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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.

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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 (Loughborough, 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-³H] 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-³H] thymidine in PBS (20 µCi, 250 µL) and 5 h later they were euthanized by inhalation of CO₂ 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 ³HTdR using a Beckman LS 6000TA β-scintillation counter. The EC3 is defined as the concentration corresponding to an SI of 3.

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 CO₂, 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 DeepSee™ femtosecond pulsed laser, operating at either 810 nm (RBITC) or 780 nm (FITC) with an integrated

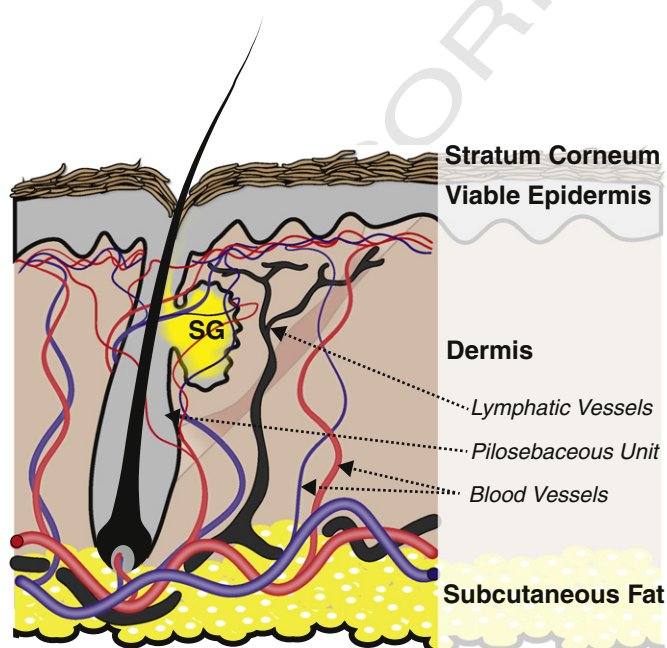


Fig. 1. Illustration of the skin and the pilosebaceous unit (SG: sebaceous gland).

Spectra-Physics 14 W Millennia® pump laser (Spectra Physics, Newport Corporation, Irvine CA). TPM images of RBITC and FITC were acquired in non-descanned mode using a red (BP565–610, RBITC channel), green (BP500–550, FITC channel) and blue (SP485, single harmonic generation (SHG) channel) filter set. Confocal images were acquired in descanned mode using a 488 nm Argon-laser and a 561 nm laser diode as excitation light sources and a 34-channel Quasar detection system with the spectral detection range set to 493–542 nm for detection of LYVE-1 + tissue, 566–703 nm for detection of RBITC + tissue or 493–542 nm for detection of FITC-dextran + vessels. The pixel dwell time was 1.58 μ s. Image acquisition was controlled via the integrated computer software ZEN2010 (Carl Zeiss MicroImaging GmbH). Images were processed, analyzed and displayed using Image J (National Institute of Health, Bethesda, MD).

Analysis of serum samples. As an indicator of percutaneous absorption, serum samples from mice exposed to RBITC (0.05% w/v) in A:DBP 1 h ($n=4$) or 24 h ($n=5$), DMSO 1 h ($n=3$) or 24 h ($n=4$) or acetone 1 h ($n=2$) or 24 h ($n=2$) were analyzed using a Spectra Max M2 fluorescence spectrometer (Molecular Devices, Sunnyvale, USA), the excitation and emission wavelengths being 544 nm and 590 nm, respectively. Untreated mice or mice exposed to vehicle alone were used as controls ($n=6$).

