

Caged Fluorescent Haptens Reveal the Generation of Cryptic Epitopes in Allergic Contact Dermatitis

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Allergic contact dermatitis (ACD) is the most prevalent form of human immunotoxicity. It is caused by skin exposure to haptens, i.e., protein-reactive, low-molecular-weight chemical compounds, which form hapten–protein complexes (HPCs) in the skin, triggering the immune system. These immunogenic HPCs are elusive. In this study a series of thiol-reactive caged fluorescent haptens, i.e., bromobimanes, were deployed in combination with two-photon fluorescence microscopy, immunohistochemistry, and proteomics to identify possible hapten targets in proteins in human skin. Key targets found were the basal keratinocytes and the keratins K5 and K14. Particularly, cysteine 54 of K5 was found to be haptenated by the bromobimanes. In addition, elevated levels of anti-keratin antibodies were found in the sera of mice exposed to bromobimanes *in vivo*. The results indicate a general mechanism in which thiol-reactive haptens generate cryptic epitopes normally concealed from the immune system. In addition, keratinocytes and keratin seem to have an important role in the mechanism behind ACD, which is a subject for further investigations.

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INTRODUCTION

Topical exposure to low-molecular-weight chemical compounds can cause allergic contact dermatitis (ACD) (Karlberg *et al.*, 2008). Affecting 15–20% of the population in the Western world, ACD constitutes the most prevalent form of human immunotoxicity (Thyssen *et al.*, 2007). Once an individual is sensitized, there is no cure, but the offending compound and any crossreacting compounds will have to be avoided. The growing list of compounds that can cause ACD (i.e., haptens) today comprises >4,350 substances (De Groot, 2008).

ACD is a T-cell-mediated delayed-type hypersensitivity reaction. The basic mechanism behind ACD was described in a seminal paper published as early as 1935 (Landsteiner and Jacobs, 1935) in which the key step in sensitization was proposed to be the formation of an immunogenic hapten–

protein complex (HPC), e.g., covalent bond formation between electrophilic haptens and carrier proteins. Since then, it has been shown that antigen-presenting cells in the skin, i.e., Langerhans cells and dermal dendritic cells, migrate to the regional lymph nodes and present epitopes of the HPCs on major histocompatibility complex molecules to naive T cells. T cells with a matching T-cell receptor become activated, proliferate, and differentiate into a specific T-cell progeny that is released into the circulatory system. These “patrolling” antigen-specific T cells are responsible for the clinical outcome in sensitized individuals (Rustemeyer *et al.*, 2001). Although ACD is one of the most studied immunologically mediated toxicities, several details about its mechanisms are still lacking. For example, major histocompatibility complex-restricted haptenated epitopes have yet to be identified. Furthermore, it is not known where in the skin the haptenation reaction occurs, if the primary cellular targets are keratinocytes or antigen-presenting cells, or if intra- or extra-cellular proteins are targeted. Also, the targeted protein sequences remain elusive. Better understanding of these processes might be helpful in the development of predictive *in vitro* tests of potentially allergenic compounds in consumer products that can replace the murine local lymph node assay (LLNA), the OECD (Organization for Economic Co-operation and Development) standard model for predictive testing (Kimber *et al.*, 2002). This is an urgent need, because of the animal testing ban of cosmetic products (2009) and the coming up marketing ban of animal-tested cosmetic products (2013) in the European Union (EU, 2003).

To decode sensitization, i.e., identify target proteins and reveal the exact position of haptenation, haptenated proteins need to be singled out with molecular precision against the

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Abbreviations: ACD, allergic contact dermatitis; C54, cysteine 54; dBBR, dibromobimane; HPC, hapten–protein complex; K14, keratin 14; K5, keratin 5; LLNA, local lymph node assay; mBBR, monobromobimane; TPM, two-photon fluorescence microscopy

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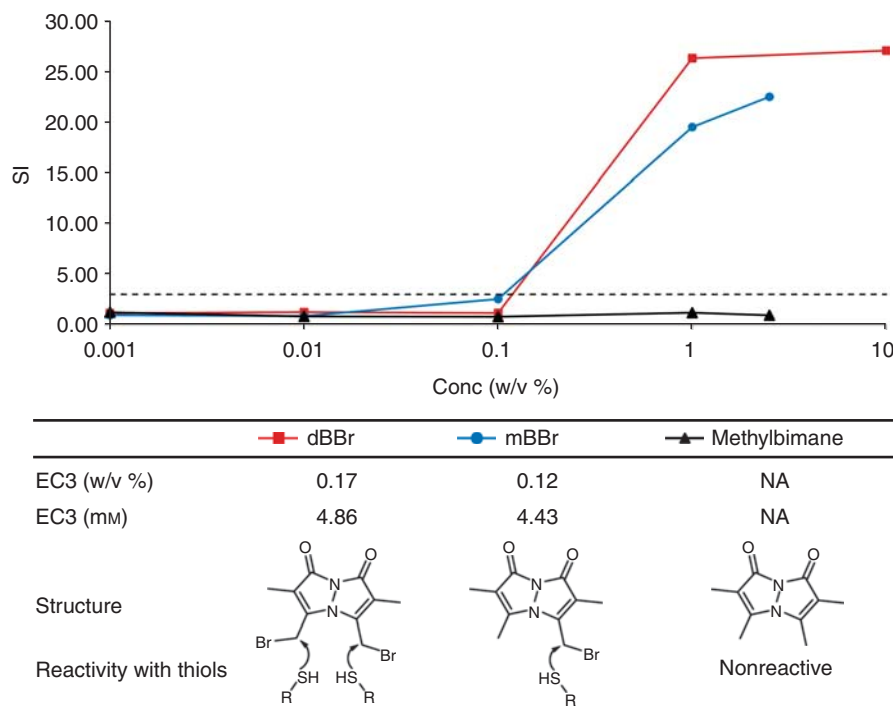


