Skin Sensitization to Fluorescein Isothiocyanate is Enhanced by Butyl Paraben in a Mouse Model

Takeshi Matsuoka,a Yukina Endo,a Kohta Kurohane,a and Yasuyuki Imai*,a

a Laboratory of Microbiology and Immunology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka City, Shizuoka 422-8526, Japan

* To whom correspondence should be addressed. e-mail: imai@u-shizuoka-ken.ac.jp
Summary

Contact hypersensitivity (CHS) to preservatives is receiving increased attention. Parabens are widely used in foods, pharmaceutics and cosmetics as preservatives. The skin sensitizing activity of parabens remains controversial but a few investigations have been made as to whether parabens could facilitate sensitization to other chemicals. We have shown that di-\textit{n}-butyl phthalate (DBP), a phthalate ester, has an adjuvant effect in a fluorescein isothiocyanate (FITC)-induced CHS mouse model. We have also demonstrated that DBP activates transient receptor potential ankyrin 1 (TRPA1) cation channels expressed on sensory neurons. Comparative studies of phthalate esters revealed that TRPA1 agonistic activity and the adjuvant effect on FITC-CHS coincide. Here we focused on two commonly used parabens, butyl paraben (BP) and ethyl paraben (EP), as to their adjuvant effects. BALB/c mice were epicutaneously sensitized with FITC in acetone in the presence or absence of a paraben. Sensitization to FITC was evaluated as the ear-swelling response after FITC challenge. BP but not EP enhanced skin sensitization to FITC, but the effect of BP was much weaker than that of DBP. Mechanistically, BP enhanced the trafficking of FITC-presenting CD11c$^+$ dendritic cells (DCs) from the skin to draining lymph nodes as well as cytokine production by draining lymph nodes. When the TRPA1 agonistic activity was measured with a cell line expressing TRPA1, BP exhibited higher activity than EP. The present study provides direct \textit{in vivo} evidence that BP causes sensitization to other chemicals by means of a mouse FITC-CHS model.

Key words  butyl paraben; dibutyl phthalate; adjuvant; contact hypersensitivity; transient receptor potential ankyrin 1; mouse
Parabens, having antimicrobial activities, are widely used as preservatives in foods, pharmaceutics, cosmetics and other goods in daily use. Commonly used compounds are methyl, ethyl, propyl and butyl parabens, and mixtures of them. They are considered to have weak sensitization properties that may lead to the contact allergies. Retrospective studies involving skin patch test in Europe revealed that the proportion of adults sensitized to paraben mixtures exceeded 1% of subjects, however, a more recent similar study suggested that sensitization to parabens is rare. To summarize the current situation, the skin sensitization potency of preservatives is gaining increasing attention while the prevalence of contact allergies to parabens has remained unchanged over the last decade.

Besides direct sensitization to parabens, parabens may enhance the sensitization to other allergens. The odds of aeroallergen sensitization were reported to be significantly high in persons with elevated urinary concentrations of propyl and butyl parabens. Mechanistically, activation or maturation of dendritic cells (DC) during co-culture of human keratinocytes and DC-like cells has been evaluated in an assay named the loose-fit coculture-based sensitization assay (LCSA). With this assay, butyl paraben (BP) exhibited a strong while ethyl paraben (EP) exhibited a weak effect on DC maturation. The authors discussed that the sensitization potencies of parabens are correlated with their side chain lengths.

Although a topic from a different research area, a study on parabens as to the sensation of nociceptive stimuli has been carried out. The results demonstrated that methyl paraben caused the activation of transient receptor potential (TRP) A1 cation channels expressed on sensory neurons. They also demonstrated that methyl, ethyl, propyl and butyl parabens also increased intracellular Ca\(^{2+}\) in HEK293 cells expressing TRPA1. However, the relative ability to activate TRPA1 of different types of parabens has not been investigated.

