

Effects of Phthalate Esters on Dendritic Cell Subsets and Interleukin-4 Production in Fluorescein Isothiocyanate-Induced Contact Hypersensitivity

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Abstract: Phthalate esters with short alkyl chains, such as di-ethyl (DEP), di-*n*-propyl (DPP), and di-butyl phthalate (DBP), have adjuvant effects on an FITC-induced contact hypersensitivity mouse model. The adjuvant effects of DPP and DBP are associated with enhanced trafficking of FITC-presenting CD11b⁺ dendritic cells (DC). DEP has relatively weak activity as to FITC-positive cell migration. Here we demonstrated that DBP and DPP also increased the number of FITC-positive CD8α⁺ DC in draining lymph nodes. We also found enhanced production of interleukin-4 in draining lymph nodes after FITC sensitization with DEP, DPP, or DBP, suggesting an additional adjuvant mechanism of phthalate esters.

Key words: Contact hypersensitivity, Dendritic cells, IL-4, Phthalate esters

Phthalate esters are commonly used in plastics such as polyvinyl chloride (PVC) as plasticizers. For example, they are used for vinyl flooring, wall coverings, blood transfusion bags, synthetic leather, soft PVC toys, solvents for cosmetics, and mosquito repellants. Some phthalate esters have been thought to be risk factors for allergies (4, 5), and to be substances that influence endocrine systems (11, 19). One phthalate ester, di-butyl phthalate (DBP), is generally used as a solvent for fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CS) models during the sensitization phase (13, 20). We previously demonstrated that DBP has an adjuvant effect through enhancement of trafficking of antigen-presenting dendritic cells (DC) from the skin to draining lymph nodes (10, 15, 20). Furthermore, we found that some other phthalate esters with short alkyl-chains, i.e. di-*n*-propyl phthalate (DPP) and di-ethyl phthalate (DEP), had adjuvant effects during the sensitization phase with FITC (10).

The presence of DBP or DPP markedly increased the number of FITC-positive DC (CD11c⁺) in the draining lymph nodes 24 hr after skin sensitization with FITC. More specifically, DBP and DPP increased the number

of FITC-positive myeloid DC or Langerhans cells (CD11c⁺/CD11b⁺) (10). However, a significant proportion of the FITC-positive cells in the draining lymph nodes was found to be CD11c⁺ but CD11b[−]. This population may represent resident DC in lymph nodes and may be involved in the uptake of FITC within the draining lymph nodes *in situ* (21). We did not determine whether the CD11c⁺ but CD11b[−] population belongs to another DC subset such as lymphoid DC or plasmacytoid DC (2, 9, 16) in our previous studies (10). Lymph node DC subsets have been classified into (i) CD11b⁺ CD207 (Langerin)⁺ DC (Langerhans cells), (ii) CD11b⁺ CD207[−] DC (myeloid DC), (iii) CD8α⁺ DC (lymphoid DC), and (iv) B220⁺ DC (plasmacytoid DC) (3, 17).

In this study, we examined whether the numbers of lymphoid DC and plasmacytoid DC were increased in the FITC-positive DC population in draining lymph nodes when FITC was applied in the presence of DBP or DPP. Specific pathogen-free female CD-1 (ICR) mice (8–12 weeks of age) from Japan SLC, Inc. (Shizuoka, Japan) were used. Animal care and experiments were performed according to the guidelines for the care and use of laboratory animals of the University of Shizuoka. FITC (DOJINDO, Kumamoto, Japan)

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Abbreviations: CS, contact hypersensitivity; DBP, di-butyl phthalate; DC, dendritic cells; DEP, di-ethyl phthalate; DPP, di-*n*-propyl phthalate; FITC, fluorescein isothiocyanate.