Analysis of RBITC-labeled cells in blood, lymph nodes, and spleen using flow cytometry. Whole blood samples from mice exposed to RBITC (0.05% w/v) 24 h in A:DBP ($n=2$) and vehicle control ($n=1$) were diluted 1:100 with 2.5% FBS in PBS and washed two times in 2.5% FBS in PBS. The cells were then resuspended in 500 μ L 2.5% FBS in PBS before analyzed by flow cytometry. Cervical lymph nodes and spleen single cell suspensions from RBITC exposed ($n=2$) and control ($n=1$) mice were prepared by mechanical disruption through BD Falcon 70 μ m nylon cell strainers (BD Biosciences, Bedford, MA) and washed in FBS/PBS. Erythrocytes in spleen samples were removed by 5 min incubation in ammonium chloride lysing solution pH 7.3 (155 mM ammonium chloride and 10 mM tris(hydroxymethyl) aminomethane in water). Cells were washed in 2.5% FBS in PBS before resuspended in 2.5% FBS in PBS and analyzed by flow cytometry. The analysis of RBITC-labeled blood, spleen and lymph node cell suspensions was performed using a BD FACS Array Bioanalyzer (BD Biosciences, Franklin Lakes, NJ). Cells were excited using a 10 mW 532 nm green solid state laser and RBITC-labeled cells were detected using a BP564–606 filter. The flow cytometry experiments were repeated twice.

Reactivity assay. A reactivity assay was performed to investigate vehicle-induced variations in the chemical reactivity of RBITC towards skin surface proteins. Benzylamine was selected as a model nucleophile, mimicking potential binding sites on proteins in the stratum corneum. RBITC (1 mM) and benzylamine (5 mM) were mixed and stirred (200 rpm) at room temperature in either A:DBP, acetone, or DMSO. Samples were taken out and analyzed directly after mixing the reagents and every 40 min until at least 50% of the initial amount of RBITC was depleted. Samples were diluted (1:5) in acetonitrile before analyzed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS). All reactivity experiments were performed in duplicates.

HPLC-MS. Samples from the reactivity assay were analyzed on a Hewlett Packard 1100 HPLC-MS system (Agilent Technologies, Santa Clara, CA), including a vacuum degasser, a column thermostat, a diode array detector and a single quadrupole mass spectrometer. Separation was performed on a HyPURITY C18 column (Thermo Scientific, Waltham, MA), at 30 °C, by gradient elution using 0.1% formic acid in ELGA ultrapure LabWater and acetonitrile (10–95% acetonitrile gradient) as mobile phases. The mass spectrometer was equipped with an

atmospheric pressure ionization electron spray interface used in positive ionization mode. The nebulizer pressure was set at 35 psig, the capillary voltage was 3000 V, the drying gas temperature and flow rate were 350 °C and 12 L/min, and the electric potential over the ionization chamber was 70 V. The mass analyzer was set to detect ions with m/z from 50 to 700. Quantification was performed by measuring the absorbance at 544 nm with the diode array detector.

Results

Sensitization potency depends on delivery vehicle

The sensitization potency of RBITC was investigated in the murine LLNA. Fig. 2 (and Supplementary Tables S1 and S2) shows the SI as a function of the concentration of RBITC in either A:DBP or DMSO. As shown by the data, the SI increased significantly when RBITC was applied in A:DBP while no increase in the SI was observed when RBITC was applied in DMSO. At an applied dose of 1.0% (w/v) there was a more than 10-fold difference in the RBITC induced proliferative response between the two formulations. The calculated EC₃ value, i.e. the concentration inducing a SI of 3 was 0.03% (w/v, 0.6 mM) when RBITC was applied in A:DBP. RBITC would thus be classified as an extreme sensitizer (EC₃<0.1%) (ECETOC, 2003) in A:DBP. No EC₃ value could be determined when RBITC was applied in DMSO up to 2.5%. The sensitization potency in acetone alone was not investigated here as it has already been shown that isothiocyanates, e.g. RBITC, are more potent sensitizers when applied in A:DBP than in acetone alone (Dearman et al., 1996; Imai et al., 2006; Matsuda et al., 2010).

Two-photon microscopy reveals a DBP-specific uptake via the pilosebaceous units

TPM revealed striking differences in the cutaneous distribution of RBITC in mouse ears depending on the vehicle (Fig. 3). When RBITC was applied in DMSO, it could be detected in the stratum corneum but not in the basal or supra basal layers of the epidermis or in the dermis (Fig. 3a, d and g). Likewise, when RBITC was applied in acetone it was mainly detected in the stratum corneum (Fig. 3b, e and h), although the signal was less intense. RBITC could also be detected deeper into the skin within the PSUs when applied in acetone as compared to DMSO, although the signal was very weak. The incubation time (1 or 24 h) did not affect the skin distribution of RBITC neither when applied in acetone nor in DMSO.

