The Cutaneous Biochemical Redox Barrier: A Component of the Innate Immune Defenses against Sensitization by Highly Reactive Environmental Xenobiotics

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The Cutaneous Biochemical Redox Barrier: A Component of the Innate Immune Defenses against Sensitization by Highly Reactive Environmental Xenobiotics

Chris Pickard, Fethi Louafi, Carolann McGuire, Kelly Lowings, Pawan Kumar, Hywel Cooper, Rebecca J. Dearman, Marie Cumberbatch, Ian Kimber, Eugene Healy, and Peter S. Friedmann

Contact allergy to environmental xenobiotics is a common and important problem, but it is unclear why some chemicals are potent sensitizers and others weak/nonsensitizers. We explored this by investigating why similar chemicals, 2,4-dinitrochlorobenzene (DNCB) and 2,4-dinitrothiocyanobenzene (DNTB), differ in their ability to induce contact hypersensitivity (CHS). DNCB induced CHS in humans, whereas at similar doses DNTB did not. However, following DNCB sensitization, DNTB elicited CHS in vivo and stimulated DNCB-responsive T cells in vitro, suggesting that differences in response to these compounds lie in the sensitization phase. In contrast to DNCB, DNTB failed to induce emigration of epidermal Langerhans cells in naïve individuals. Examination for protein dinitrophenylation in skin revealed that DNCB penetrated into the epidermis, whereas DNTB remained bound to a thiol-rich band within the stratum corneum. DNTB reacted rapidly with reduced glutathione in vitro and was associated with a decrease in the free thiol layer in the stratum corneum, but not in the nucleated epidermis. By contrast, DNCB required GST facilitation to react with glutathione and, following penetration through the stratum corneum, depleted thiols in the viable epidermis. Chemical depletion of the thiol-rich band or removing it by tape stripping allowed increased penetration of DNTB into the epidermis. Our results suggest that the dissimilar sensitizing potencies of DNCB and DNTB in humans are determined by a previously undescribed outer epidermal biochemical redox barrier, a chemical component of the innate immune defense mechanisms that defend against sensitization by highly reactive environmental chemicals. The Journal of Immunology, 2009, 183: 7576–7584.

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3 Abbreviations used in this paper: DNFB, 2,4-dinitrofluorobenzene; GSH, reduced glutathione; LC, Langerhans cell; MBB, monobromobimane; RU, relative unit; TCC, T cell clones.

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include their capacity to bind to proteins, their properties as irritants, and their ability to penetrate into the viable epidermis. Being an irritant may facilitate generation of appropriate danger signals that in turn are believed to augment the Ag-presenting capacity of DCS, and hence, to enhance the initiation of immune responses.

We thus wished to investigate possible mechanisms relating to potential differences in the irritant and/or cutaneous penetrating properties of DNCB and DNTB. We therefore examined the interactions of both these compounds in relation to their capacity to induce emigration of Lcs, to induce cytokine expression, and to penetrate through the stratum corneum. We show that contrary to being a tolerizer, DNTB is a weak sensitizer in humans and exposure to DNTB can actually augment a subsequent response to DNCB. In addition, and in contrast to DNCB, DNTB fails to induce significant emigration of Lcs in human skin. This difference in sensitizing potential appears to be the result of different chemical interactions within the skin. DNCB permeates the viable layers of epidermis, whereas DNTB reacts with a sulfydryl-rich biochemical barrier in the stratum corneum and is therefore predominantly retained within this upper epidermal layer. We propose that the different sensitizing potencies of these two chemicals in humans are determined by the epidermal biochemical barrier, a previously unrecognized component of the innate immune defense against reactive environmental chemicals.

Materials and Methods

Volunteers

Adults of both sexes gave written informed consent to participate in these studies. The project was approved by the local research ethics committee (LREC 130/00).

Chemicals

DNCB was purchased from Sigma-Aldrich. DNTB was obtained from Lancaster Synthesis as a 95% pure preparation.

