Dibutyl Maleate and Dibutyl Fumarate Enhance Contact Sensitization to Fluorescein Isothiocyanate in Mice

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In 1985, a dermatitis outbreak occurred in an envelope-making factory where di-n-butyl maleate (DBM)-containing polyvinyl acetate glue was used.1 In 2009, a case of allergic dermatitis due to occupational exposure to DBM was reported.2 A 47-year-old woman working as a batch mixer in a factory producing gelcoats and specialty paints developed eczematous lesions until she was withdrawn from work. Interestingly, among the North American Contact Dermatitis Group standard series, plastics, glues, adhesives as well as preservatives for oils and fats, but the risk of their use for dermatitis development has not been established.4 DBM, dibutyl maleate (DBM), widely used as a plasticizer for industrial application, has been reported to cause dermatitis in humans. DBM is a butyl alcohol ester of di-carboxylic acid that represents a part of the DBP structure, while di-n-butyl fumarate (DBF) is a trans isomer of DBM. We examined whether DBM or DBF exhibits an adjuvant effect like DBP does.5 When BALB/c mice were epicutaneously sensitized with FITC in the presence of DBM or DBF, the FITC-specific CHS response was enhanced, as we have observed for DBP. As to underlying mechanisms, DBM and DBF facilitated the trafficking of FITC-presenting CD11c+ dendritic cells (DCs) from skin to draining lymph nodes and increased the cytokine production by draining lymph nodes. In conclusion, DBM and DBF may have an effect that aggravates contact dermatitis through a skin sensitization process.

Key words dibutyl maleate; dibutyl fumarate; adjuvant; contact hypersensitivity; dendritic cell trafficking; cytokine production

In 1985, a dermatitis outbreak occurred in an envelope-making factory where di-n-butyl maleate (DBM)-containing polyvinyl acetate glue was used.1 In 2009, a case of allergic dermatitis due to occupational exposure to DBM was reported.2 A 47-year-old woman working as a batch mixer in a factory producing gelcoats and specialty paints developed eczematous lesions until she was withdrawn from work. Interestingly, among the North American Contact Dermatitis Group standard series, plastics, glues, adhesives as well as products in the workplace, DBM was the only chemical that gave a positive reaction in the patch test. DBM is a colorless, clear, and oily liquid used for industrial applications. DBM consists of an alkenyl di-carboxylic acid with two butyl alcohol esters of cis configuration.

Di-n-butyl fumarate (DBF) is an isomer of DBM with butyl alcohol esters of trans configuration. There has been no report on dermatitis caused by DBF. However, dimethyl fumarate (DMF) has been banned for the use in consumer products in the European Union (EU), because it caused an unprecedented large-scale dermatitis outbreak.5 DBM and DBF have been used as plasticizers, raw materials for polymer syntheses, and preservatives for oils and fats, but the risk of their use for dermatitis development has not been established.4 DBM represents a part of the structure of di-n-butyl phthalate (DBP), an aromatic dibutyl ester. DBP is widely used as a plasticizer for consumer products such as paints, varnishes, paper coatings and cosmetics.6,7 Our previous studies demonstrated that DBP contributed to contact hypersensitivity (CHS) to isothiocyanate hapten as adjuvant chemicals.8 Thus, we hypothesized that DBM might exhibit an enhancing effect through a similar mechanism as observed for DBP. We also examined whether or not DBF, an isomer of DBM, produced such an enhancing effect.

In this study, we investigated whether DBM and DBF could have an enhancing effect on a fluorescein isothiocyanate (FITC)-induced mouse CHS model. As underlying mechanisms, we examined the trafficking of FITC-presenting dendritic cells (DCs) from skin to draining lymph nodes as well as cytokine production by lymph nodes. The results showed that not only DBM but also DBF had an enhancing effect, which might contribute to the development of contact dermatitis. MATERIALS AND METHODS