On the contrary, when RBITC was applied in A:DBP, we observed an intense signal from the hair follicles and the sebaceous glands deeper into the skin (Fig. 3c, f and i). The signal in the stratum corneum was not as strong as in the samples from animals exposed

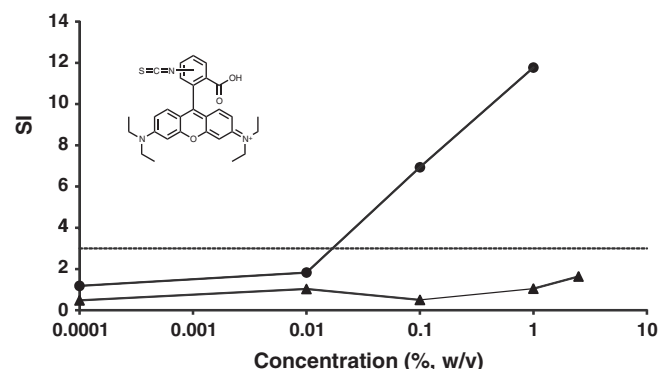


Fig. 2. LLNA dose response curves for RBITC topically applied in A:DBP or in DMSO. The calculated EC₃ value, the concentration corresponding to an SI of 3 (marked by the dashed horizontal line), was 0.03% (w/v, 0.6 mM) when RBITC was applied in A:DBP. RBITC applied in DMSO did not induce a significant lymph node cell proliferative response at the concentrations tested.

to the two other vehicles. TPM images also revealed fluorescent vascular-like structures, probably blood or lymphatic vessels, (Fig. 3l) and RBITC-labeled thinner threadlike structures spreading out from the PSUs in the dermis (Fig. 3m). These structures likely correspond to nerve- or elastin fibers. Uptake via the PSU and the vascular-like structures were clearly visible in all samples exposed to RBITC in A:DBP, both 1 and 24 h after application, while the thinner threadlike structures were only detected after 24 h. FITC, another fluorescent isothiocyanate, which has previously been shown to be an extreme sensitizer in A:DBP (Samuelsson et al., 2009), was also specifically absorbed via the PSUs (Fig. 3k, 1 h observation). The ability of the PSU to be a depot for RBITC following in vivo application in combination with DBP was also investigated. The result revealed that significant amounts of RBITC remained in the PSUs up to at least 7 days following a single topical application in A:DBP (Fig. 3j). In a control experiment rhodamine B, a structural analogue to RBITC lacking the reactive isothiocyanate functional group, was applied in either A:DBP or DMSO. Contrary to RBITC, the results indicated that rhodamine B was effectively absorbed into the skin by both vehicles (Supplementary Fig. S1).

Altogether, the results indicate that DBP induces a rapid PSU-targeted delivery of isothiocyanates, resulting in an enhanced cutaneous and percutaneous absorption compared to the absorption obtained with the other investigated vehicles. Additional data from the TPM investigations are presented in Supplementary Fig. S1 and Supplementary Video 1 and a summary of features observed in the TPM images, and the number of samples investigated is presented in Supplementary Table S3.