Experimental design

The overall approach was as follows (Fig. 1): 1) to establish responses to a standard sensitization regimen with DNCB; 2) to see whether DNTB could induce and elicit contact sensitivity to itself; 3) to see whether pre-exposure to DNTB could alter subsequent responses to DNCB; and 4) to see whether DNTB could elicit responses in individuals initially sensitized with DNCB.

The basic approach was to apply a sensitizing exposure of chemical (either DNCB or DNTB) and then, 4 wk later, to apply an eliciting challenge of four graded doses (6.25, 8.8, 12.5, and 17.7 μg/cm²) of the same chemical applied on 7-mm-diameter Finn chambers. For the experiments designed to see whether pre-exposure to DNTB would modify subsequent sensitization by DNCB, after the initial DNTB exposure, reactivity to DNCB would be elicited 4 wk later by challenge with four doses of DNCB, as above. At 48 h, lack of response to this challenge might reflect absence of sensitization or tolerization, whereas increased responses would indicate low level (subclinical) immunological priming. To distinguish between these possibilities, subsequent experimental design made use of the fact that the DNCB challenge regimen of four doses is itself an effective sensitizing dose (12). Therefore, a second challenge with four doses of DNCB applied 4 wk later (at a different site) would show whether the sensitizing capacity of the first DNCB challenge had been modified by pre-exposure to DNTB. The necessary control group received the four DNCB challenge doses as the initial sensitizing exposure, and the responses to an elicitation challenge with the same four doses of DNCB applied 4 wk established the response against which other responses could be compared (Fig. 1).

Sensitization and elicitation of contact sensitivity with DNCB

For group 1 (n = 12), sensitization to DNCB was induced by application of the regimen normally used to elicit contact sensitivity (12). This comprised four doses of DNCB (6.25, 8.8, 12.5, and 17.7 μg/cm²) applied on separate 7-mm aluminum chambers (Finn chamber; Epitests) to the upper inner arm for 6 h. Four weeks later, the strength of sensitization was assessed by a second application of the four-dose challenge regimen, and responses were quantified at 48 h as skinfold thickness with Harpenden calipers with one spring removed to reduce compression (13).

Sensitization and elicitation of contact sensitivity with DNTB

Groups 2 (n = 12) and 3 (n = 11) received an initial dose of DNTB of 70 or 125 μg/cm², respectively, on an 11-mm-diameter Finn chamber, applied for 48 h to the upper inner arm. Four weeks later, sensitization was elicited with four challenge doses of DNTB (6.25, 8.8, 12.5, and 17.7 μg) as for DNCB above.

Effect of priming with DNTB on subsequent responses to DNCB

Group 4 (n = 10) received an initial priming dose of 70 μg/cm² DNTB on an 11-mm Finn chamber; 4 wk later, they were challenged with four doses of DNCB, which were assessed 48 h later for evidence of sensitization induced by DNTB. After additional 4 wk, they received the four-dose challenge regimen with DNTB to assess whether the pre-exposure to DNTB had modified the sensitization by the first challenge with DNCB. Responses were assessed and measured with calipers 48 h after each challenge.

Elicitation of sensitivity with DNTB

For group 5, six individuals from group 1 (sensitized and challenged with DNCB) received an additional challenge with four doses of DNTB at various times (usually more than 8 wk later).

Effect of addition of an irritant (croton oil) to DNTB for induction of sensitization

Group 6 (n = 12) received an initial sensitizing dose of DNTB (70 μg/cm²) plus 3% croton oil. Four weeks later, the presence of sensitization was detected by challenge with four doses of DNTB (as above). Responses were assessed clinically and measured with calipers at 48 h.

Presentation of results

The responses at each of the four elicitation challenge sites were measured at 48 h as skinfold thickness with Harpenden calipers. The responses (minus unchallenged control skin) at the four sites were summed to give an approximation of the area under the curve.