was dissolved at 0.5% (w/v) in acetone, acetone/DBP (A/DBP), or acetone/DPP (A/DPP). Acetone and phthalate esters were mixed in a ratio of 1:1 (v/v). DBP and DPP were purchased from Wako Pure Chemicals (Osaka, Japan) and Kanto Chemical (Tokyo), respectively. Mouse forelimbs were shaved using small animal clippers 1 day before sensitization. The FITC solution (160 μ l) was applied to the shaved forelimbs. After 24 hr, brachial lymph nodes were obtained. Single-cell suspensions were prepared in Dulbecco's MEM (Nissui Pharmaceutical, Tokyo) from lymph nodes of each mouse by gentle teasing using needles. After washing in PBS containing 0.1% BSA (Sigma, St. Louis, Mo., U.S.A.) and 0.1% NaN_3 (B-PBS), the lymph node cells (1×10^6) were incubated with 1 μ g of biotin-conjugated rat anti-CD8 α (clone 53-3.7, IgG2a; eBioscience, San Diego, Calif., U.S.A.), biotin-conjugated rat anti-B220 (RA3-6B2, IgG2a; eBioscience), or biotin-conjugated isotype control (clone R35-95, IgG2a; BD Biosciences, San Jose, Calif., U.S.A.) for 30 min at 4 C in 100 μ l of B-PBS. After washing in B-PBS twice, the cells were incubated with 0.2 μ g of R-phycoerythrin (PE)-conjugated anti-CD11c (clone HL3, hamster IgG1; BD Biosciences) and 0.12 μ g of PerCP-Cy5.5-conjugated streptavidin (BD Biosciences) for 30 min at 4 C in 100 μ l of B-PBS. Cells were washed in B-PBS twice and then examined with a flow cytometer (EPICS XL; Beckman Coulter) with appropriate filter sets, as follows: FITC (FL-1), PE (FL-2), and PerCP-Cy5.5 (FL-4). A total of 2×10^5 cells were examined using gates for forward and side light scattering to collect signals of cell-associated fluorescence.

Twenty-four hours after epicutaneous application of FITC, FITC-positive cells were detected in the draining lymph nodes. When FITC was applied in the presence of DBP or DPP, the percentage of FITC-positive cells in the draining lymph nodes was confirmed to be increased as compared with FITC in acetone (Fig. 1A). We previously demonstrated that FITC sensitization with DBP or DPP increased the number of FITC-positive CD11c $^+$ /CD11b $^+$ cells in draining lymph nodes (10). We examined whether FITC-positive cells in other DC subset also increased when DBP or DPP was used. Cells exhibiting FITC fluorescence above the background level (a relative fluorescence intensity of 10 in Fig. 1A) were gated, and then the expression of CD8 α or B220 together with CD11c was examined by three-color flow cytometry. The percentages of CD11c $^+$ /CD8 α $^+$ cells and CD11c $^+$ /B220 $^+$ cells relative to total FITC-positive cells were determined by comparison with the results of experiments involving an isotype control (Fig. 1B). For example, under A/DBP conditions, 15.2% of the FITC-positive cells were

found in the region of CD11c $^+$ /CD8 α $^+$ upon staining with anti-CD11c and anti-CD8 α (panel *e*), whereas 2.1% were found in the corresponding region upon staining with anti-CD11c and the isotype control (panel *d*). Thus 13.1% were taken to be the CD11c $^+$ /CD8 α $^+$ population. The number of FITC-positive CD11c $^+$ /CD8 α $^+$ cells per mouse was calculated for each condition by means of this population analysis, the percentage of FITC-positive cells in the draining lymph nodes (Fig. 1A), and the total lymph node cell number. The results of three independent experiments are summarized in Fig. 1C. The numbers of FITC-positive CD11c $^+$ /CD8 α $^+$ cells were significantly increased under A/DBP and A/DPP conditions as compared with acetone alone. The number of FITC-positive CD11c $^+$ /B220 $^+$ cells was also increased under A/DBP conditions. However, this population constitutes a minor subset of FITC-positive DC (Fig. 1B, panel *f*). There was an increase in FITC-positive CD11c $^+$ /B220 $^+$ cells under A/DPP conditions (Fig. 1C), but the increase was not statistically significant in the present experiments.

The results demonstrated that FITC was incorporated into lymphoid DC (CD8 α $^+$) and plasmacytoid DC (B220 $^+$) after epicutaneous application of FITC. The number of FITC-positive lymphoid DC in the draining lymph nodes increased in the presence of DBP or DPP. The increase in the number of FITC-positive lymphoid DC was much more evident under A/DBP conditions (Fig. 1C). The present results are in contrast to our previous finding that the increase in the number of FITC-positive myeloid DC or Langerhans cells (CD11c $^+$ /CD11b $^+$) was more evident with A/DPP than A/DBP (10).