Figure 1. Local lymph node assay (LLNA) dose-response curves and chemical structures for monobromobimane (mBBR), dibromobimane (dBBR), and methylbimane. All compounds were tested in DMSO. Cell proliferation in the lymph nodes following topical application of test chemicals was determined by measuring the concentration of $^3\text{HTdR}$ by β -scintillation counting. The results are given as mean disintegrations per minute (d.p.m.). mBBR and dBBR were strong sensitizers, whereas methylbimane was a nonsensitizer at the concentrations tested. NA, no value available.

entire backdrop of the proteome. To meet this challenge, we have deployed caged thiol-reactive haptens (bromobimanes, Figure 1) that are nonfluorescent before an bimolecular nucleophilic substitution displacement of the bromine atom (Kosower *et al.*, 1979). Excised human skin has been exposed to the bromobimanes *in vitro*. The locations of the fluorescent products have been visualized using two-photon fluorescence microscopy (TPM), and the protein targets analyzed using mass spectrometry (experimental flowchart, Supplementary Figure S1 online). In contrast to typical caged probes (Ellis-Davies, 2007), uncaging of bromobimanes is not in the hands of the researcher, but a consequence of biology occurring preferably with thiols at physiological pH (Kosower *et al.*, 1979). Cysteines are thus potential endogenous nucleophiles in the formation of uncaged HPCs. Preferential reactivity toward thiols is advantageous as many clinical haptens are thiol reactive (Gerberick *et al.*, 2004). Also, the question of how to relate haptenation reactions by monodentate haptens (one reactive group) to those of bidentate haptens (two reactive groups) is addressed. Bromobimanes with one (monobromobimane (mBBR)) or two (dibromobimane (dBBR)) reactive groups were thus deployed with the goal of identifying hapten targets in the skin and increasing knowledge regarding the development of ACD.

RESULTS

Bromobimanes as haptens

Using the murine LLNA, the bromobimanes were found to be strong sensitizers with EC3 values (the concentration giving a stimulation index of three in the LLNA) in the range of 4 mm

(Figure 1). They are thus ideal chemical probes for addressing the molecular pathway of ACD. The nonreactive, permanently fluorescent analog methylbimane was nonsensitizing, in accordance with the postulated need for covalent hapten-protein binding to form the immunogenic HPCs (Landsteiner and Jacobs, 1935).

The EC3 values for mBBR and dBBR were found to be virtually identical (Figure 1), although the numbers of reactive groups differ, i.e., the monodentate mBBR reacts with one thiol group, whereas the bidentate dBBR links two sulfhydryl groups. As their molecular weight and theoretical average $\log P$ values are comparable (dBBR: 1.87 ± 0.86 , mBBR: 1.45 ± 0.85) (<http://www.vcclab.org>, accessed 2009) (Tetko *et al.*, 2005), the penetration profiles of the compounds in skin are expected to be similar. *In vitro* experiments verified that both mBBR and dBBR react swiftly with glutathione, despite the difference in the number of reactive groups (Supplementary Figures S2–S5 online). In skin, there are numerous disulfide linkages between the cysteines in proteins to ensure the mechanical integrity. dBBR was not able to react with oxidized sulfhydryls (disulfides) or to reduce disulfides *in vitro* (data not shown), indicating that reduced sulfhydryls are bimane targets. The time to complete reaction with sulfhydryls was similar for mBBR and dBBR, as was the slow rate of hydrolysis after 24 hours of incubation at pH 7 (data not shown).

Basal cells are hapten targets

The localization of fluorescent HPCs in skin was visualized by TPM to enable detection without compromising the