Generation of DNCB-specific T cell clones (TCC) and EBV-transformed B cells

PBMCs from one presensitized individual were stimulated with DNCB (1–5 μM) for 7 days. The cells were then subjected to a second round of Ag challenge, in conjunction with irradiated (3000 rad) autologous PBMC. DNCB-specific clones were generated by limiting dilution assay, and growing cells were expanded further using 20 U/ml IL-2 (PeproTech) and 1 μg/ml phytohemagglutinin (Sigma-Aldrich).

For the measurement of the Ag-specific proliferation of TCC, 10⁵ cells were incubated with 5 × 10⁴ irradiated (6000 rad) autologous EBV-transformed B cells, and 2 μM DNCB or 2 μM DNTB in a U-bottom 96-well plate for 72 h. Proliferation was determined by measuring the incorporation of [³H]thymidine.

Isolation of human primary keratinocytes

Keratinocytes were isolated from healthy foreskins obtained at circumcision. Approval for this study was granted by Southampton and South West Hampshire Local Ethics Committee (Submission 241/01). The skin was incubated overnight with 2 U/ml dispase (In vitrogen) in PBS at 37°C. The epidermis was separated with fine forceps and dissociated in 0.05% trypsin/0.02% EDTA solution (Invitrogen) for 15 min. The resulting cells were centrifuged and cultured in serum-free keratinocyte-specific growth medium (In vitrogen). Cell purity was evaluated by immunocytochemical detection of cytokeratin. All cell preparations used in these studies were within the first or second passage.

Staining of epidermal sheets for CD1a

Three sites (3 × 2 cm) on the lower back of each volunteer were treated with DNCB or DNTB (40 μl of 0.5% = 200 μg; 33.3 μg/cm²) or vehicle control (acetone). Sites were occluded for 2 h after application of the chemicals. At 18 h, a 6-mm punch biopsy sample was taken from each site and placed in 0.02 M EDTA (Sigma-Aldrich) for 30 min at 37°C to allow the separation of the epidermis. The epidermal sheets were stained with an anti-human CD1a-FTTC (1/100; Dako Cytomation), and LCs were counted.

Amplification of cytokine transcripts using SYBR Green real-time RT-PCR assay

Normal human keratinocytes were incubated with 5 μM DNCB or DNTB in DMEM and incubated at 37°C with 5% CO₂ for 6 h. Total RNA was
extracted using RNA-Easy columns (Qiagen), and cDNA was obtained by reverse transcription (Primer Design). TNF-α, IL-1β, IL-6, IL-8, IL-12, and IL-18 transcripts were amplified using a SYBR Green PCR assay master mix and primer sets designed by Primer Design. PCR were performed in duplicate and amplified on an iCycler real-time detection system (Bio-Rad). Resulting cycle threshold values for the DNCB- and DNTB-treated samples were normalized to 28S ribosomal RNA and expressed as relative units (RU) above the vehicle-treated samples.

**Interaction of DNCB or DNTB with glutathione**

DNCB and DNTB (200 μM) were incubated with equimolar concentrations of reduced glutathione (GSH; Sigma-Aldrich) in PBS (pH 7.4) at room temperature. The absorption spectrum was read immediately and following a 60-min incubation with GST (5 U/ml; Sigma-Aldrich), using a SPECTRAmax spectrophotometer (Molecular Devices).

**Staining of frozen sections**

DNCB, DNTB (50–400 μg/cm²), and vehicle (acetone) were applied to skin explants mounted in Franz’s diffusion chambers for 24 h. Frozen sections were stained with a polyclonal goat anti-dinitrophenyl Ab (Sigma-Aldrich; 1/2000) for 1 h at room temperature, followed by the addition of a FITC-labeled anti-goat secondary Ab (Sigma-Aldrich; 1/400). Staining was detected using two-channel microscopy at x40 magnification on a Zeiss confocal laser microscope.

**Glutathione depletion assay**

Detection of free thiol groups in human skin cryosections and in human blood-derived monocytes was performed using monobromobimane (MBB; Sigma-Aldrich). Skin sections exposed to 200 μg/cm² for 24 h and human monocytes treated for 10 min with 5 μM DNCB and DNTB were incubated with 90 μM MBB for 20 min. Positive staining of live monocytes, indicating the presence of free thiols, was determined using flow cytometry, and positive MBB staining of skin sections, counterstained with To-Pro-3, was determined using a laser-inverted confocal microscope (Leica Tcs SP5).