Langerhans cells and myeloid DC are major antigen-presenting cells in the skin. These cells are thought to migrate from local skin sites to the draining lymph nodes after antigen presentation (12, 24). Lymphoid DC are sometimes reported to have tolerogenic activity (7, 8). Lymphoid DC are reported to induce a high level of T helper 1 (Th1)-type cytokines and to lead to Th1 differentiation (14, 18). Considering the fact that T helper 2 (Th2)-type response is required for the initiation of FITC-induced CS (6, 22, 23), the FITC-positive lymphoid DC could play regulatory roles. Under A/DPP conditions, the FITC-positive cell population included more myeloid DC (10) but less lymphoid DC (Fig. 1C) as compared with under A/DBP conditions. This difference may reflect that the adjuvant effect was much more pronounced under A/DPP than A/DBP conditions, as observed upon CS elicitation (10). The role of each FITC-presenting DC population in the sensitization may have to be directly determined by sorting each

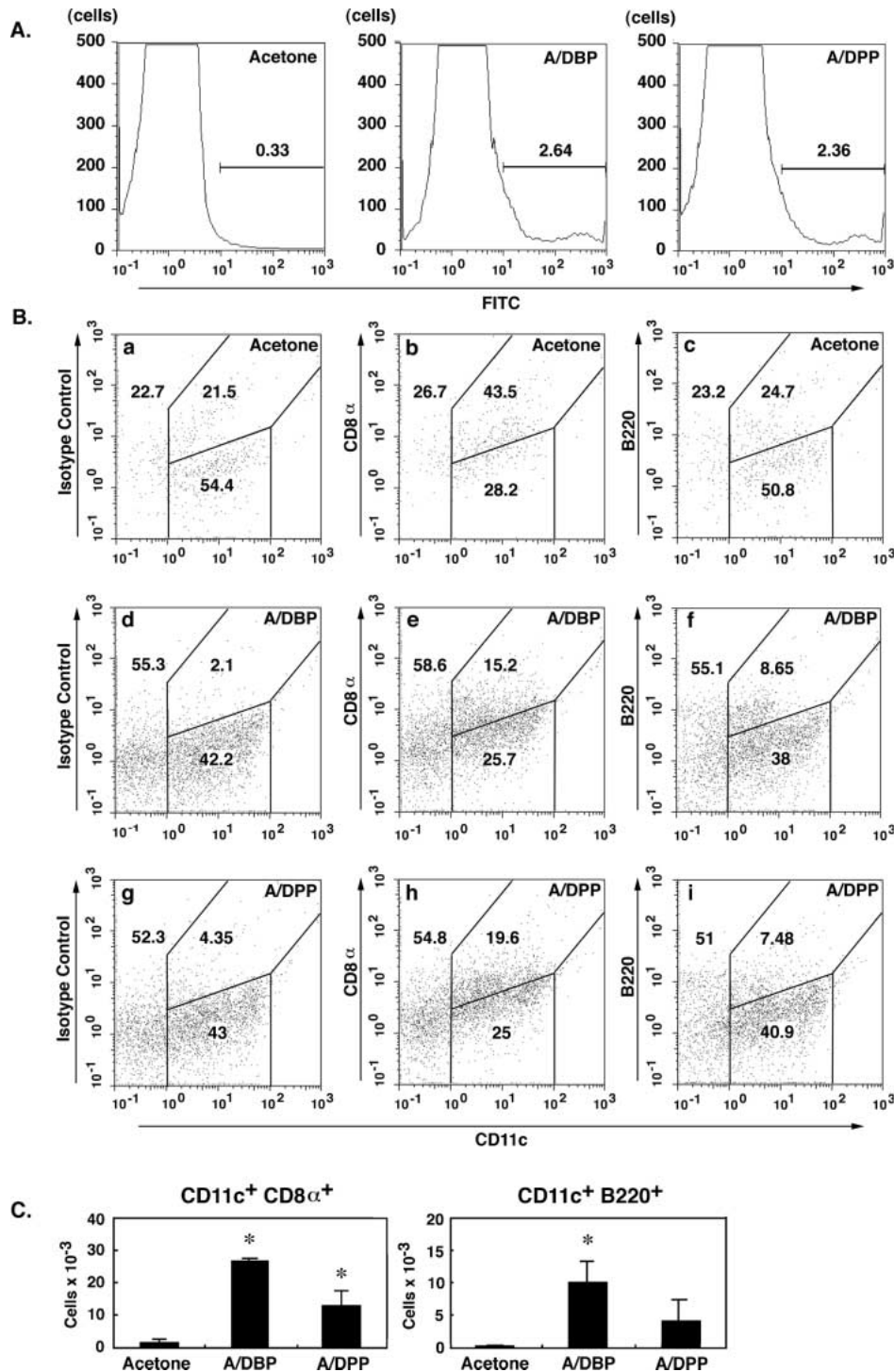


Fig. 1. Effects of phthalate esters on FITC-presenting DC subsets in draining lymph nodes. Brachial lymph nodes were prepared from individual ICR mice at 24 hr after epicutaneous sensitization with FITC in acetone, A/DBP, or A/DPP. The solvent conditions are given in each panel. (A) Appearance of FITC-positive cells in draining lymph nodes. Histograms of FITC fluorescence. The abscissa represents the fluorescence intensity on a log scale and the ordinate represents the cell number. The percentage of FITC-positive cells (>10 in fluorescence intensity) is indicated by a number. (B) Lymph node cells were stained with anti-CD11c and the isotype control (panels a, d, and g), anti-CD11c and anti-CD8 α (panels b, e, and h), or anti-CD11c and anti-B220 (panels c, f, and i). FITC-positive cells are gated and analyzed for CD11c (abscissa) together with CD8 α or B220 (ordinate) expression. The numbers in the panels are the percentages of cells in the gates relative to total FITC-positive cells. (C) Summary of the FITC-presenting DC subsets. Based on the results shown in (A) and (B), the number of FITC-positive CD11c⁺/CD8 α ⁺ cells (lymphoid DC) and CD11c⁺/B220⁺ cells (plasmacytoid DC) were calculated per mouse for each solvent condition. Each bar represents the mean $\pm$ SD of three independent experiments. Statistical significance (compared with the acetone control) was analyzed by means of one-way ANOVA followed by Dunn's test ($*P < 0.01$).