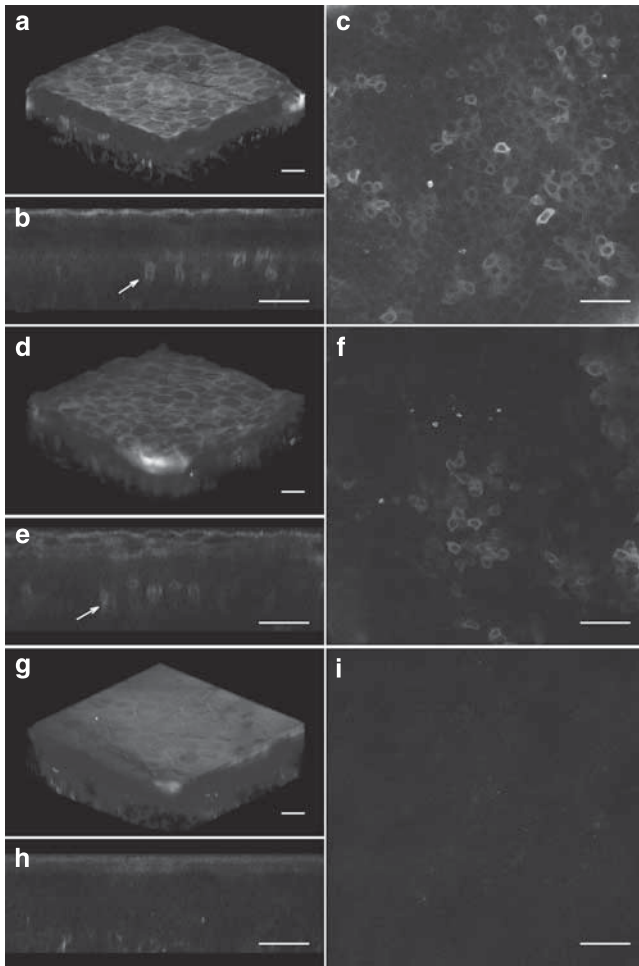


Figure 2. Two-photon fluorescence microscopy (TPM) images of human skin incubated with bromobimanes. The skin samples were exposed to (a–c) monobromobimane (mBBr), (d–f) dibromobimane (dBBr), or (g–i) methylbimane, using an *in vitro* skin penetration system for 20 hours. (Left panel) Three-dimensional (3D) representations and optical cross-sections. (Right panel) The xy-image planes of epidermis after incubation with mBBr (c, $z = 52 \mu\text{m}$), dBBr (f, $z = 62 \mu\text{m}$), and methylbimane (i, $z = 50 \mu\text{m}$). Scale bar = $50 \mu\text{m}$ for all images. Arrows indicate bimane-labeled cells present in the basal layer of the epidermis.

integrity of the tissue. Full-thickness human skin was incubated with bimanes (5–20 mM) for 20 hours in a skin permeation system and consecutively imaged by TPM. Also, bromobimane-exposed skin was cryosectioned and imaged with laser scanning confocal microscopy to confirm the localization seen with TPM.

TPM showed an intense labeling of the stratum corneum (Figure 2), as earlier observed for other reactive compounds (Samuelsson *et al.*, 2009). This is in accordance with the biological role of stratum corneum as a protective physical barrier with a significant amount of degraded proteins and free, and thus unhindered, amino acids available for reaction (Sylvestre *et al.*, 2010). Intriguingly, covalent binding of the bromobimanes to proteins in the suprabasal cell layers was less frequent. Instead, the haptens penetrated down to the basal layer, where reaction readily occurred. The reactivity pattern of the bromobimanes can be explained by the availability of protein targets, e.g., free thiols, in combination with changes in the cell membrane integrity, e.g., formation of cornified envelope across the epidermis (Figure 3; Sun and Green, 1978; Candi *et al.*, 2005). Also, because of the lipid composition of the basal cell membrane (Hachisuka *et al.*, 1990), it has a relatively high fluidity, facilitating cell penetration and reaction with intracellular free thiols, forming HPCs. This distribution correspond to that observed in skin biopsies obtained from human volunteers exposed to 1-chloro-2,4-dinitrobenzene, a very potent thiol-reactive hapten (EC3 = 0.05% (w/v; Gawkrödger *et al.*, 1989; Kimber *et al.*, 2002)).

The reaction pattern of the bromobimanes also revealed labeling of clusters of cells within the basal layer (Figure 2). This phenomenon was reproducible throughout several independent experiments ($n = 13$) using tissues from different donors ($n = 6$). The epidermis consists of roughly 95% keratinocytes, whereas immature antigen-presenting Langerhans cells make up 1–3% of the epidermal cells (Bauer *et al.*, 2001). The scarcity of the labeled cells suggested that Langerhans cells were targets. However, this was ruled out by laser scanning confocal microscopy colocalization studies of bromobimane-exposed tissue with CD1a antibodies

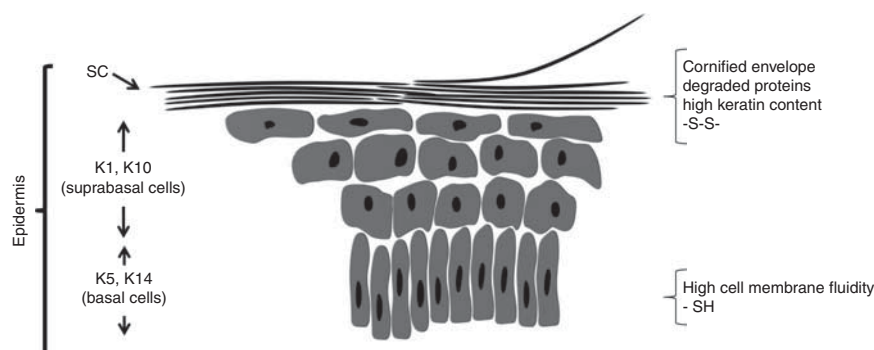


Figure 3. Changes in skin composition and structure during keratinocyte differentiation, and the availability of protein targets, i.e., free thiols, in the epidermis. While the keratin content increases toward the skin surface, the cysteines of the keratins in stratum corneum (SC) are highly crosslinked by intermolecular disulfide bonds. In addition, the cornified envelope, formed during the terminal differentiation of the keratinocytes, results in more rigid, thicker, and less permeable cell membranes. In SC, degraded proteins and free amino acids could be potential hapten targets. Free thiols are abundant inside the cells in the layers of stratum spinosum and basale. However, in the suprabasal layers, K1 and K10 are extensively bundled and crosslinked, hiding the cysteines. The relatively high membrane fluidity of the basal cells may facilitate cell penetration and reaction with intracellular cysteines of K5 and K14.