To confirm the importance of a thiol-rich layer within the stratum corneum in reducing DNTB penetration, 90 μM MBB was added epicutaneously to ex vivo skin. After 1 h, DNTB (50 μg/cm²) was added, and the skin was incubated overnight, snap frozen, sectioned, and stained with anti-DNP Abs (as above). In addition, the stratum corneum was removed from ex vivo skin by 20 repeated strips with adhesive tape, and DNTB penetration was assessed using anti-DNP Abs; successful removal of the stratum corneum was confirmed by a H&E stain.

**Statistical analysis**

Statistical significance was assessed by use of ANOVA and Student’s t test (GraphPad statistical package).

**Results**

**Contact sensitivity responses to DNCB and DNTB; DNTB is a weak sensitizer**

Twelve individuals (group 1) received an initial exposure to DNCB comprising four different doses, as used in the elicitation challenge. When the challenge was repeated 4 wk later on the opposite upper arm, all 12 reacted to the DNCB (Figs. 1 and 2a) consistent with our previous experience of this potent sensitizer (12). By contrast, DNTB appeared to be a very weak or nonsensitizer. The groups primed with either 70 or 125 μg/cm² DNTB (groups 2 and 3) gave completely negative responses to the four elicitation doses of DNTB applied 4 wk later (Figs. 1 and 2a). Group 3 was given a repeat of the elicitation challenge of DNTB after a further 4 wk, and 2 of 11 showed weak reactivity (Figs. 1 and 2a). An initial exposure to DNTB (70 μg/cm²), followed by a first challenge/sensitization with DNCB and then 4 wk later a rechallenge with DNCB, failed to demonstrate DNTB-induced tolerance to DNCB (Fig. 1; group 4). By contrast, the initial exposure to DNTB significantly augmented the subsequent sensitization by DNCB (group 1 vs group 4; p = 0.014; Student’s t test).

**DNTB can elicit T cell memory responses induced with DNCB**

When six individuals from group 1 who had been sensitized and challenged with DNCB were rechallenged with DNTB, positive responses were obtained in all six (group 5; Fig. 2, A and B). The mean responses were similar to those obtained with the DNCB challenge (Fig. 2, A and B). In addition, TCC (n = 7; 1 CD4+ clone and 6 CD8+ clones) expanded from the blood of a DNCB-sensitized individual responded upon challenge in vitro with DNTB in the presence of autologous EBV-transformed B cell lines (Fig. 3). Although four clones showed somewhat stronger responses to DNCB than to DNTB, there was no significant difference overall (p > 0.05; Student’s t test).

The above results suggest first that DNTB is inefficient at activating a primary immune response to itself; however, once immunological memory has been established with DNCB, the T cell response recognizes DNTB similarly. This also indicates that DNTB penetrates in sufficient quantities to elicit established memory responses.

**Effects on epidermal LCs**

To explore potential reasons why DNTB was not good at activating a primary immune response, the cellular responses in the epidermis were examined. When biopsies were taken from sites challenged 18 h previously with either DNCB, DNTB (33.3 μg/cm²),
or vehicle, overall, there was a significant reduction in LC numbers only following challenge with DNCB (Fig. 4; \( p = 0.0005, n = 8 \) subjects). The mean percentage reduction in LCs following DNCB was 18% (SE \( \pm 3.3 \)), which is similar to that seen previously in normal volunteers following topical application of the potent chemical contact allergen diphenycyprone (14) or the intradermal administration of cytokines such as TNF-\( \alpha \) (15).