We have been focusing on enhanced skin sensitization by several types of phthalate esters
by means of a fluorescein isothiocyanate (FITC)-specific contact hypersensitivity (CHS) mouse model. In this model, di-$n$-butyl phthalate (DBP) facilitates sensitization to FITC. Importantly, DBP does not exhibit antigenicity but acts as an adjuvant for FITC. We have compared phthalate esters with different side chains as to their adjuvant effects. We found phthalate esters with side chains of carbon numbers C2 to C7 enhanced skin sensitization. Interestingly, TRPA1 activation by phthalate esters with C2 to C6 has also been demonstrated. We propose a correlation between the ability of TRPA1 activation and the adjuvant effect on FITC-CHS.

In this study, we compared BP, which may enhance the allergic responses to other antigens, and EP, which may have little effect, using the FITC-CHS model. We found that BP is more potent than EP as to the activation of TRPA1 using a cell line expressing TRPA1. BP but not EP exhibited an adjuvant effect on FITC-CHS through enhancement of DC-trafficking to the draining lymph nodes and of cytokine production by lymph nodes.

**MATERIALS AND METHODS**

**Chemicals and Reagents** Acetone, 2-2’-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), di-$n$-butyl phthalate (DBP; CAS No.: 84-74-2), dimethyl sulfoxide (DMSO), kanamycin and RPMI 1640 medium were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); butyl paraben (BP; CAS No.: 94-26-8) and ethyl paraben (EP; CAS No.: 120-47-8) from Tokyo Chemical Industry (Tokyo, Japan); FITC and Fluo 4-AM from Dojindo Laboratories (Kumamoto, Japan); and bovine serum albumin (BSA; fraction V) and probenecid from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium were purchased from Nissui Pharmaceuticals.
(Tokyo, Japan); fetal bovine serum (FBS) from Hyclone (South Logan, UT, U.S.A.); ionomycin from LKT Laboratories, Inc. (St Paul, MN, U.S.A.); and HC-030031 from Cayman Chemical (Ann Arbor, MI, U.S.A.). Phycoerythrin (PE)-conjugated hamster anti-mouse CD11c monoclonal antibodies (mAbs) (clone HL3; immunoglobulin G1 (IgG1)) and a PE-conjugated hamster IgG1 isotype control (clone G235-2356) were purchased from BD Biosciences (San Jose, CA, U.S.A); purified rat anti-mouse interferon-\(\gamma\) (IFN-\(\gamma\)) mAbs (clone AN-18), biotin-conjugated rat anti-mouse IFN-\(\gamma\) mAbs (clone R4-6A2), and recombinant mouse IFN-\(\gamma\) from BioLegend (San Diego, CA, USA); purified rat anti-mouse IL-4 mAbs (clone 11B11), biotin-conjugated rat anti-mouse IL-4 mAbs (clone BVD6-24G2), and recombinant mouse IL-4 from eBioscience (San Diego, CA, U.S.A.); and horseradish peroxidase (HRP)-avidin from Life Technologies (Carlsbad, CA, U.S.A.).

**Cells**  
Chinese hamster ovary (CHO) cells expressing human TRPA1 (TRPA1-CHO) were used as described previously.\(^{10,11,14,15}\)

**Animals**  
Specific pathogen-free female BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan) at 7 weeks of age and held for 1 week before use for acclimatization. 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 and water *ad libitum*. Animal care and experiments were performed in accordance with guidelines for the care and use of laboratory animals published by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and those of the University of Shizuoka. The plans of animal studies were reviewed and approved (approval numbers: 146135, 166199 and 176237) by the Institutional Animal Care and Use Committee of the University of Shizuoka.

**TRPA1 Activation in Vitro**  
TRPA1-CHO cells were labeled with a calcium ion-sensitive fluorescent probe, Fluo 4-AM. After automatic addition of a test sample.
(prepared in DMSO) in the presence or absence of a TRPA1 antagonist, HC-030031, the intracellular Ca\textsuperscript{2+} level was measured over time with a fluorometric imaging plate reader, FLEXstation II (Molecular Devices, Sunnyvale, CA, U.S.A.) at 37°C as described previously.\textsuperscript{12–14} The maximal Ca\textsuperscript{2+} level was determined using 5 μM ionomycin.