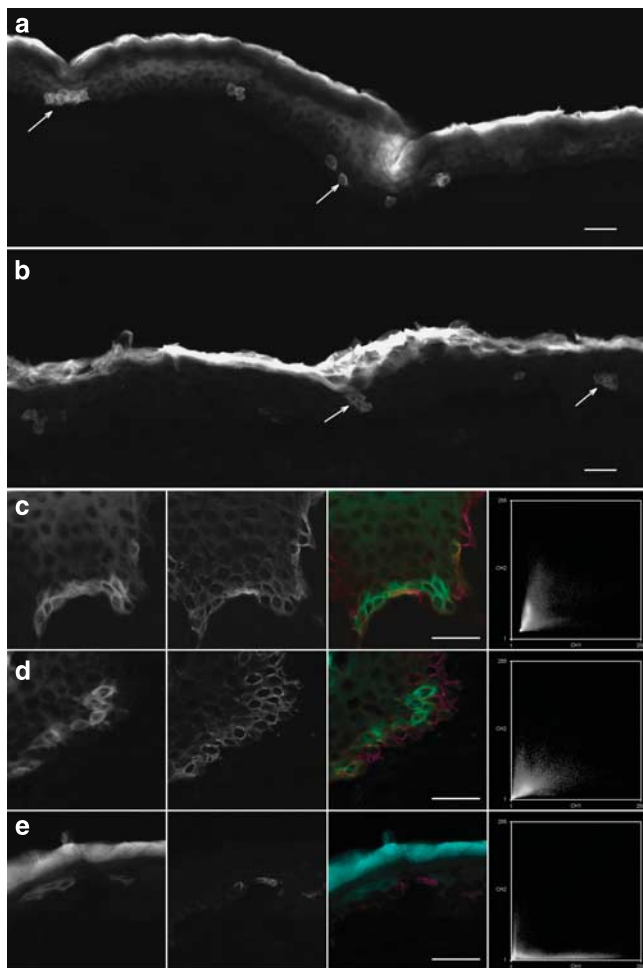


Figure 4. Confocal and immunohistochemistry images of skin exposed to bromobimanes. (a) Skin incubated with monobromobimane (mBBBr) and (b) dibromobimane (dBBBr). Immunofluorescence using (c) keratin 5 (K5), (d) keratin 14 (K14), and (e) CD1a antibodies of skin exposed to mBBBr. Panels from left to right: mBBBr fluorescence images, immunofluorescence images, merged fluorescence images (cyan channel = mBBBr and magenta channel = immunofluorescence), and intensity correlation plots. The Manders overlap coefficients for mBBBr and immunofluorescence were 0.92, 0.80, and 0.40 for K5, K14, and CD1a, respectively. Arrows highlight clusters of cells with elevated fluorescence. Scale bars = 30 μ m for all images.

expressed by Langerhans cells (Figure 4). Instead, colocalization with keratinocyte-specific antibodies showed that the labeled cells were keratinocytes in the basal layer, as shown in the figure.

The basal layer is the only mitotic cell layer in the epidermis and it is home to the stem cell (progenitor) keratinocytes that afford continuous renewal of the squamous epidermis. The progenitor cells make up $\sim 10\%$ of the basal cells, and have been shown to form clusters, the so-called stem cell niches, in *in vivo* clonal-labeling experiments (Clayton *et al.*, 2007). The clusters correspond to the localization pattern of the uncaged HPCs in our study. This raises the interesting notion that the targeted keratinocytes might be the stem cells.

Basal cell keratins are targeted in tissue

To pinpoint the hapten-protein targets, labeled proteins present in the skin pieces were visualized by their bimane fluorescence following SDS-PAGE separation (Figure 5a). Incubation with mBBBr and dBBBr resulted in prominent fluorescent protein bands. The haptenation did not simply reflect the amount of a protein, as can be seen by comparing the Coomassie-stained gel (Supplementary Figure S6 online) with detection through bimane fluorescence. Thus, some proteins seem especially prone to reaction with thiol-reactive haptens.

The molecular weights of the most intense fluorescent band for mBBBr (Figure 5a) suggested that a protein with a molecular weight of ~ 60 kDa could be a target. In keratinocytes, obligatory heterodimeric keratin pairs (type I and type II) make up the cytoskeletal keratin intermediate filament network. Epithelial tissues express different pairs of keratins depending on the cell type and the stage of differentiation (Eichner *et al.*, 1986; Steinert, 1993; Fuchs and Cleveland, 1998). The progenitor keratinocytes in the basal layer express the keratin 5/keratin 14 (K5/K14), which constitutes 25–35% of the total protein content of the cells. This pair is replaced by the K1/K10 pair in the suprabasal layers (Sun and Green, 1978), where it accounts for as much as 80–85% of the total cellular content of a fully differentiated keratinocyte. The cysteines of K1 and K10 are hidden through extensive bundling and crosslinking of K1/K10 in the suprabasal layers (Moll *et al.*, 2008), which limits their potential to react with the bromobimanes. As TPM revealed bimane localization to the basal layer (Figure 2), and the molecular weight of K5 (58 kDa) corresponds to the most fluorescently intense protein band in the SDS-PAGE (Figure 5a), K5 is a likely target for haptenation. Western blot experiments performed with polyclonal antibodies directed against K5 showed that the fluorescent band colocalized with K5 (Figure 5b). In contrast, tissue exposed to dBBBr revealed a prominent fluorescent band around ~ 100 kDa (Figure 5a), raising the issue whether this band could correspond to a crosslinking of two K14 monomers (100 kDa), two K5 monomers (116 kDa), and/or a K5/K14 heterodimer (108 kDa) by the bidentate hapten. This notion was confirmed through a western blot with polyclonal antibodies directed against K5 or K14, respectively (Figure 5b). As K5 and K14 are present in a 1:1 stoichiometry, the localization pattern of mBBBr and dBBBr should appear very similar at the resolution of TPM and confocal microscopy, in accordance with our results. In addition, this agrees with a study on cultured human keratinocytes exposed to bidentate derivatives of mustard gas, demonstrating multimerization of K5/K5, K14/K14, and K5/K14 (Dillman *et al.*, 2003). This suggests that K5 and K14 are important targets for bidentate haptens also (here dBBBr), providing a molecular explanation for the observed immunological phenomenon; matching EC3 values (Figure 1) and the location of reaction in tissue (Figure 2) using the bromobimanes. In tissue experiments, the anti-K5 and anti-K14 antibodies label the basal keratinocytes and the stratum corneum but not the suprabasal layers in the xy-sections of skin (data not shown). This result is likely because