Irritant croton oil augments sensitization by DNTB

The difference in LC migration in response to DNCB and DNTB raised the question of whether the two compounds were inducing different patterns or amounts of cytokine expression within the epidermis. We had noticed that DNCB was clearly irritant at the higher concentrations because it caused erythema in nonsensitized individuals; DNTB was completely nonirritant at all concentrations, including 125 \( \mu \)g/cm\(^2\). We therefore investigated whether the lack of induction of sensitization by DNTB was related to its nonirritancy and, hence, lack of danger signaling. Previously, we had observed that 3% croton oil elicited a moderate erythematous irritant response in all normal volunteers tested (data not shown). Therefore, 3% croton oil was added to the acetone solution of DNTB, and a primary sensitizing dose of 70 \( \mu \)g/cm\(^2\) was applied to 15 volunteers (group 6). When challenged with four doses of DNTB 4 wk later, positive responses were observed in 12 of 15 and were significantly greater than those responses previously observed in group 2 (Fig. 2A; \( p = 0.025 \), Student’s \( t \) test). Thus, adding an irritant stimulus appeared to convert DNTB into a moderate sensitizer.

**DNCB and DNTB elicit similar expression of proinflammatory cytokines**

The role of irritancy in the sensitization potential of chemical hapteners has been well described in murine models, particularly with respect to the release of proinflammatory cytokines such as TNF-\( \alpha \) and IL-1\( \beta \) from resident skin cells, which are important in stimulating the migration of LCs to the draining lymph nodes (15–17). We therefore compared the effects of DNCB and DNTB on IL-1\( \beta \), IL-6, IL-8, IL-12, IL-18, and TNF-\( \alpha \) RNA transcription by primary human keratinocytes. Following exposure of keratinocytes to DNCB, there was an increase in the expression of IL-1\( \beta \), IL-6, IL-8, and TNF-\( \alpha \) (Fig. 5). However, there was no significant difference overall in the responses evoked by DNTB to those produced by DNCB. Moreover, the DNTB-treated keratinocytes showed greater transcription of IL-8, (DNCB median RU, 1.27; interquartile range, 1.06; DNTB median, 6.72; interquartile range, 4.4), although this did not reach statistical significance (\( p = 0.09 \)).

**FIGURE 4.** DNTB fails to induce LC migration. Human volunteers were challenged with DNCB, DNTB, or diluent control, and biopsied after 18 h. Epidermis was removed and stained with an anti-CD1a Ab, and LCs were counted and expressed as number/mm\(^2\). The bar indicates the mean migration for each group.
Penetration of DNCB and DNTB into human skin is different

DNCB is significantly more lipid soluble than DNTB; hence, another reason it may be a more potent sensitizer could be due to differences in skin penetration. It would be expected that, following penetration into the epidermis, both chemicals would react with proteins, dinitrophenylating them. After application of each compound to the outer epidermal surface of skin explants mounted in Franz’s diffusion chambers, a polyclonal Ab that recognizes the dinitrophenyl compound of both chemicals, regardless of the protein carrier, was used to stain skin cryosections. It was observed that DNCB dinitrophenylated proteins throughout the epidermis and along the basement membrane (Fig. 6B; \( n = 10 \)). By contrast, following treatment with DNTB, there was markedly less dinitrophenylation of proteins within the viable epidermis; however, there was much stronger staining of the stratum corneum (Fig. 6C; \( n = 10 \)). To ensure that the observed difference in the staining patterns was not due to differences in the specificity of the Ab for the two chemicals, primary keratinocytes were incubated with either vehicle alone or 5 \( \mu \)M DNTB or DNCB for 10 min. Subsequent staining with the anti-DNP Ab showed that there was extensive dinitrophenylation of intracellular proteins by both chemicals and that a greater intensity of staining was apparent in the keratinocytes that had been treated with DNTB (Fig. 7; \( n = 4 \)).