Vehicle-dependent hapten reactivity

It was hypothesized that the vehicle-induced variations in the reactivity of RBITC towards proteins in the upper stratum corneum could act as a regulating factor of the skin penetration and thus the skin sensitization potency of the hapten. To estimate variations in the rate of the reaction between RBITC and stratum corneum proteins, the reactivity of RBITC in the actual vehicles towards the model nucleophile benzylamine was investigated in vitro. The pseudo first order rate constant for the formation of RBITC-benzylamine adducts and the half-life of the reaction ($t_{1/2}$) were determined by measuring the depletion of RBITC in the reaction mixtures (Fig. 4). The rate constant in A:DBP was found to be $1/167 \text{ min}^{-1}$ and $t_{1/2}$ was 118 min. The rate of the reaction in acetone was slightly lower, rate constant = $1/250 \text{ min}^{-1}$ and $t_{1/2}$ = 169 min. Interestingly, the depletion of RBITC in DMSO was so fast that the rate constant could not be determined using this assay. In DMSO, all RBITC in the reaction mixture was consumed within less than 3 min, i.e. approximately the time it takes to mix the reagents and inject the sample into the HPLC-MS. In the absence of benzylamine no significant depletion of RBITC was detected in either vehicle up to 5 h (data not shown); thus RBITC was stable in the reaction mixtures within the time frame of the experiment. Furthermore, the depletion of RBITC correlated with the formation of a peak having a mass-to-charge ratio corresponding to the benzylamine-RBITC adducts. The results indicate that a rapid formation of hapten-protein conjugates in the uppermost layer of the stratum corneum leads to a reduced skin penetration of RBITC when applied in DMSO. On the contrary, in A:DBP, RBITC is