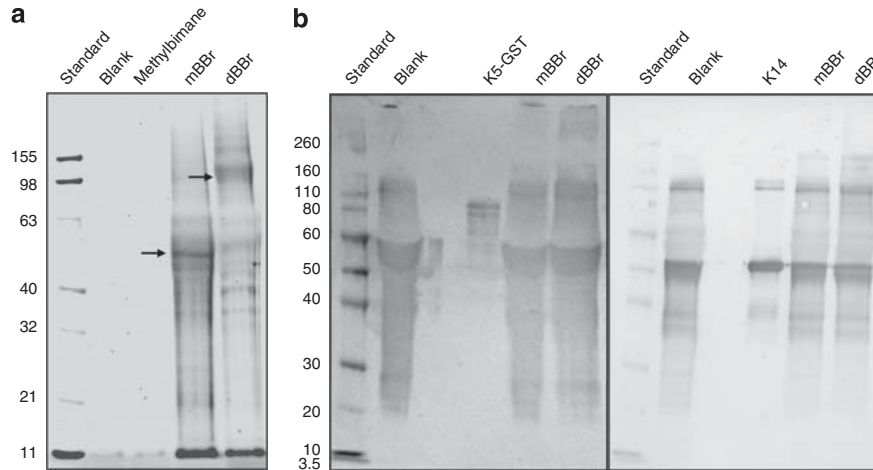


Figure 5. SDS-PAGE and western blot of epidermis samples. (a) SDS-PAGE of epidermis samples exposed to bromobimanes visualized by fluorescence (excitation: 480 nm, emission: 530 nm). The arrows point at the protein bands at 58 kDa and ~100 kDa that were excised for liquid chromatography-mass spectrometry (LC-MS) analysis. (b) Western blot of epidermis samples. Left panel: anti-keratin 5 (K5) primary antibody. Right panel: anti-keratin 14 (K14) primary antibody. Blank refers to skin sample subjected to buffer (PBS) only. The K5-positive control carries a glutathione *S*-transferase (GST) tag, explaining the significantly higher molecular weight. All numbers indicate $M_R \times 10^{-3}$. dBBR, dibromobimane; mBBR, monobromobimane.

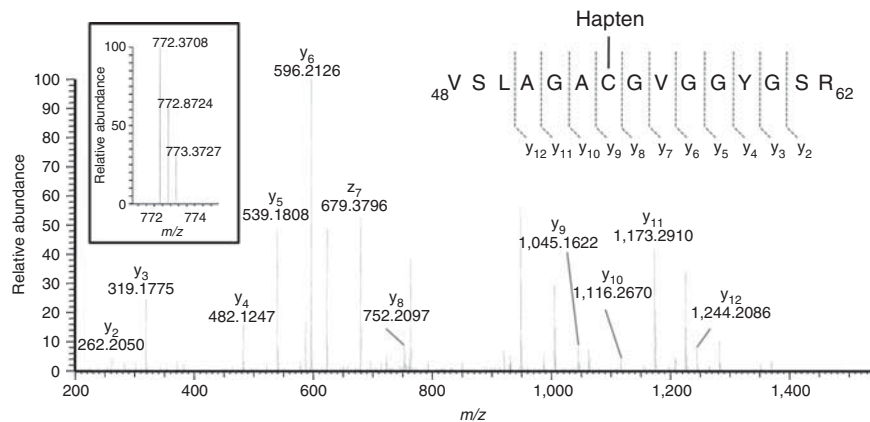


Figure 6. Tandem mass spectrometry (MS/MS) fragmentation spectra. Tryptic fragment V48–R62 of keratin 5 (K5), modified by monobromobimane (mBBR) at residue cysteine 54 (C54). Inset shows the M^{2+} ion with a m/z of 772.37 (see Supplementary Figure S8 online for details).

of the partial degradation of proteins in the outermost layers of the skin (Sylvestre *et al.*, 2010), leading to exposure of epitopes that are otherwise hidden through bundling and crosslinking of K1/K10 in the suprabasal layers. Taken together, these results imply that K5 and K14 are important protein targets when human skin is exposed for thiol-reactive haptens.