**FITC-Induced CHS** Mouse contact hypersensitivity experiments involving FITC as a hapten were performed as described previously,\textsuperscript{9,16} with modifications. In brief, BALB/c mice were epicutaneously sensitized with 0.5% (w/v) FITC (160 μL) on shaved forelimbs on days 0 and 7. FITC was dissolved in one of the following solvents: acetone alone or acetone containing a test sample, i.e., BP, EP or DBP. On day 14, 0.5% FITC in acetone containing 10% DBP was applied on the right auricle to elicit inflammation, while the solvent alone was applied on the left auricle as a control. To distinguish the effect on the sensitization from the elicitation process, all mice were challenged with FITC under the same 10% DBP in acetone conditions. If the challenge was done in the absence of DBP, only a weak ear-swelling response was observed (unpublished results). To obtain enough responses upon challenge, we included 10% DBP in the solvent for elicitation in all experimental groups. Ear thickness was measured before and 24 h after elicitation by means of a dial thickness gauge (Mitutoyo, Kanagawa, Japan). Ear swelling is defined as the difference in the thicknesses of the right and left auricles as described previously.\textsuperscript{16}

**Dendritic Cell (DC) Trafficking** Trafficking of FITC-presenting DC from the skin to draining lymph nodes was examined as described previously.\textsuperscript{16} In brief, mouse forelimbs were epicutaneously treated with 0.5% FITC in acetone in the presence or absence of a test sample. Twenty-four hours after treatment, single-cell suspensions of brachial lymph nodes were prepared, and stained with PE-conjugated anti-CD11c mAbs (2 μg/mL) or isotype control mAbs (2 μg/mL). A total of $5 \times 10^5$ cells was analyzed with a flow cytometer (BD FACS
Canto II; BD Biosciences, San Jose, CA, U.S.A.). An isotype control was used to monitor non-specific binding of antibodies.\textsuperscript{9) Data were analyzed with FACSDiva software.

**Cytokine Production by Draining Lymph Nodes** Cytokine production by draining lymph nodes after FITC sensitization was determined as described previously.\textsuperscript{12,14,16) Mice were sensitized on forelimbs with 0.5% FITC in acetone in the presence or absence of a test sample on days 0 and 7. Twenty-four hours after the second sensitization, a single cell suspension of brachial lymph nodes was individually prepared from each mouse. The cells were cultured (2.5 × 10\textsuperscript{6} cells/mL) in the wells of flat-bottomed 96-well culture plates (Falcon\textsuperscript{®} #353072) for 72 h in RPMI 1640 supplemented with 10% FBS and 60 μg/mL kanamycin at 37°C under a humidified atmosphere of 5% CO\textsubscript{2}/95% air. Culture supernatants were collected at 72 h to measure the levels of IL-4 and IFN-γ. Cytokine levels were determined by a sandwich ELISA, in which two different mAbs were used to capture and detect each cytokine as described previously.\textsuperscript{16) The detection mAbs were biotin-conjugated ones, and their binding was revealed by means of HRP-avidin. Recombinant IL-4 and IFN-γ were used as standards.

**Statistics** Multiple comparisons were performed using one-way ANOVA followed by the Tukey test or Dunnett’s test with GraphPad Prism 7 (version 7.03; Graphpad software, San Diego, CA, U.S.A.). For experiments including data lower than the detection limits, Fisher’s exact test was employed to judge the difference between two groups.

**RESULTS**

**Butyl Paraben Activates TRPA1** The structures of DBP, BP and EP are shown in Fig. 1. DBP has two butyl side chains, while parabens have a single side chain and a hydroxyl
group on the benzene ring. BP has a butyl side chain while EP has an ethyl one.