population. However, the percentage of FITC-positive DC in the draining lymph nodes was too small for us to isolate a sufficient number of DC for testing antigen-presenting activity directly. A main focus of our present study is the demonstration that DBP and DPP also increase the number of FITC-presenting DC subsets other than myeloid DC or Langerhans cells in the draining lymph nodes.

Lymphoid DC are thought to be non-migratory cells (1), and plasmacytoid DC have been found to enter lymph nodes from the blood circulation through high endothelial venules (25). That is, it is possible that FITC-conjugated self-antigens reach lymph nodes through afferent lymphatics, and then they are incorporated into lymphoid and plasmacytoid DC within the lymph nodes. Alternatively, migrating DC could transfer FITC-modified antigens to lymphoid DC (1). We can not exclude the possibility that FITC-conjugated self-antigens could be incorporated *in situ* by myeloid DC. Recent reports suggested such a possibility by showing that CD11b⁺ cells in lymph nodes incorporated FITC-dextran that had been injected intradermally 90 min beforehand (21). However, it was not determined whether or not those cells also expressed CD11c in that study.

Another question we could not address in our previous studies is that despite that DEP has some adjuvant effect, though less than those of DBP and DPP, it did not enhance FITC-positive DC trafficking efficiently (10). When FITC was epicutaneously applied under A/DEP conditions, the increase in the number of FITC-positive cells in the draining lymph nodes was confirmed to be relatively low as compared with under A/DBP or A/DPP conditions (data not shown). We assumed that DEP has some other activity that facilitates sensitization against FITC. Because the Th2 response has been shown to be required for the initiation of FITC-induced CS (6, 22, 23), we measured interleukin-4 (IL-4) production in the draining lymph nodes after epicutaneous application of FITC under different conditions. There is a report that IL-4 was detected in the culture supernatant of lymph node cells from FITC-sensitized mice after activation by concanavalin A (6). Another report indicated that the production of IL-4 was detected by ELISPOT assay in the draining lymph node cells from FITC-sensitized mice after *in vitro* pulse with FITC (23). However, these studies were performed under the standard condition of FITC sensitization: that is in the presence of DBP. No comparison had been made until we demonstrated that DBP facilitated the production of IL-4 from the draining lymph nodes (15). In the present study, we examined whether DEP and DPP could enhance IL-4 production as well.