Haptens target cysteine 54 (C54) of K5

In order to find the exact site of haptenation on keratin, the two most prominent fluorescent protein bands on the SDS gel (Figure 5a) were excised and subjected to tryptic digestion with subsequent liquid chromatography–tandem mass spectrometry analysis. In accordance with the western blot results, positive identification of K5 in the band of 58 kDa, and K5 plus K14 in the band of ~100 kDa, were afforded in the MASCOT (Matrix Science, London, UK) search of all the tandem spectra (Supplementary Figure S7 online). In the following manual analysis of the mass spectrometry spectra,

no dBBR-modified peptide fragment could be observed in the band of ~100 kDa. This is probably because of the fact that crosslinking agents give rise to fragments that are too large for detection in the mass spectrometer. In the 58 kDa protein band, the tryptic fragment of K5 containing C54 (amino acids V48 to R62; Figure 6) could not be found. However, a fragment corresponding to the weight of the C54 fragment plus the weight of one bound mBBR could be detected as the M^{2+} ion with a m/z of 772.37. The tandem mass spectrometry fragmentation pattern was in accordance with a C54-bimane-derivatized tryptic fragment (V48 to R62; Figure 5). We thus found evidence that the thiol-reactive hapten mBBR modifies C54 of K5 in intact, full-thickness human skin. To our knowledge, exact hapten reaction sites in skin are previously unreported.

Haptens provoke production of K14 antibodies *in vivo*

To this point, the hapten targets were studied in excised human tissue *ex vivo*. To investigate the clinical relevance of

the finding that keratins are the hapten targets for the thiol-reactive bromobimanes, the hypothesis had to be tested in an *in vivo* setting. A modified LLNA experiment was performed where serum samples from mice subjected to mBBr or dBBr were collected. To investigate if the sera contained anti-keratin antibodies, the serum samples were analyzed by an anti-K14 ELISA. Indeed, elevated levels of anti-keratin antibodies were found in the sera of mice exposed to mBBr and dBBr compared with irritant control (Supplementary Figure S9 online). This suggests that exposure to the bromobimanes *in vivo* generates cryptic epitopes normally concealed from the immune system, which supports our *ex vivo* findings.

DISCUSSION

Using thiol-reactive caged fluorophores, i.e., bromobimanes, as model haptens, this study demonstrates that keratins K5 and K14 in keratinocytes may have an important role in the mechanism of ACD. As keratinocytes make up ~95% of the epidermis, their importance in ACD is not surprising. Furthermore, the key role of keratins in sensitization and elicitation can be considered to be a matter of yield, as keratins make up $\geq 80\%$ of the total cellular protein in fully differentiated keratinocytes, and up to 35% of the basal keratinocytes.

The identified hapten target, i.e., amino acid C54 of K5, is located in the head region of the protein, a region that makes crucial contacts with desmoplakin in the desmosomes (Kouklis *et al.*, 1994). The basal cells in epithelia are welded tightly together by bundling keratins together into strong keratin intermediate filaments. This ensures a high effective local concentration of the peptide stretch containing C54, and thus strong anchoring to desmoplakin, strengthening the epithelial integrity. This position reduced C54 in a high effective concentration just inside the cell membranes, constituting an ideal target for incoming electrophilic haptens. Thus, as our results demonstrate that C54 is a key target of thiol-reactive haptens, this implies that the formed HPC may cause a malfunction of the desmoplakin anchoring. This could result in destruction of the desmosomes, and the release of cryptic epitopes from dying keratinocytes. These cryptic epitopes could then be taken up by the antigen-presenting cells of the skin and be presented to naive T cells in the lymph nodes, sensitizing the exposed individual. Furthermore, as anti-K14 antibodies were detected in the serum of hapten-exposed mice, this indicates that hapten-specific T cells provide the necessary help to B cells in recognizing K14 to become activated and produce anti-K14 antibodies.

Many clinically relevant haptens are thiol reactive (Gerberick *et al.*, 2004; Natsch and Gfeller, 2008). Thus, the bromobimanes, which are selectively reacting with thiols, serve as good model compounds for clinically relevant haptens. It should be noted that haptens with different chemical reactivity might lead to binding to other protein targets (Meschkat *et al.*, 2001; Gerberick *et al.*, 2007; Karlsson *et al.*, 2009). Indeed, other research groups have described other hapten targets, e.g., using human serum

albumin and K14 as model proteins *in vitro* (Thierse *et al.*, 2004; Heiss *et al.*, 2005; Aleksic *et al.*, 2008; Dietz *et al.*, 2010; Jenkinson *et al.*, 2010). However, this study is, to the best of our knowledge, the only report on the identification of a haptenation site in human skin tissue, obtained with the complete set of potential skin protein targets available for reaction.

A limitation of the study is the lack of viable cells in the experiments performed on excised skin tissue *ex vivo*. Nevertheless, serum samples from mice topically exposed to the haptens confirm the formation of recurrent cryptic epitopes in living organisms. This supports our *ex vivo* findings, and further work on cell cultures is in progress.

Emerging from this work is a theory where recurring neoepitopes are formed upon hapten exposure. The generated knowledge that keratinocytes might take an active role in the development of ACD could be helpful in the development of alternative methods replacing animal models for allergy testing in the future. Based on these findings, the generality of the proposed theory is now subject for further investigations.