There has been a report showing parabens activate TRPA1 cation channels.\textsuperscript{7} We compared the TRPA1 agonistic activities of BP and EP by means of an intracellular Ca\textsuperscript{2+} increase in TRPA1-CHO cells. BP caused a dose-dependent increase of intracellular Ca\textsuperscript{2+}, which was inhibited in the presence of a TRPA1-specific inhibitor, HC-030031 (Fig. 2A). TRPA1 activation was also observed for EP, but higher concentrations were required. TRPA1 activation by EP was also inhibited by HC-030031 (Fig. 2B). The 50\% effective concentration (EC\textsubscript{50}) values for BP and EP were 19 \(\mu\)M and 120 \(\mu\)M, respectively. These results indicate that both BP and EP cause TRPA1 activation, but BP has higher activity than EP.

**Effects of Butyl Paraben and Ethyl Paraben on Skin Sensitization to FITC**

Our previous studies revealed a correlation between TRPA1 activation and the adjuvant effect on FITC-CHS, phthalate esters with different side chain lengths being used.\textsuperscript{9,10} Because BP exhibits higher activity as a TRPA1 agonist, we expected BP might exhibit a stronger adjuvant effect than EP. Mice were epicutaneously sensitized with FITC in the presence of a test sample. After ear auricles of the sensitized mice had been treated with FITC, ear-swelling responses were measured at 24 h (peak response time). As a positive control, mice sensitized in the presence of 2\% DBP exhibited higher ear-swelling responses as compared with acetone controls (Fig. 3A). Because sensitization to FITC with neither 2\% BP nor 2\% EP increased the ear-swelling response (data not shown), we compared them at 20\% concentration. The ear-swelling responses of mice sensitized under 20\% BP conditions but not those under 20\% EP ones were significantly enhanced (Fig. 3A). Another set of experiments involving different concentrations of BP showed that 20\% but not 2\% BP increased the sensitization (Fig. 3B). These results indicated that BP but not EP exhibited an adjuvant effect on
**Butyl Paraben Enhanced DC-Trafficking to Draining Lymph Nodes** During the process of skin sensitization, antigen-presenting DC migration from the skin to draining lymph nodes is involved. We examined the appearance of FITC\(^+\)CD11c\(^+\) DC in the draining lymph nodes 24 h after FITC application on the skin by flow cytometry. In cytograms (Fig. 4A), signals from FITC\(^-\)CD11c\(^+\) cells reside in the area (a) and those from FITC\(^+\)CD11c\(^+\) in the area (b). As positive controls, FITC application with 2% or 20% DBP increased the number of FITC\(^+\)CD11c\(^+\) cells as compared with under the FITC in acetone alone conditions. The FITC\(^+\)CD11c\(^+\) cell trafficking was enhanced under the 20% BP conditions but not under the 20% EP ones as compared with under the acetone alone conditions (Fig. 4). It should be noted that the effect of BP was relatively weaker than that of DBP.

**Butyl Paraben Enhanced Cytokine Production by Draining Lymph Nodes** We next examined whether the increased DC-trafficking is reflected by the cytokine production by draining lymph nodes. Mice were sensitized with FITC twice in the presence of a test sample. Lymph node cells were collected 24 h after the second sensitization and then cultured. Cytokines accumulated during 72-h of culture were measured by ELISA. Lymph node cells from mice sensitized with FITC in acetone did not produce IL-4 or IFN-\(\gamma\) above the detection limit (3.9 pg/mL). As a positive control, mice sensitized with FITC in 2% DBP/acetone exhibited significant production of IL-4 and IFN-\(\gamma\). In the case of the 20% BP conditions, five of six mice produced IL-4 (Fig. 5A), and six of six mice produced IFN-\(\gamma\) (Fig. 5B) above the detection limits. Under the 20% EP conditions, IL-4 production in none of six mice, and IFN-\(\gamma\) production in two of six mice were above the detection limits. These results indicated that significant elevation in the cytokine production by lymph nodes was observed in the presence of BP. This is consistent with the finding that the 20% BP conditions but not the
20% EP ones yielded elevated ear-swelling responses.

