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# The pilosebaceous unit—a phthalate-induced pathway to skin sensitization

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ABSTRACT

Allergic contact dermatitis (ACD) is caused by low-molecular weight compounds called haptens. It has been shown that the potency of haptens can depend on the formulation in which they are applied on the skin. Specifically the sensitization potency of isothiocyanates, a group of haptens which can be released from e.g. adhesive tapes and neoprene materials, increases with the presence of phthalates; however, the underlying mechanisms are not clear. A better understanding of the mechanisms governing the potency of haptens is important, e.g. to improve the risk assessment and the formulation of chemicals in consumer products. In this study we have explored phthalate-induced effects on the sensitization potency, skin distribution, and reactivity of fluorescent model isothiocyanate haptens using non-invasive two-photon microscopy to provide new insights regarding vehicle effects in ACD. The data presented in this paper indicate that the sensitization potency of isothiocyanates increases when applied in combination with dibutylphthalate due to a specific uptake via the pilosebaceous units. The results highlight the importance of shunt pathways when evaluating the bioavailability of skin sensitizers. The findings also indicate that vehicle-dependent hapten reactivity towards stratum corneum proteins regulates the bioavailability, and thus the potency, of skin sensitizers.



## Introduction

Allergic contact dermatitis (ACD) is a T-cell-mediated contact hypersensitivity reaction in the skin caused by low-molecular weight chemical compounds called haptens (Karlberg et al., 2008). Due to e.g. occupational exposure to haptens as well as exposure to haptens in consumer products, contact allergy has become a common health problem, affecting 15–20% of the population in the western world (Thyssen et al., 2007). The sensitization potency of haptens depends essentially on intrinsic chemical properties related to the molecular structure, e.g. reactivity, lipophilicity and molecular weight (Landsteiner and Jacobs, 1935; Gerberick et al., 2007); however, other factors, such as the vehicle in which the hapten is applied on the skin, can also have a significant effect (Kligman, 1966; Wright et al., 2001; Madsen et al., 2010).

Contact allergy caused by isothiocyanates has been reported following exposure to neoprene materials and adhesive tapes (Fregert et al., 1983; Samuelsson et al., 2011). Interestingly, isothiocyanates, e.g. phenyl isothiocyanate, fluorescein isothiocyanate (FITC), and rhodamine B isothiocyanate (RBITC), have been shown to be more potent sensitizers when applied in combination with phthalates, e.g. dibutylphthalate (DBP) (Dearman et al., 1996; Imai et al., 2006; Matsuda et al., 2010). It has been proposed that phthalates act as adjuvant, or alternatively affect the skin penetration; however, the mechanism has not been investigated in detail. Even though clinical evidence is lacking, it cannot be ruled out that phthalates could increase the risk of sensitization to isothiocyanates in consumer products. For example, phthalates are commonly used as plasticizers and solubilizing agents in plastics, drugs, and cosmetics (Hyun and Byung, 2004; Heudorf et al., 2007). Improved understanding of the vehicle-dependent sensitization potency of haptens is important to improve predictive testing and formulation of chemicals in consumer products.

In previous work, we have gained novel insights in the mechanisms involved in ACD with respect to the potency, uptake and distribution of fluorescent model haptens by the combination of sensitization experiments and non-invasive two-photon microscopy (TPM) (Samuelsson et al., 2009; Simonsson et al., 2011a; Simonsson et al., 2011b). The aim of the present study was to explore vehicle-induced effects regulating the sensitization potency of isothiocyanates, specifically when

*Abbreviations:* ACD, allergic contact dermatitis; DBP, dibutylphthalate; A:DBP, dibutylphthalate in acetone; DMSO, dimethylsulfoxide; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; LLNA, local lymph node assay; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; MS, mass spectrometry; PBS, phosphate buffer saline; PSU, pilosebaceous unit; RBITC, rhodamine B isothiocyanate; TPM, two-photon microscopy.