MATERIALS AND METHODS

Chemicals

Both dBBr and methylbimane were obtained from Sigma-Aldrich (St Louis, MO) and mBBr from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

LLNA

Sensitization potential of the compounds was tested in DMSO using the LLNA. The experiments were approved by the local ethics committee and performed as described elsewhere (Samuelsson *et al.*, 2009).

Two-photon microscopy

Full-thickness human skin obtained from breast reduction surgery, stored at -70°C and used within 3 months from surgery, was mounted in vertical (Franz-type) skin penetration systems (Laboratory Glass Apparatus, Berkely, CA) and exposed to vehicles prepared from stock solutions of bimanes in ACN, by dilution with phosphate-buffered saline (PBS), giving a final concentration of 5–20 mM in PBS/ACN (9:1). The vehicle (0.5 ml) was added to the donor compartment and the acceptor compartment was filled with PBS. The skin was incubated for 20 hours at 32°C . The bimane-exposed skin samples were rinsed with PBS and mounted in imaging chambers. TPM was performed (see Supplementary Materials and Methods online for details).

Immunohistochemistry and confocal microscopy

Skin exposed to bimane solutions were subject to vertical cryosectioning. The samples were incubated in normal nonimmune goat serum (Invitrogen, Carlsbad, CA) before incubation with primary antibodies: CD1a mouse anti-human monoclonal antibody (Santa Cruz Biosciences, Santa Cruz, CA) or K5 and K14 guinea pig anti-human polyclonal antibodies (Lifespan Biosciences, Seattle, WA); and secondary antibodies: tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-mouse (Santa Cruz Biosciences) or

Alexa Fluor 633-labeled goat anti-guinea pig antibodies (Invitrogen). The sections were thereafter imaged using laser scanning confocal microscopy (see Supplementary Materials and Methods online for details).

SDS-PAGE

The skin pieces exposed to bimanos were tape stripped to remove stratum corneum and immersed in warm water (60 °C) for 60 seconds followed by separation of epidermis and dermis. Proteins were extracted by heating the samples in SDS/Tris-buffer, pH 8.5. The concentration of proteins in each sample was determined by BCA Protein Assay (Pierce, Rockford, IL).

The samples were diluted with XT Sample Buffer 4X (Bio-Rad, Hercules, CA) and Milli-Q grade water (Millipore, Billerica, MA). Dithiothreitol (1 µl; 2 M) was added to each sample followed by heating at 96 °C for 2 minutes before application on 10% Bis-Tris precast polyacrylamide gels (Invitrogen).

Electrophoresis was performed using 3-(N-morpholino)propane-sulfonic acid running buffer. The gels were fixed, scanned for fluorescence (excitation 400 nm, emission 530 nm) using a 2920 2D MasterImager (Amersham Pharmacia Biotech, Uppsala, Sweden), and stained with Bio-Safe Coomassie G-250 (Bio-Rad).

Western blot

Unfixed gels were blotted onto polyvinylidene difluoride membranes (Bio-Rad). The blots were blocked for 2 hours in buffer containing 50 mM Tris-HCl, 0.2 M NaCl, and 3% nonfat dry milk (Bio-Rad), incubated for 1 hour with primary K5 or K14 guinea pig anti-human polyclonal antibodies (1:5,000; Lifespan Biosciences), and incubated for 30 minutes with a peroxidase-labeled rabbit anti-guinea pig IgG (H+L) secondary antibody (Invitrogen; 1:2,000). The blots were washed 3 × 10 minutes with buffer consisting of 50 mM Tris-HCl, 0.2 M NaCl, and 0.05% Tween-20 between each step and developed with 3,3'-diaminobenzidine.

In-gel trypsinization, extraction of proteins, and nanoflow liquid chromatography-tandem mass spectrometry

Selected protein bands were excised from the coomassie-stained gel and analyzed with nanoflow liquid chromatography-tandem mass spectrometry (see Supplementary Materials and Methods online for details).