DISCUSSION

Parabens are preservatives that may be a cause of contact hypersensitivity, although this is still controversial. In addition to a direct role as allergens, it should be noted that some forms of paraben could act as adjuvants during sensitization to other allergens. Parabens commonly used as preservatives have a short side chain such as methyl, ethyl, propyl, and butyl parabens. Among them, an association was reported between the urinary levels of propyl paraben as well as BP and an increase in the odds of aeroallergen sensitization. The aeroallergens tested in that study included ones of animal, plant, arthropod and fungal origin. Sensitization was judged as to the presence of serum IgE against aeroallergens.

Regarding experimental studies of the adjuvant effects of chemicals, we have demonstrated that some phthalate esters enhance skin sensitization to FITC in a mouse model. Interestingly, both the adjuvant effect on FITC-CHS and the agonistic activity toward TRPA1 nociceptive cation channels were observed for several phthalate esters with short side chains. In the present study on parabens, we demonstrated that BP is more potent than EP as to the activation of TRPA1. In addition, we showed that BP but not EP exhibited an adjuvant effect on FITC-CHS.

As to TRPA1 activation, the EC50 value for methyl paraben was reported to be 4.4 mM. In the present study, EC50 was determined to be 19 μM for BP and 120 μM for EP. The results indicated that BP exhibits higher affinity towards TRPA1 than EP does. In addition, concentration–response curves plotted against concentrations of EP or BP shifted toward high concentrations of parabens with the increasing concentrations of HC-030031, a TRPA1
antagonist. These results suggest that HC-030031 competitively inhibits EP- and BP-induced TRPA1 activation. With 10 μM of HC-030031, the effect of EP but not that of BP was completely inhibited. This may be due to that BP has higher affinity than EP toward TRPA1 as revealed by a lower EC$_{50}$ value for BP. Thus, 10 μM HC-030031 is not sufficient to inhibit the effect of BP of high concentrations tested in this study. We do not have mechanistic explanations for steeper concentration–response curves of BP in the presence of HC-030031 at present. The shape of concentration–response curves in the presence of HC-030031 could vary with agonists. We observed steeper curves in the presence of HC-030031 when allyl isothiocyanate was used as an agonist.$^{11}$

The ability of TRPA1 activation is reflected by the adjuvant effect on FITC-CHS. In the case of phthalate esters, the EC$_{50}$ for DBP was shown to be 7.3 μM$^{15}$ indicating that DBP has much higher affinity than BP. In this regard, the relative weaker adjuvant effect of BP than that of DBP is consistent with low affinity towards TRPA1 (Fig. 3). However, our previous study demonstrated that butyl benzoate (BBz) has an EC$_{50}$ of 87 μM.$^{15}$ Despite this, BBz exhibited a strong adjuvant effect that is comparable to that of DBP.$^{15}$ The relatively weak adjuvant effect of BP and the lack of an effect of EP suggested that other factors contribute to the adjuvant effect on FITC-CHS. One possibility is that the presence of a hydroxyl group on the benzene ring might attenuate the adjuvant effect in vivo, but has no effect on TRPA1 activation in the case of parabens.

We then examined the mechanistic basis of the adjuvant effect of BP and the lack of such an effect of EP. First, after skin application of FITC, DCs that present FITC haptens need to migrate toward draining lymph nodes to initiate helper T cell activation. Because FITC exhibits fluorescence, FITC-presenting DCs can be detected in draining lymph nodes after staining with PE-labeled anti-CD11c antibodies (DC marker) with a flow cytometer. As
expected from the adjuvant effect, DBP significantly facilitated DC migration, at both 2% and 20% concentration, as compared with FITC in acetone (Fig. 4). In the case of 20% BP, a weak but significant increase in DC migration was observed. On the other hand, 20% EP did not enhance the DC migration as compared with FITC in acetone conditions. These results are consistent with that BP but not EP exhibits an adjuvant effect on FITC-CHS.