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applied in the presence of DBP, in vivo in mice. Vehicle-induced variations in sensitization potency were investigated using the murine local lymph node assay (LLNA) and the cutaneous absorption of the investigated isothiocyanates was visualized using TPM. Furthermore vehicle-induced variation in chemical reactivity of isothiocyanates was investigated.

The results reveal a phthalate-induced cutaneous absorption of hapten via the pilosebaceous units (PSU, Fig. 1), which could explain the enhanced sensitization potency of isothiocyanates. We also present data indicating that vehicle-dependent hapten reactivity towards stratum corneum proteins regulates the bioavailability, and thus the potency, of skin sensitizers.

## Materials and methods

*Mice.* Female CBA/Ca mice, Scanbur AB (Sollentuna, Sweden) were housed in HEPA-filtered air-flow cages and handled according to approvals by the local ethics committee.

*Chemicals and reagents.* Ammonium chloride, ammonium thiocyanate, DBP, DMSO, FITC, FITC-Dextran (70 kDa), Fluoromount aqueous mounting medium, formic acid, phosphate buffer saline (PBS), rhodamine B, RBITC, trichloroacetic acid, tris(hydroxymethyl)aminomethane, benzylamine, and Triton X were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Acetonitrile was purchased from Fischer Scientific (Lougborough, UK), fetal bovine serum (FBS) from VWR International (Stockholm, Sweden), and acetone from Merck (Darmstadt, Germany). Isoba Vet isofluran was purchased from Schering-Plough Animal Health (Boxmeer, The Netherlands), Domitor Vet from Orion Pharma (Espoo, Finland), Ketalar from Pfizer AB (Sollentuna, Sweden), and [methyl-$^3$H] thymidine from Amersham Biosciences (Little Chalfont, UK). Scintillation liquid was purchased from EcoLume INC Radiochemicals (Irvine, CA), Alexa Fluor 488 conjugated goat anti-rabbit IgG F(ab′)$_2$ fragments and normal goat serum from Invitrogen (Carlsbad, CA), and polyclonal lymphatic vessel endothelial hyaluronan receptor 1 (LYVE)-1 antibodies from Santa Cruz Biosciences (Santa Cruz, CA).

*Local lymph node assay (LLNA).* The sensitization potency of RBITC in acetone:DBP (A:DBP, 1:1, v/v) and RBITC in DMSO was investigated using a slightly modified version of the murine LLNA (OECD, 2002). Briefly, 4–5 groups of CBA/Ca mice (3 mice per group) were treated with RBITC either in A:DBP or in DMSO by topical application on the dorsal side of the ears (25 μL/ear). A control group of 3 mice received topical treatment with vehicle alone, i.e. either A:DBP or DMSO. The formulations were applied once daily for 3 consecutive days. All formulations were prepared within 2 h before each application. Two days after the last application mice were given an intravenous injection of [methyl-$^3$H] thymidine in PBS (20 μCi, 250 μL) and 5 h later they were euthanized by inhalation of $CO_2$ while anesthetized with isofluran. Cervical lymph nodes were excised and lymph node single cell suspensions were prepared, pooled by group, and washed in PBS before incubation in 5.0% (w/v) trichloroacetic acid over night at 4 °C. The precipitates were collected by centrifugation and suspended in trichloroacetic acid and scintillation liquid. The relative cell proliferation in each group compared to the control group, i.e. the stimulation index (SI), was determined by analyzing the incorporation of $^3$HTdR using a Beckman LS 6000TA β-scintillation counter. The EC3 is defined as the concentration corresponding to an SI of 3.