Chemicals and Reagents Acetone, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), DBP (CAS No: 84-74-2), hydrogen peroxide, polyoxyethylene (20) sorbitan monolaurate (Tween 20), kanamycin sulfate, and RPMI 1640 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); DBF (CAS No: 105-75-9) and DBM (CAS No: 105-76-0) from Tokyo Chemical Industry (Tokyo, Japan); bovine serum albumin (BSA; fraction V) from Sigma-Aldrich (St. Louis, MO, U.S.A.); Dulbecco’s modified Eagle’s medium (DMEM) from Nissui Pharmaceuticals (Tokyo, Japan); fetal bovine serum (FBS) from Hyclone (South Logan, UT, U.S.A.); FITC from Dojindo Laboratories (Kumamoto, Japan); and pentobarbital from Kyoritsu Seiyaku Corporation (Tokyo, Japan). Phycocerythrin (PE)-conjugated hamster anti-CD11c monoclonal antibodies (mAb) (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-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, U.S.A.); 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, U.S.A.). Horse-radish peroxidase (HRP)–avidin was purchased from Zymed Laboratories, Inc. (San Diego, CA, U.S.A.).
Laboratories Inc. (South San Francisco, CA, U.S.A.).

**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 Law Concerning the Protection and Control of Animals, and with the guidelines for the care and use of laboratory animals of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and those of the University of Shizuoka. The plans of animal studies were reviewed and approved (approval numbers: 136031 and 146135) by the Institutional Animal Care and Use Committee of the University of Shizuoka.

**FITC-Induced CHS** FITC-induced contact hypersensitivity experiments were performed as described previously. Briefly, on days 0 and 7, mice were topically sensitized on shaved forelimb skin with an FITC solution (0.5% [w/v]) in acetone, DBM (2% [v/v] in acetone), DBF (2% [v/v] in acetone), and DBP (2% [v/v] in acetone). The concentration of 2% DBP has been determined as a minimum level of concentration that produces stable and reproducible results in the FITC-induced CHS. We chose the same concentration for other chemicals as well. On day 14, mice were challenged by painting with 20 µL of an FITC solution (0.5% [w/v] in acetone–DBP [1:1]) on the right auricle. The left auricle was painted with 20 µL of acetone–DBP (1:1) alone as a control. Ear thickness was measured with a dial thickness gauge (Mitutoyo, Kanagawa, Japan) before elicitation (0 h), and 24, 48, and 72 h after elicitation. Ear swelling at Xh is defined as follows: [thickness of the right ear – thickness of the left ear] at Xh – [thickness of the right ear – thickness of the left ear] at 0 h.

**DC-Trafficking** Trafficking of FITC-presenting DC from the skin to draining lymph nodes was examined as described previously. In brief, mice were epicutaneously treated with 160 µL of an FITC solution in acetone alone, or in acetone containing 2% DBM, 2% DBF or 2% DBP on forelimbs. Twenty-four hours after sensitization, brachial lymph nodes were collected and pooled for each condition to prepare single-cell suspensions. After washing in phosphate-buffered saline containing 0.1% BSA and 0.1% NaN₃, the cells were adjusted to 2×10⁶ cells/100 µL for staining with PE-conjugated anti-CD11c mAb (2 µg/mL) or isotype control mAb for 15 min at 4°C as described previously. After washing with the same buffer and filtering through a cell strainer (70-µm pore, nylon; Corning, Union City, CA, U.S.A.), a total of 5×10⁵ cells were analyzed with a flow cytometer (BD FACS Canto II; BD Biosciences, San Jose, CA, U.S.A.) using gates for forward and side scatters 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. The threshold for FITC-positive cells was set in such a way that no more than 0.01% of total lymph node cells exceed this threshold when lymph nodes were obtained from mice that had been treated with 2% DBP in acetone without FITC.

**Cytokine Production by Draining Lymph Nodes** Cytokine production by lymph nodes upon skin sensitization to FITC was determined as described previously. In brief, on days 0 and 7, BALB/c mice were sensitized on shaved forelimbs with 160 µL of an FITC solution under anesthesia. The solvents were acetone alone, or acetone containing 2% DBM, 2% DBF or 2% DBP. Twenty-four hours after the second sensitization, lymph node cells were collected, single-cell suspensions were prepared, and then the cells were cultured (2.5×10⁶ cells/mL) for 72 h in RPMI 1640 supplemented with 10% FBS and 60 µg/mL kanamycin at 37°C under a humidified atmosphere of 5% CO₂/95% air. Culture supernatants were collected at 24, 48, and 72 h to evaluate the production of IL-4 and IFN-γ.

**Cytokine Concentration Measurement** The concentrations of IL-4 and IFN-γ in each culture supernatant were determined by means of a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously. In brief, cytokines in the supernatants were captured with immobilized purified anti-IL-4 or anti-IFN-γ antibodies. To detect the captured IL-4 or IFN-γ, biotin-conjugated anti-IL-4 or anti-IFN-γ was applied followed by HRP-avidin. ABTS and H₂O₂ were used as enzyme substrates. Recombinant IL-4 and IFN-γ were used to generate standard curves. The cytokine level in each sample was determined from the respective standard curve.