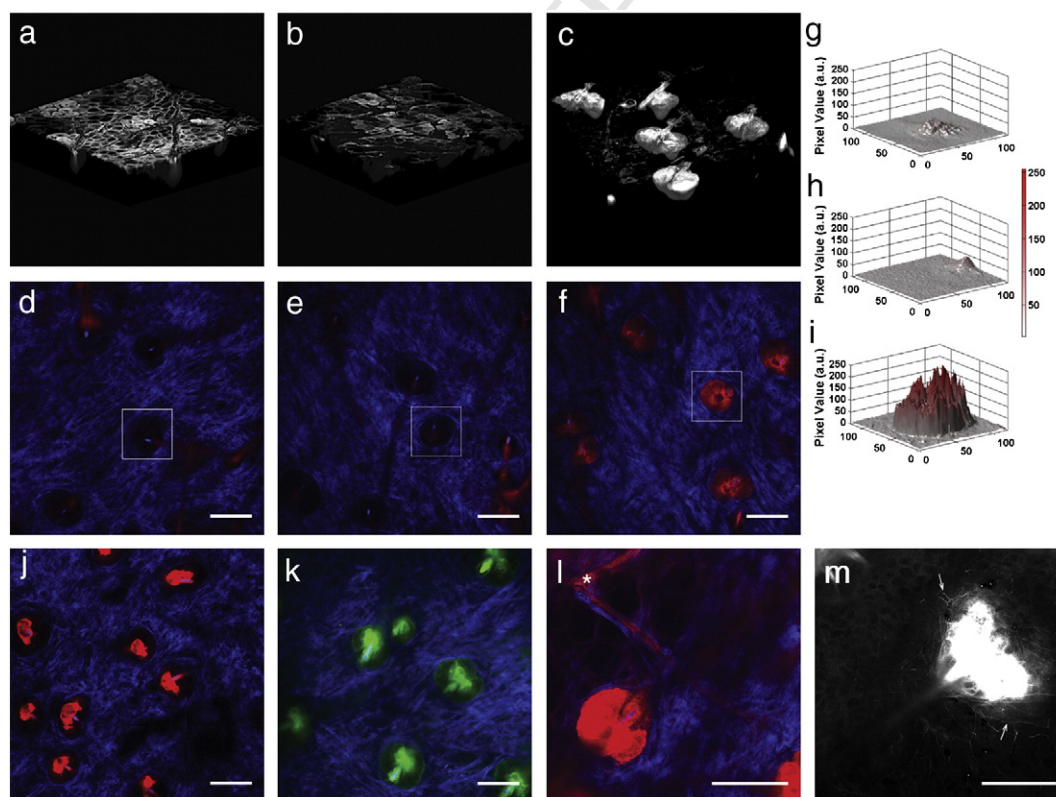


Fig. 3. Non-invasive TPM visualization of the cutaneous distribution of RBITC after a single topical application on the dorsal side of the ears of mice in vivo. (a–c) Three-dimensional visualization of skin samples after application (24 h after exposure) in DMSO (a), acetone (b) or in A:DBP (c). (d–f) The lateral distribution of RBITC (skin depth approximately 40 μm) after application (1 h after exposure) in DMSO (d), acetone (e) or A:DBP (f). (g–i) The relative amount of RBITC (pixel values from the red channel) in and adjacent to the pilosebaceous units (PSUs, white squares, g=d, h=e and i=f). (j) RBITC detected in the PSUs 7 days following a single topical application. (k) Fluorescein isothiocyanate (FITC) specifically absorbed by the PSUs when applied in A:DBP (1 h after exposure). (l) Unidentified RBITC-labeled vessel (*) in the dermis 1 h after application in A:DBP. (m) Thin RBITC-labeled threadlike structures (arrows) radiating out from the hair follicles in the dermis when RBITC was applied in A:DBP (24 h after exposure). Red or grey-scale signal = RBITC, blue signal = second harmonic generation from e.g. collagen fibers. Scale bars = 100 μm (d, e, f, j, k and l) and 50 μm (m).

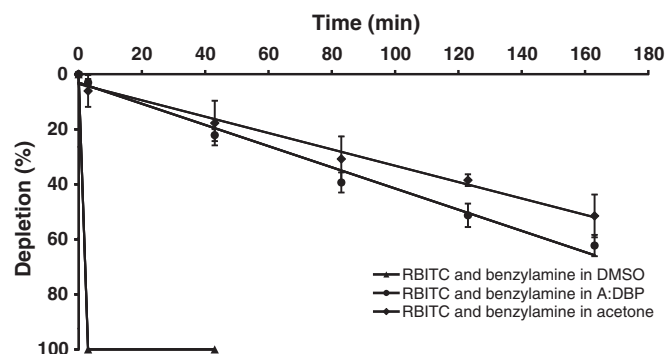


Fig. 4. The RBITC-depletion rate in the reactivity assay with the model nucleophile benzylamine *in vitro* is dependent on the vehicle. The pseudo first order rate constant for the formation of RBITC-benzylamine adducts in A:DBP was $1/167 \text{ min}^{-1}$ and the half-life of the reaction ($t_{1/2}$) was 118 min. The rate constant for adduct formation in acetone was slightly lower, $1/250 \text{ min}^{-1}$ and $t_{1/2}$ was 169 min. The rate constant for adduct formation in DMSO could not be determined as the reaction rate was too fast to be resolved by the assay.

less reactive and thereby has a higher probability of reaching the viable layers of the skin before reacting with proteins.

Rapid absorption of RBITC by the blood and lymphatic's

The RBITC-labeled vascular-like structures visualized by TPM were further investigated by histochemical analysis of isolated dermal sheets. Lymphatic vessels were identified using an anti-LYVE-1 antibody, and blood vessels by intravenous injection of FITC-dextran shortly before euthanizing the animals. The results revealed both RBITC-labeled lymphatic vessels (Fig. 5a–c) and blood vessels (Fig. 5d–f) in the skin 1 h after application of the hapten in A:DBP. The RBITC-labeled thin threadlike structures observed in the TPM images were also confirmed to be present in the dermal sheets (Supplementary Fig. S1). The uptake of RBITC in the blood was confirmed by analysis of serum samples, showing enhanced levels of RBITC in sera from mice treated with A:DBP compared to sera from mice receiving RBITC in either acetone or DMSO (Fig. 6). However, using flow cytometry, RBITC-labeled cells could only be detected in lymph nodes 24 h after application in A:DBP, and not on cells in the blood or spleen (Supplementary Fig. S2). Since the TPM images revealed a specific localization of RBITC to the PSUs, these units seem to act like DBP-induced pathways for the isothiocyanates to enter the skin, the lymphatics, and the blood vessels.

