg/mL)

Acetone  DBP  DIA

* **
Fig. 5.