**Statistics** Differences between the group treated with FITC in acetone and each experimental group were analyzed using one-way ANOVA followed by Dunnett's test with Graphpad Prism 5 (Version 5.02; Graphpad Software, San Diego, CA, U.S.A.).

**RESULTS**

**Effects of DBM and DBF on Skin Sensitization to FITC, as Revealed by the Ear-Swelling Test** The structures of DBM, DBF and DBP are shown in Fig. 1. To determine whether DBM and DBF enhance skin sensitization to FITC,
On days 0 and 7, BALB/c mice were sensitized with 0.5% FITC dissolved in acetone containing 2% DBM, 2% DBF or 2% DBP (positive control), or in acetone alone. One week after the second sensitization, mice were challenged on the right auricle with 0.5% FITC dissolved in 50% DBP in acetone, while 50% DBP in acetone was applied to the left auricle. To differentiate the effects of chemicals on the sensitization and elicitation processes, all mice were challenged with FITC in the presence of 50% DBP. The ear-swelling responses at 24 h (A), 48 h (B), and 72 h (C) after challenge were shown for each individual mice that had been sensitized with FITC in acetone alone (circles: n=6), in acetone–DBP (squares: n=7), in acetone–DBF (triangles: n=6), or in acetone–DBM (inverted triangles: n=6). Horizontal bars represent the means for the groups. The p values denote significance compared with the group sensitized with FITC in acetone alone. Reproducibility was confirmed by two independent experiments.

Compared with sensitization with FITC in acetone alone, sensitization with FITC in the presence of DBP (positive control), DBM or DBF caused an enhanced ear-swelling response (Fig. 2). The ear-swelling peaked at 24 h and then decreased with time. For each time point, the responses of the DBP, DBM and DBF groups were significantly higher than that of the acetone alone group.

Enhanced DC-Trafficking to Draining Lymph Nodes from Sensitized Skin in the Presence of DBM or DBF

Trafficking of FITC-bearing cutaneous DC to draining lymph nodes is involved in the initial phase of FITC-induced CHS. We have demonstrated that DBP facilitated DC trafficking from skin to draining lymph nodes. 8,10) Twenty-four hours after epicutaneous treatment with FITC in the presence of 2% DBP, 2% DBM or DBF, the number of FITC+CD11c+ cells in brachial lymph nodes was increased as compared with the FITC treatment with acetone alone (Fig. 3). The increase in DC trafficking was in the following order: DBP>DBM>DBF conditions.
Effect on Cytokine Production by Draining Lymph Nodes

The effect of DBP on the FITC-induced CHS has been related to polarization toward T helper type 2 (Th2) responses. In fact, we have demonstrated that FITC-sensitization in the presence of DBP enhanced IL-4 production by draining lymph nodes. However, our recent studies revealed that enhanced production of IFN-γ rather than IL-4 was involved in the adjuvant effect of an alternative plasticizer, diisopropyl adipate (DIA). To evaluate the roles of cytokines on FITC-sensitization in the presence of DBM or DBF, draining lymph nodes were collected 24 h after the second sensitization with FITC. At this point, FITC-primed T cells as well as FITC-presenting DC (Fig. 3) are present within the draining lymph nodes. The cytokine levels in the culture supernatants of the draining lymph node cells were compared (Fig. 4).

Under the FITC in 2% DBP-acetone condition, IL-4 as well as IFN-γ accumulated over time. In contrast, only a marginal level of IL-4 or IFN-γ was detected under the FITC in acetone condition at any time point. As for the FITC in DBM–acetone condition, the IL-4 level was significantly higher than that under the FITC in acetone condition in the 72 h supernatant. In contrast, the IFN-γ level was significantly higher under the FITC in DBM–acetone condition in both the 48 and 72 h supernatants. As for the FITC in DBF–acetone condition, IL-4 production was not significantly elevated, while IFN-γ was significantly higher in the 72 h supernatant.