The positive effect on DC-trafficking of BP but not of EP reminds us of an in vitro study involving human keratinocytes and a DC-like cell culture testing the effects of parabens on DC maturation. In that setting, a strong effect on DC maturation of BP but a weak effect of EP were reported. The stronger effect of BP on the DC maturation, which will contribute to the T cell activation, coincided with our in vivo results showing an enhanced DC trafficking with BP.

After a second FITC application on the skin, FITC-specific memory T cells and FITC-presenting DC meet within the draining lymph nodes to initiate immune responses. To evaluate such immune responses, accumulating cytokine levels in lymph node cell cultures were measured. In response to a supply of FITC-presenting DC, IL-4 and IFN-γ were produced under the conditions with DBP (Fig. 5). In contrast, no cytokine production above the detection limit was observed for FITC-sensitization under acetone alone conditions. This assay demonstrated that BP significantly enhanced cytokine production while EP did not. These results are consistent with the supply of FITC-presenting DC from the skin (Fig. 4), and the resulting ear-swelling responses after challenge (Fig. 3).

FITC-CHS is believed to be a type 2 helper T (Th2) cell-driven mouse model as long as 50% DBP is included in the solvent for the skin sensitization with FITC. IL-4 production is a typical feature of Th2 responses. This is supported by the lack of sensitization in signal transducer and activator of transcription 6 (STAT6)-deficient mice, which have a defect in
Th2 differentiation.\textsuperscript{17} However, diisopropyl adipate (DIPA; an alternative plasticizer) was found to induce only marginal IL-4 but abundant IFN-γ production by draining lymph nodes in BALB/c mice.\textsuperscript{12} In the present study, DBP was confirmed to augment both IL-4 and IFN-γ production (Fig. 5). The effect of BP was weak on both IL-4 and IFN-γ production, but all individual mice produced IFN-γ above the detection limit but not all mice did so in the case of IL-4 (Fig. 5). This may imply that BP exhibits an adjuvant effect, which is more similar to DIPA than DBP.

A literature reported that methyl, ethyl, propyl, isopropyl, butyl, isobutyl and benzyl parabens are used in cosmetic products.\textsuperscript{18} Upper limit of concentrations are suggested to be 0.4% as single paraben and 0.8% as mixtures of parabens. The limit value of 0.4% was also adopted for EP and BP in another literature.\textsuperscript{19} Cosmetic, Toiletry and Fragrance Association (CTFA) submitted data to the Cosmetic Ingredient Review (CIR) Expert Panel in 2003, in which BP was found to be used up to 0.54% and EP up to 0.98%.\textsuperscript{18} Our mouse model demonstrated that 20% but not 2% BP exhibited an adjuvant effect and EP did not show it even at 20%. Actual conditions of paraben usage may suggest that parabens would not pose a risk that enhance contact hypersensitivity. As to relative risks, one can estimate that BP has a higher risk than EP from our results. However, when parabens are used in combination with other chemicals that activate TRPA1, parabens may also contribute to the adjuvant effect to some extent. For example, structurally related BBz exhibited an adjuvant effect at 2%.\textsuperscript{15} Therefore, the risk for adjuvant effect should be collectively considered as a mixture of chemicals beyond a specific group such as parabens.

In conclusion, we obtained direct \textit{in vivo} evidence that BP may facilitate sensitization to other chemicals by means of a mouse FITC-CHS model. The effect of BP was mechanistically supported by the enhancement of DC-trafficking from the skin to draining
lymph nodes as well as that of cytokine production by lymph nodes. Among parabens suspected to have skin sensitization activity, BP but not EP showed an adjuvant effect. For the two parabens, TRPA1 agonistic activity was shown to correlate with the adjuvant effect.