ELISA

Sera obtained from mice exposed to bimanos or SDS for three consecutive days were collected and pooled for each group ($N=3$) and analyzed by an anti-K14 ELISA. An antibody directed toward the immunoglobulin classes IgA, IgM, and IgG was used (see Supplementary Materials and Methods online for details).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Aleksic M, Pease CK, Basketter DA *et al.* (2008) Mass spectrometric identification of covalent adducts of the skin allergen 2,4-dinitro-1-chlorobenzene and model skin proteins. *Toxicol In Vitro* 22:1169–76
- Bauer J, Bahmer FA, Worl J *et al.* (2001) A strikingly constant ratio exists between Langerhans cells and other epidermal cells in human skin. A stereologic study using the optical disector method and the confocal laser scanning microscope. *J Invest Dermatol* 116:313–8
- Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* 6:328–40
- Clayton E, Doupe DP, Klein AM *et al.* (2007) A single type of progenitor cell maintains normal epidermis. *Nature* 446:185–9
- De Groot A (2008) *Patch Testing. Test Concentrations and Vehicles for 4350 Chemicals*, 3rd edn. Acdegroot Publishing: Waperveen
- Dietz L, Esser PR, Schmucker SS *et al.* (2010) Tracking human contact allergens: from mass spectrometric identification of peptide-bound reactive small chemicals to chemical-specific naive human T-cell priming. *Toxicol Sci* 117:336–47
- Dillman III JF, McGary KL, Schlager JJ (2003) Sulfur mustard induces the formation of keratin aggregates in human epidermal keratinocytes. *Toxicol Appl Pharmacol* 193:228–36
- Eichner R, Sun TT, Aebi U (1986) The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. *J Cell Biol* 102:1767–77
- Ellis-Davies GCR (2007) Caged compounds: photorelease technology for control of cellular chemistry and physiology. *Nat Methods* 4:619–28
- EU (2003) Directive 2003/15/EC of the European Parliament and of the Council of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of laws on the Member States relating cosmetic products. *OJ L0 66* 2003:26–35
- Fuchs E, Cleveland DW (1998) A structural scaffolding of intermediate filaments in health and disease. *Science* 279:514–9
- Gawkrodger DJ, Haftek M, Botham PA *et al.* (1989) The hapten in contact hypersensitivity to dinitrochlorobenzene-immuno-electron microscopic and immunofluorescent studies. *Dermatologica* 178:126–30
- Gerberick GF, Vassallo JD, Bailey RE *et al.* (2004) Development of a peptide reactivity assay for screening contact allergens. *Toxicol Sci* 81:332–43
- Gerberick GF, Vassallo JD, Foertsch LM *et al.* (2007) Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. *Toxicol Sci* 97:417–27
- Hachisuka H, Nomura H, Mori O *et al.* (1990) Alterations in membrane fluidity during keratinocyte differentiation measured by fluorescence polarization. *Cell Tissue Res* 260:207–10
- Heiss K, Junkes C, Guerreiro N *et al.* (2005) Subproteomic analysis of metal-interacting proteins in human B cells. *Proteomics* 5:3614–22
- Jenkinson C, Jenkins RE, Aleksic M *et al.* (2010) Characterization of p-phenylenediamine-albumin binding sites and T-cell responses to hapten-modified protein. *J Invest Dermatol* 130:732–42
- Karlberg AT, Bergström MA, Börje A *et al.* (2008) Allergic contact dermatitis - formation, structural requirements, and reactivity of skin sensitizers. *Chem Res Toxicol* 21:53–69
- Karlsson I, Hillerstrom L, Stenfeldt AL *et al.* (2009) Photodegradation of dibenzoylmethanes: potential cause of photocontact allergy to sunscreens. *Chem Res Toxicol* 22:1881–92

- Kimber I, Dearman RJ, Basketter DA *et al.* (2002) The local lymph node assay: past, present and future. *Contact Dermatitis* 47:315–28
- Kosower NS, Kosower EM, Newton GL *et al.* (1979) Bimane fluorescent labels: labeling of normal human red cells under physiological conditions. *Proc Natl Acad Sci USA* 76:3382–6
- Kouklis PD, Hutton E, Fuchs E (1994) Making a connection: direct binding between keratin intermediate filaments and desmosomal proteins. *J Cell Biol* 127:1049–60
- Landsteiner K, Jacobs J (1935) Studies on the sensitization of animals with simple chemical compounds. *J Exp Med* 61:643–56
- Meschkat E, Barratt MD, Lepoittevin JP (2001) Studies of the chemical selectivity of hapten, reactivity, and skin sensitization potency. 2. NMR studies of the covalent binding of the C-13-labeled skin sensitizers 2-C-13 - and 3- C-13 hex-1-ene- and 3- C-13 hexane-1,3-sultones to human serum albumin. *Chem Res Toxicol* 14:118–26
- Moll R, Divo M, Langbein L (2008) The human keratins: biology and pathology. *Histochem Cell Biol* 129:705–33
- Natsch A, Gfeller H (2008) LC-MS-based characterization of the peptide reactivity of chemicals to improve the *in vitro* prediction of the skin sensitization potential. *Toxicol Sci* 106:464–78
- Rustemeyer T, van Hoogstraten IMW, Blomberg BME *et al.* (2001) Mechanisms in allergic contact dermatitis. In: *Textbook of Contact Dermatitis*. (Rycroft RJG, Menné T, Frosch PJ, Lepoittevin J-P, eds) 3rd edn. Springer: New York, pp 15–58
- Samuelsson K, Simonsson C, Jonsson CA *et al.* (2009) Accumulation of FITC near stratum corneum-visualizing epidermal distribution of a strong sensitizer using two-photon microscopy. *Contact Dermatitis* 61:91–100
- Steinert PM (1993) Structure, function, and dynamics of keratin intermediate filaments. *J Invest Dermatol* 100:729–34
- Sun TT, Green H (1978) Keratin filaments of cultured human epidermal cells. Formation of intermolecular disulfide bonds during terminal differentiation. *J Biol Chem* 253:2053–60
- Sylvestre JP, Bouissou CC, Guy RH *et al.* (2010) Extraction and quantification of amino acids in human stratum corneum *in vivo*. *Br J Dermatol* 163:458–65
- Tetko IV, Gasteiger J, Todeschini R *et al.* (2005) Virtual computational chemistry laboratory - design and description. *J Comput-Aided Mol Des* 19:453–63
- Thierse HJ, Moulon C, Allespach Y *et al.* (2004) Metal-protein complex-mediated transport and delivery of Ni²⁺ to TCR/MHC contact sites in nickel-specific human T cell activation. *J Immunol* 172:1926–34
- Thyssen JP, Linneberg A, Menné T *et al.* (2007) The epidemiology of contact allergy in the general population - prevalence and main findings. *Contact Dermatitis* 57:287–99