DISCUSSION

Phthalate esters are used as plasticizers for industrial applications to impart flexibility to consumer products, especially those made of polyvinyl chloride. One of the phthalate esters, DBP, is ubiquitously present in environments, and its potential toxicity is gaining attention increasingly. Some reports have revealed the risk to reproductive health of the uptake of DBP. In addition, our previous studies have demonstrated that DBP plays the role of an adjuvant in an FITC-induced CHS mouse model. We hypothesized that DBM and DBF exposure might cause similar responses for the following reasons. Structurally, DBM and DBF consist of an aliphatic di-carboxylic acid with a single double bond with two butyl alcohol esters of cis and trans configuration, respectively (Fig. 1). The former represents a part of DBF, which is an aromatic dibutyl ester.

To test enhancing effect, BALB/c mice were epicutaneously sensitized with FITC in the absence or presence of 2% DBM, 2% DBF, or 2% DBP (positive control), and then FITC-dependent inflammation was measured as the ear-swelling response. In contrast to the low-level of sensitization with FITC in acetone, DBM and DBF enhanced the sensitization to FITC to similar levels of sensitization in the presence of DBP (Fig. 2). The enhanced ear-swelling responses were significantly elevated even on day 3 after elicitation. These results suggested DBM as well as DBF exhibited an enhancing effect on skin sensitization to other immunogenic chemicals such as FITC.

As a mechanism initiating the enhanced sensitization, we investigated the trafficking of FITC-presenting CD11c+ DC from skin to draining lymph nodes. Epicutaneous treatment with FITC in the presence of 2% DBM or 2% DBF facilitated DC-trafficking to a similar level of positive control, 2% DBP (Fig. 3). In FITC-induced CHS, in which 50% DBP is present in the solvent, production of IL-4 was shown to be required for sensitization by the lack of sensitization in signal transducer and activator of transcription 6 (STAT6) deficient mice. On the other hand, an alternative plasticizer, DIA, which has an adju-
vant effect on the FITC-induced CHS, augmented the produc-
tion of IFN-γ while marginal increase in the IL-4 production was observed.\(^\text{10}\) Thus, it is reasonable to assess the production of IL-4 as well as IFN-γ as markers following the hapten-
specific T cell activation.

We epicutaneously sensitized with FITC two times with one
week interval. Twenty-four hours after the second treatment, dr
aining lymph nodes are expected to contain both FITC-
presenting DC and FITC-specific helper T cells. Thus, cells from
draining lymph nodes were cultured and the cytokines
secreted into the supernatant were quantified by cytokine-
specific ELISA. We have repeatedly demonstrated that 50% DBP
enhanced the production of not only IL-4 but also IFN-
\(\gamma\).\(^\text{9,10}\) In the present experiments, we showed that 2% DBP also
enhanced the production of IL-4 as well as IFN-\(\gamma\) (Fig. 4).

Regarding IL-4, FITC-sensitization in the presence of 2%
DBM significantly augmented the IL-4 accumulation com-
pared with under the acetone alone condition as seen in the
72h supernatant. The IL-4 concentrations under the acetone
alone condition were marginal, which is consistent with the
paucity of FITC-presenting DC in draining lymph nodes and
with weak ear-swelling responses. The IL-4 level under the
2% DBF condition was above the detection limit at 72h,
but was not significantly higher than that under the acetone
alone condition. On the other hand, the IL-4 accumulation
under the 2% DBP condition was evident in 48 and 72h
supernatant. Thus, we think the order of IL-4 production is
DBP > DBM > DBF.

Regarding IFN-\(\gamma\), FITC-sensitization caused an early ac-
cumulation of IFN-\(\gamma\) in the supernatant, especially under the
2% DBP condition after 24h of culture. In the 48h superna-
tant, the 2% DBM but not the 2% DBF condition gave rise to
significant accumulation of IFN-\(\gamma\). In the 72h supernatant, the
2% DBM as well as the 2% DBF condition caused significant
production of IFN-\(\gamma\). Thus, we think the order of IFN-\(\gamma\) pro-
duction is also DBP > DBM > DBF.

It should be mentioned that the immunogenicity of DBM
and DBF to mouse might complicate the interpretation of the
enhanced cytokine production. Thus, cells from draining
lymph nodes could have produced cytokines due to the DBM
or DBF-specific immune responses rather than FITC-specific
ones. If this is the case, bystander immune responses in the
draining lymph nodes facilitated the FITC-specific response.
However, in preliminary experiments, when mice were epici-
taneously treated with 2% DBM in acetone twice followed by
elicitation with 2% DBM in acetone, we did not observe any
ear swelling response (data not shown). This may suggest the
lack of immunogenicity of DBM to mouse at least under our
current experimental conditions. Immunogenicity of DBM and
DBF to mouse should be evaluated in further experiments.