Discussion

According to the seventh amendment of the cosmetic directive "The safety of finished cosmetic products can already be ensured on the basis of knowledge of the safety of the ingredients that they contain" (EU, 2003). In contrast, the present study reveals that interactions between hapten and vehicle, including vehicle-induced target specific delivery and hapten reactivity, can have a significant effect on the sensitization potency of haptens such as isothiocyanates. The fluorescent model hapten, RBITC was here found to be an extreme sensitizer ($EC_{0.03} = 0.03\%$) when applied in A:DBP, while being a non-sensitizer in DMSO up to a concentration of 2.5%, clearly demonstrating the effect of the vehicle on sensitization potential. In correlation with our results, increased sensitization potencies of isothiocyanates in combination with phthalates in murine models of contact hypersensitivity have been reported by others (Dearman et al., 1996; Imai et al., 2006; Matsuda et al., 2010). However, a full chemical and biological explanation for the 'adjuvant effect' is lacking. A better insight into these mechanisms is important to improve the safety assessment of chemical allergens.

Using TPM we have identified a phthalate-induced, rapid, localized uptake and an accumulation of RBITC in the PSUs, which could be major contributing factor for the observed enhanced sensitization potency. We had earlier seen indications of a similar uptake of FITC, another extreme sensitizer when applied in A:DBP, in excised human skin (Samuelsson et al., 2009). In the present *in vivo* investigation, FITC was also specifically absorbed via the PSUs, implying that this is a general penetration pathway for isothiocyanates when applied together with DBP. When RBITC was applied in acetone alone, it was mainly observed in the stratum corneum, and only minor amounts of RBITC could be detected within the region of the PSUs. Thus, our results give a rational explanation for previously reported enhanced responses to isothiocyanates in the combination with phthalate in murine models of contact hypersensitivity (Dearman et al., 1996; Imai et al., 2006; Matsuda et al., 2010).

The PSU comprises more than 20 different cell populations (Patzelt et al., 2008), including a relatively high fraction of stem cells (Fuchs, 2007) and immune cells, e.g. dendritic cells, T-cells, macrophages and mast cells (Moresi and Horn, 1997; Christoph et al., 2000). It has a dense network of perifollicular capillaries (Knorr et al., 2009), supporting effective systemic resorption. Whether the appendages are important for the overall uptake of topically applied compounds have been disputed; however, several recent studies show that follicular transport has been underestimated (Lauer et al., 1996; Knorr et al., 2009). The relatively high density of immune cells, e.g. dendritic cells, in and around the follicular infundibulum in combination with the reduced barrier properties of the lower infundibulum (Moresi and Horn, 1997; Christoph et al., 2000; Knorr et al., 2009) is likely to affect the sensitization potency of haptens applied in vehicles targeting the PSUs.

When assessing the bioavailability of haptens one should also consider the reservoir capacity of the PSUs. Compounds trapped in the hair follicles generally persist in the skin for longer periods of time compared to compounds trapped in the stratum corneum (Lademann et al., 2006). Indeed, RBITC could be detected in the PSUs in a mouse euthanized 7 days after a single topical application in A:DBP. Primary ACD reactions, i.e. elicitation reactions after a single dose application without challenge, have been reported as a consequence of the persistence of hapten in the skin (Saint-Mezard et al., 2003). It is likely that haptens trapped in PSUs could elicit such reactions. One could also speculate that repeated exposure to low concentrations of hapten could build up the concentration in the PSU to a level that is high enough to induce sensitization.

To broaden the investigation of the role of hapten bioavailability and skin penetration pathways on the sensitization potency of isothiocyanates, DMSO was applied as a control vehicle. DMSO was chosen as it has been shown to effectively improve the cutaneous absorption of a wide range of compounds (Franz et al., 2006). Hence, it was assumed that this vehicle would effectively deliver the hapten into skin. Unexpectedly, RBITC did not induce any cell proliferation in the LLNA when applied in DMSO, and initially it was believed that bioavailability was not a major regulating factor controlling the sensitization potency of RBITC. However, TPM images of the exposed skin samples revealed that the result could be explained by a limited cutaneous absorption of RBITC when applied in DMSO. This result was contradicting earlier reports on the penetration enhancing properties of this vehicle. When we applied rhodamine B, a compound with the same overall structure as RBITC but lacking the isothiocyanate functional group in DMSO, it was effectively absorbed by the skin.