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Conflict of Interest The authors declare no conflict of interest.
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Figure Legends

Fig. 1. Structures of Parabens and Di-n-butyl Phthalate

Di-n-butyl phthalate (DBP), butyl paraben (BP), and ethyl paraben (EP) were used in this study.

Fig. 2. TRPA1 Activation is Stronger with Butyl Paraben (BP) than with Ethyl Paraben (EP)

Various concentrations (abscissa) of BP (A) or EP (B) were added to TRPA1-CHO cells. The increase in intracellular Ca$^{2+}$ was recorded in the presence or absence of a TRPA1 antagonist, HC-030031, at the indicated concentrations. The percentages of the maximal calcium level (ionomycin) are shown on the ordinate. Each datum is the mean ± standard error ($n = 4$). Error bars underneath the symbols are not visible.

Fig. 3. Adjuvant Effect of Butyl Paraben on Skin Sensitization to FITC

(A) BALB/c mice ($n = 6$) were sensitized with 0.5% FITC in acetone (open circles, negative control), or in acetone with 2% DBP (open diamonds, positive control), 20% BP (open squares), or 20% EP (open triangles) on days 0 and 7. (B) Another series of experiments showing a dose response of BP regarding the adjuvant effect. The sensitization conditions were FITC in acetone (open circles), or in acetone with 2% DBP (open diamonds), 2% BP (closed squares), or 20% BP (open squares). On day 14, the mice were challenged on an ear auricle with 0.5% FITC in acetone containing 10% DBP. The ear-swelling response at 24 h after challenge in an individual mouse (each point) and the means (horizontal bars) are
shown. Statistical significance was tested by ANOVA followed by the Tukey test. 

\[ ***P < 0.001, ****P < 0.0001, \text{ns (not significant)} \]

compared with FITC in acetone conditions.

Fig. 4. Increased DC-Trafficking Induced by Butyl Paraben

Twenty-four hours after skin application of 0.5% FITC in acetone alone, or in acetone containing 2% DBP, 20% DBP, 20% BP or 20% EP, cells in draining lymph nodes were analyzed as to the levels of FITC (abscissa) and CD11c (ordinate) with a flow cytometer. (A) Representative cytograms. Cells in the area (a) are assigned to FITC−CD11c+ cells, and those in the area (b) are assigned to FITC+CD11c+ cells. Thus, cells in the areas (a) or (b) belong to total CD11c+ cells. (B) Summary of experiments. The percentages of FITC+CD11c+ cells relative to total CD11c+ cells are shown (means ± SEM, \( n = 3 \)). Statistical significance was tested by ANOVA followed by Dunnett’s test. *\( P < 0.05; **P < 0.01; \) ns (not significant) compared with FITC in acetone conditions. Reproducibility was confirmed by two independent experiments.

Fig. 5. Cytokine Production by Draining Lymph Nodes after Skin Sensitization to FITC with Butyl Paraben

BALB/c mice were sensitized with 0.5% FITC in acetone containing 20% BP (open squares), 20% EP (open triangles), or 2% DBP (open diamonds; positive control), or in acetone alone (open circles; negative control) 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 72 h, and then the concentrations of IL-4
(A) and IFN-γ (B) in the supernatants were determined by ELISA. Each data point corresponds to the result for an individual mouse. Data were collected through two experiments and pooled (n = 6). Bars represent the means for the data exceeding the detection limit (horizontal broken lines) in each group. The statistical significance between acetone conditions and 20% BP conditions was examined by means of Fisher’s exact test. * $P < 0.05$; ** $P < 0.01$. 
Fig 1.

DBP

BP

EP
Fig 2.

(A) butyl paraben

(B) ethyl paraben
Fig 3.

(A) Ear swelling (µm)

(B) Ear swelling (µm)
Fig 4.

(A) acetone 2% DBP 20% DBP 20% BP 20% EP

(B) % FITC$^+$ CD11$c^+$ cells relative to total CD11$c^+$ cells

** ** * ns
Fig 5. 

(A) IL-4

(B) IFN-γ