We do not exclude a possibility that physical properties of
DBM, DBF and DBP are involved in the enhanced sensitiza-
tion to FITC through an increase in skin retention and/or in
skin permeability. However, our previous results revealed
that lipophilicity does not necessarily determine the enhance-
ment. For example, di-n-heptyl phthalate (DHP) enhanced sensiti-
zation to FITC, but di(2-ethylhexyl)phthalate (DEHP) did not.\(^\text{8,17}\) DEHP has a similar but slightly higher value in the
octanol–water partition coefficient (\(log P_{\text{ow}}\)) than that of
DHP.\(^\text{18,19}\) Another example is diethyl phthalate (DEP),\(^\text{18,19}\)
DEP enhanced sensitization to FITC as well as IL-4 produc-
tion upon application to the skin with FITC.\(^\text{8,12}\) Log\(P_{\text{ow}}\) for
DEP is even lower than that of DIA, suggesting that molecules
with relatively low lipophilicity, such as DIA and DEP, can
enhance sensitization to FITC.\(^\text{8,10}\) We have demonstrated a
correlation of the adjuvant effect with the ability to activate
transient receptor potential ankyrin 1 (TRPA1) cation chan-
nels.\(^\text{10,17,20}\) We are currently under investigation whether DBM
and DBF activate TRPA1.

According to several case studies, frequent cases of der-
matitis occurred in factories making envelopes where DBM-
containing polyvinyl acetate glue was used.\(^\text{3}\) Another study
showed a case of allergic dermatitis reaction to DBM in a
factory that produced liquid resins containing DBM.\(^\text{21}\)
Considering a low-grade result in the patch test (+1 positive; light
erithma, nonvesicular) recommended by the International
Contact Dermatitis Research Group upon exposure to 5%
DBM, we suspected that DBM may not only function as a hapten by itself but may enhance sensitization to other haptons
through a combined effect.

Regarding the combined effects of chemicals, it is impor-
tant to know how these chemicals are used. DBM was also
studied as a promising oxygenating agent for diesel fuel.\(^\text{22}\)
As to transdermal drug delivery, incorporation of DBM into the
synthesis of a new type of membrane was shown to be useful
to achieve controlled drug release from the membrane.\(^\text{23}\)

Although 2% DBM may be a high dose as we regularly
contact in our daily life, we think it is not out of range for us
to contact. A transdermal therapeutic system such as a mem-
brane-stabilizing drug was reported to include DBM mon-
omers in a ratio by weight of 20%.\(^\text{23}\) Of course it is important
to assess the actual concentration of DBM leaching out from
such membrane. On the other hand, relatively high dose (5%)
of DBM was required to obtain a low-grade positive reaction
in a sensitized person with patch test,\(^\text{21}\) suggesting that a cer-
tain level of concentration is required in experimental settings.
Another report revealed that DBM was detected inside furni-
ture boxes with a disposable desiccant. The DBM concen-
trations in the box wall materials were reported to range from 29
to 720 mg/kg.\(^\text{24}\)

It is not known whether DBF induces contact allergies.
However, a similar diester with two methyl groups instead of
butyl ones, DMF, recovered from furniture upholstery, was
identified as a potent contact sensitizer.\(^\text{25}\) Thus, DMF was
banned from use in consumer products in the EU because of a
large-scale outbreak of dermatitis.\(^\text{3}\)

DBF is supposed to be a good control because it is the
trans isomer of DBM. In this study, DBF was also found to
enhance FITC-induced CHS. The results may also suggest
that other short chain fumarate diesters might enhance sensiti-
zation to other haptons.

In conclusion, FITC-induced CHS models revealed that a
combined effect of DBM or DBF with other immunogenic
haptons might contribute to an aggravation of contact derma-
titis through a sensitization process. As underlying mecha-
nisms, facilitated trafficking of FITC-presenting DC as well
as cytokine production by draining lymph nodes were shown.
Although more detailed quantitative analyses are needed to
estimate the relative risk, these results suggest that not only
phthalate esters but also alkenyl carboxylic acid diesters may
exhibit an enhancing effect regardless of whether of cis or
trans configuration.
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Conflict of Interest  The authors declare no conflict of interest.

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