It was speculated that the limited skin penetration of RBITC in DMSO could be due to a reduced stability of the isothiocyanate in this vehicle, e.g. by dimerization, or alternatively, an increased chemical reactivity of RBITC towards proteins in the upper SC when dissolved in DMSO as compared to solutions in A:DBP or acetone. The dimerization effect was excluded, as RBITC was stable for at least 5 h in all three formulations. However, the reactivity assay

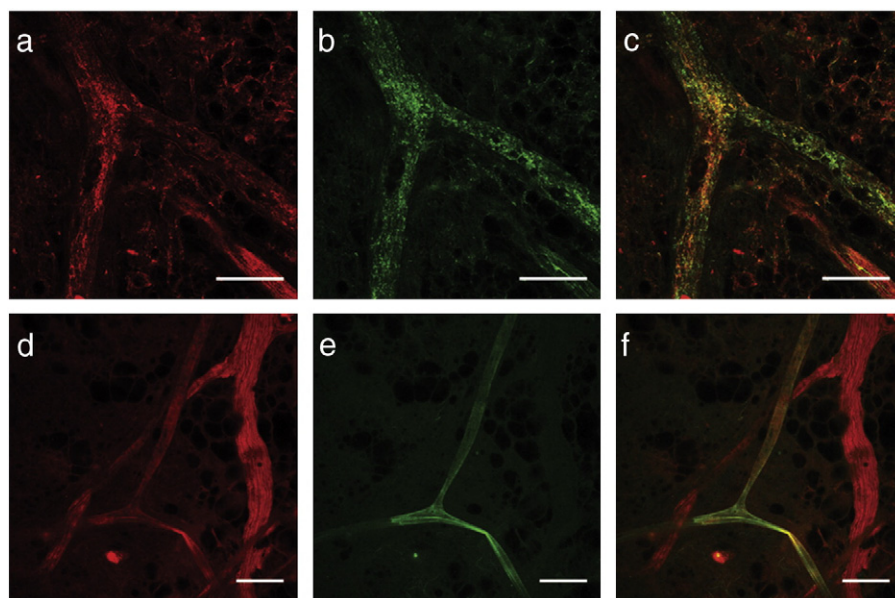


Fig. 5. Identification of RBITC-labeled vessels in dermal sheets of mouse ears after topical application in vivo, imaged by confocal microscopy. (a) Vessel-like structures observed in a skin sample exposed to RBITC (1 h) in A:DBP. (b) The same tissue labeled using a lymphatic vessel endothelial hyaluronan receptor 1 (LYVE)-1 antibody. (c) Corresponding merged channels showing colocalisation of RBITC and LYVE-1 confirming the presence of RBITC in lymphatic vessels. (d) Vessel-like structures observed in another skin sample exposed to RBITC (1 h) in A:DBP. (e) Corresponding tissue showing blood vessels visualized by intravenous injection of FITC-dextran shortly before euthanization (minutes). (f) Corresponding merged channels showing colocalisation of RBITC and FITC-dextran confirming the presence of RBITC in blood vessels.

revealed significant formulation-dependent differences in the rate of the reaction between RBITC and the model nucleophile benzylamine. While the reaction proceeded relatively slow in A:DBP as well as in acetone, with $t_{1/2}$ between 2 and 3 h, it was almost instantaneous in DMSO, i.e. 100% RBITC was depleted in less than 3 min. The reactivity assay thus indicates that application of RBITC in DMSO results in a rapid conjugation of the hapten to proteins in stratum corneum, reducing the skin penetration and thus the sensitization potency. The reactivity of a compound in different vehicles seen in relation to bio-availability and sensitization potency is to our knowledge a parameter that has previously been largely neglected. This finding further emphasizes the importance of considering not only the individual ingredients, but also the vehicle when evaluating biological impacts of topically applied compounds. It also demonstrates the ability of the skin to act as a barrier preventing the uptake of reactive xenobiotics.