Specific pathogen-free female BALB/c mice (8–12 weeks of age) were obtained from Japan SLC, Inc. FITC (160 µl of 0.5% solution) in acetone, A/DEP, A/DPP, or A/DBP was epicutaneously applied on mouse forelimb skin on days 0 and 7. On day 8, brachial lymph nodes were collected and pooled for each experimental group (3 mice per group). Single-cell suspensions were prepared. The lymph node cells (5×10^5 cells/200 µl/well) were cultured for 72 hr in the wells of a flat-bottomed 96-well culture plate (Falcon 3072) in RPMI 1640 (Nissui) supplemented with 10 mM HEPES (pH 7.2), 10% fetal bovine serum (FBS; Hyclone, South Logan, Ut., U.S.A.), and 30 µg/ml kanamycin at 37 C under a humidified atmosphere of 5% CO₂/95% air. After 72 hr incubation, the concentrations of IL-4 in the culture supernatants were determined by sandwich ELISA. The wells of an ELISA plate (Costar 9018; Corning, Inc., Corning, N.Y., U.S.A.) were coated with 0.1 µg of purified anti-IL-4 (clone 11B11; eBioscience) in 100 µl of PBS and then kept for 16 hr at 4 C. After washing in PBS containing 0.05% Tween 20 (PBS-Tween), 100 µl aliquots of culture supernatants or recombinant mouse IL-4 (eBioscience) diluted in 10% FBS-RPMI 1640 were added to the wells. After the plate had been incubated for 1 hr at 20 C, the wells were washed three times in PBS-Tween. The bound cytokines were detected with 0.1 µg of biotin-conjugated anti-IL-4 (clone BVD6-24G2; eBioscience) diluted in 100 µl of 0.1% BSA-0.05% Tween 20-PBS (BSA-TPBS). After the plate had been incubated for 1 hr at 20 C, the wells were washed three times in PBS-Tween. To detect the binding of biotinylated antibodies, 0.1 µg of horseradish peroxidase-avidin (Zymed, South San Francisco, Calif., U.S.A.) diluted in 100 µl of BSA-TPBS was added to the wells and then the plate was incubated for 30 min at 20 C. After the wells had been washed three times in PBS-Tween, 100 µl of 1 mM ABTS (Nacalai Tesque, Kyoto, Japan) dissolved in 0.1 M citrate buffer (pH 4.2) containing 0.03% H₂O₂ was added. After 30 min incubation at 20 C, absorbance readings were made with a microplate reader (TECAN SPECTRA Readers; TECAN, Salzburg, Austria) at 405 nm. The IL-4 concentration in each culture supernatant was determined based on a dose response curve generated with recombinant IL-4.

Mice were sensitized twice to generate FITC-specific memory T cells, and the draining lymph nodes were obtained 24 hr after the secondary sensitization. This protocol was chosen considering that both circulating FITC-specific T cells and FITC-presenting DC that had migrated from skin were present in the lymph node cell suspensions. After *in vitro* incubation for 72 hr, IL-4 accumulated in culture supernatants was determined.

When mice had been sensitized with FITC in acetone, no measurable IL-4 production was observed (<3 pg/ml). In contrast, lymph node cells from mice sensitized with FITC in A/DEP, A/DBP or A/DPP produced a significant and comparable amount of IL-4 (Fig. 2). It should be noted that comparable or slightly increased numbers of FITC-positive cells were found in the draining lymph nodes under the A/DEP conditions as compared with the acetone conditions (10). It is not clear what mechanisms are responsible for the increased IL-4 production under the A/DEP conditions at present. It could be systemic effects of DEP that influence the environment of lymph nodes or it could be enhanced trafficking of dermal macrophages that has been demonstrated under the A/DBP conditions previously (20).

In conclusion, FITC is incorporated into CD8 α^+ DC upon epicutaneous sensitization in the presence of DBP or DPP. The incorporation of FITC into B220 $^+$ DC was also notable with DBP. The enhancement of IL-4 production in draining lymph nodes may constitute an additional mechanism that explains the adjuvant effects

of phthalate esters including DEP, DPP, and DBP.

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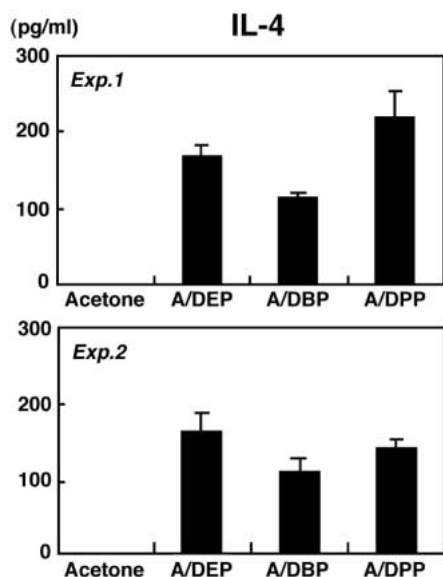


Fig. 2. Production of IL-4 in draining lymph node cells after skin sensitization with FITC in the presence of phthalate esters. BALB/c mice were sensitized on forelimb skin with FITC in acetone, A/DEP, A/DBP, or A/DPP on days 0 and 7. On day 8 (24 hr after sensitization), brachial lymph nodes (3 mice per group) were collected and single cell suspensions were prepared. After cell culture for 72 hr, IL-4 accumulated in the culture supernatants was determined by ELISA. Significant levels of IL-4 were detected after sensitization in the presence of phthalate esters while the amount of IL-4 was below the detection limit after sensitization with acetone alone. The results of two independent experiments were shown. Bars represent the means $\pm$ SD for triplicate cultures.

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