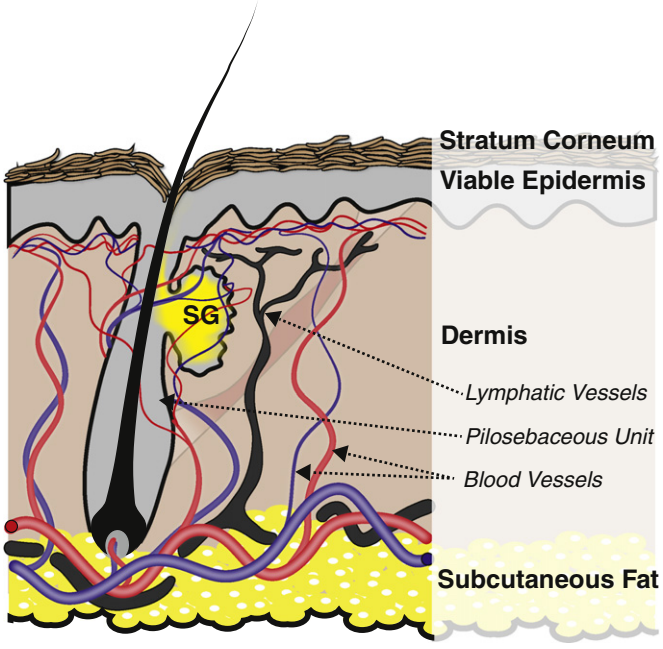


**Fig. 1.** Illustration of the skin and the pilosebaceous unit (SG: sebaceous gland).

*Topical application of test formulations and collection of tissue and serum.* Mice were anesthetized by inhalation using Isoba Vet isofluran or by intraperitoneal injection of Ketalar and Domitor Vet. Anesthetized animals received a single topical treatment of test formulations, i.e. RBITC in either A:DBP, DMSO or acetone, rhodamine B in either A:DBP or DMSO, or FITC in A:DBP, on the dorsal side of both ears (25 μL/ear). Untreated mice or mice exposed to vehicle alone were used as controls. Applied concentrations and the number of analyzed mice per treatment group are specified together with the description of the analytical methods below. All formulations were prepared within 2 h before each application. Mice were kept under anesthesia for 1 h after application of formulations and then either euthanized directly by inhalation of $CO_2$, or euthanized at a later time point, i.e. 24 h or 7 days after application. FITC-Dextran (100 μL, 1.25%, w/v) was injected via the tail-vein to visualize the dermal blood vessels. Ears and serum samples were collected and stored at −70 °C prior to further analysis. For flow cytometry analysis, whole blood, spleen and cervical lymph nodes were collected and single cell suspensions prepared.

*Tissue preparation and immunohistochemistry.* For TPM, intact ear samples were mounted in imaging chambers in Fluoromount aqueous mounting medium. For confocal microscopy, dermal sheets were prepared (Tschachler et al., 2004), washed in PBS and incubated in blocking solution, consisting of 0.3% Triton X and 5% normal goat serum in PBS. Lymphatic vessels were labeled using LYVE-1 antibodies and counterstained with Alexa Fluor 488 conjugated goat anti-rabbit IgG F(ab′)$_2$ fragments. The tissue was mounted in Fluoromount aqueous mounting medium and imaged by laser scanning confocal microscopy.

*Two-photon and laser scanning confocal microscopy.* Ear skin from mice exposed to RBITC (0.05% w/v) in A:DBP 1 h ($n=3$), 24 h ($n=3$) or 7 days ($n=1$), RBITC (0.05% w/v) in DMSO 1 h ($n=3$) or 24 h ($n=3$), RBITC (0.05% w/v) in acetone 1 h ($n=3$) or 24 h ($n=3$), FITC (0.2% w/v) in A:DBP 1 h ($n=1$), rhodamine B in A:DBP 1 h ($n=1$) or 24 h ($n=1$) or rhodamine B in DMSO 1 h ($n=1$) or 24 h ($n=1$) was analyzed by TPM to investigate the presence of the fluorescent compounds. Ears from untreated mice or mice exposed to vehicle alone were used as controls ($n=3$). Imaging was performed using a LSM710 laser scanning system (Carl Zeiss MicroImaging GmbH, Jena, Germany) in combination with an Axio Examiner upright microscope (Carl Zeiss MicroImaging GmbH) and a W Plan-Apochromat 20×/1.0 DIC D=0.17 M27 75 mm lens (Carl Zeiss MicroImaging GmbH). TPM was achieved using a MaiTai DeepSeeTM femtosecond pulsed laser, operating at either 810 nm (RBITC) or 780 nm (FITC) with an integrated