In the sensitization phase of ACD skin resident dendritic cells take up hapten-modified proteins and migrate to draining lymph nodes for presentation of modified peptides to T-lymphocytes (Vocanson et al., 2009). In addition to the relatively slow cell-mediated transport of antigen to the draining lymph nodes, it has been shown that free hapten or soluble antigen can be transported via the lymph or the

blood to the secondary lymphoid organs, where they can be taken up and presented by resident antigen presenting cells (Pior et al., 1999; Sixt et al., 2005). The rapid uptake of RBITC in A:DBP, within 1 h or possibly less, by the blood and lymphatics correlates with previous results presented by Pior et al. (1999) and implies a direct transport of RBITC or soluble RBITC-protein complexes towards secondary lymphoid organs. On the contrary, using flow cytometry we could only detect RBITC-labeled cells in the draining lymph nodes first after 24 h, and not at all on cells in the spleen, indicating that a cell-mediated transport of antigen to the draining lymph nodes dominates. However, the reason for not detecting RBITC in lymph nodes and spleen at earlier time points, theoretically coming via the blood or lymph, could be due to the relatively low concentration applied (Pior et al., 1999).

As phthalate esters like DBP are used in many consumer products, e.g. as plasticizers and solubilizing agents in plastics, drugs and cosmetics, the results presented here could have a clinical relevance. Specifically, this might be relevant for products targeting body regions with a high hair follicle density, e.g. deodorants (Hyun and Byung, 2004). However, it is important to remember that the presented data were obtained in a mouse model, thus in a skin type

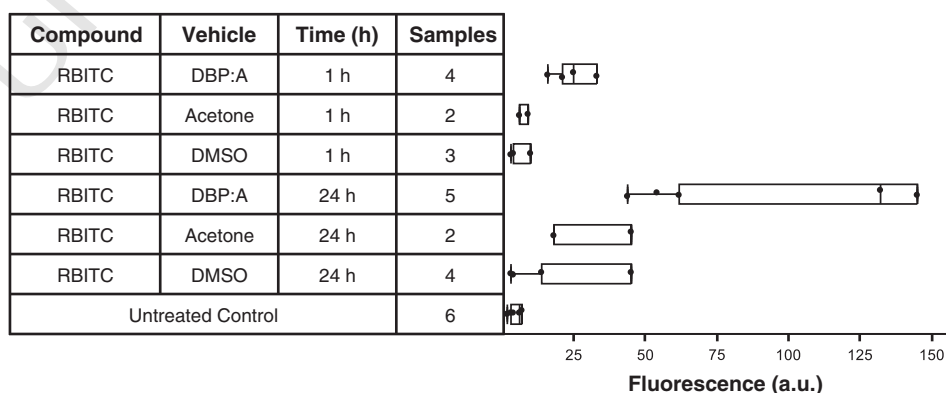


Fig. 6. Relative RBITC concentration in serum samples taken 1 and 24 h after application of test formulations, analyzed by fluorescence spectrometry.

with a significantly higher follicular density than in humans. Furthermore, although we have not specifically measured DBP concentrations in the skin, it is possible that it is absorbed equally efficiently by the PSUs as RBITC. Thus, the absorption and accumulation of phthalates in the PSU and potential adverse health effects thereof should be investigated further.

In conclusion, we have in the present study demonstrated the importance of considering vehicle-dependent skin penetration pathways to better understand the potency of a compound to cause contact allergy. Moreover, our results indicate that vehicle-dependent hapten reactivity towards stratum corneum proteins regulates the bioavailability, and thus the potency, of skin sensitizers. Our results imply that the increased sensitization potency of isothiocyanates in combination with DBP is due to an elevated cutaneous absorption via PSUs, which acts like pathways for the hapten into the skin. The results highlight the complexity of the skin barrier and the uptake of xenobiotics. The study also demonstrates the potential of utilizing the PSU pathway for the transdermal delivery of drugs.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2012.07.023>.

Conflict of interest

None.

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