The data therefore suggest that whereas DNTB penetrates very well into cultured keratinocytes, it is less able to penetrate through the stratum corneum, which in turn may explain the poor sensitization potency of the chemical. This raised the possibility that the augmented sensitization by DNTB supplemented with 3% croton oil (Fig. 2A; group 6) may simply have reflected enhanced penetration of the DNTB as a result of damage to the skin barrier by the croton oil. This was examined in human skin explants treated epicutaneously with DNTB in acetone with 3% croton oil. Increased

![Figure 5](http://www.jimmunol.org/)  
**FIGURE 5.** Challenge of primary keratinocytes with DNCB and DNTB induces the transcription of proinflammatory cytokines. Human primary keratinocytes isolated from skin biopsies were challenged with an equimolar concentration of both DNCB and DNTB. RNA was extracted, and transcripts for proinflammatory cytokines were amplified using quantitative RT-PCR. Expression is shown as RU above untreated cells, with the bar indicating the median response for each group.

![Figure 6](http://www.jimmunol.org/)  
**FIGURE 6.** The differential penetration of human skin by DNCB and DNTB. Normal human breast skin, mounted in Franz’s diffusion chambers, was challenged with vehicle alone (A), DNCB (B), DNTB (C), and DNTB and croton oil (D) for 24 h. Cryosections were obtained and stained with an anti-DNP Ab to detect protein dinitrophenylation (shown in green). Nuclear staining was conducted using To-Pro-3 (shown in blue), and the scale bar represents 50 \( \mu \)m. Figures show a representative stain of 10 independent experiments.
staining of dinitrophenylated proteins within the viable epidermis was observed in four of seven samples (Fig. 6D), suggesting enhanced penetration of the DNTB in the presence of the irritant substance.

**DNCB and DNTB react differently with free thiols (glutathione)**

The different patterns of dinitrophenylation of epidermal proteins could reflect not only penetration, but also a different chemical interaction with the stratum corneum, which is rich in sulfur-containing proteins (18). Indeed, it was noted in our earlier in vivo work in humans that DNTB, in contrast to DNCB, caused a yellowish discoloration of the skin immediately after its application. We therefore examined the interaction of the two chemicals with free thiols by examining their interaction with glutathione. The reactions between equimolar concentrations (200 μM) of GSH, as a source of free thiol, and DNTB or DNCB were followed spectrophotometrically and were noted to be substantially different. DNCB reacted very slowly on its own, but after the addition of 1 U of GST, there was rapid formation of a compound with maximal absorption at ~340 nm (Fig. 8). By contrast, DNTB reacted very rapidly with GSH to form a yellow compound with maximal absorption at 408 nm (Fig. 8); this latter reaction was virtually complete within minutes, and there was very little further increase in absorption at 408 nm over time. The reaction between GSH and DNTB was unaffected by the addition of 1 U of GST at the start of the reaction.

To explore the ability of the two compounds to bind to, and therefore deplete, intracellular glutathione, human monocytes and skin explants were exposed to DNCB and DNTB, and the levels of glutathione were quantified using MBB. In the monocytes, DNTB caused a significantly greater decrease of intracellular glutathione than DNCB at 5 and 30 min (Fig. 9; n = 3, Student’s t test, p = 0.028 and 0.048, respectively). In the studies of skin explants, MBB staining of cryosections revealed that DNTB dramatically reduced the levels of glutathione in the stratum corneum only, whereas DNCB depleted it throughout the epidermis, but did not completely reduce the glutathione levels in the stratum corneum (Fig. 10).

Epicutaneous application of MBB to ex vivo skin confirmed the presence of a thiol-rich layer within the stratum corneum (Fig. 11), situated immediately above the nucleated epidermis, and colocalized to the region of intense DNTB staining observed in Fig. 6B. This thiol-rich layer prevented the penetration of both the MBB and the DNTB into the epidermis, as evidenced by the increased penetration of DNTB into the epidermis following the removal of the stratum corneum by repeated tape stripping of the skin (Fig. 12). In addition, epicutaneous pretreatment of skin with 90 and 900 μM MBB (n = 3) for 1 h saturated the thiol-rich area and greatly enhanced the penetration of DNTB into the epidermis (Fig. 12). Similarly, application of higher concentrations of DNTB (up to 400 μg/cm²) also saturated the thiol-rich region, allowing greater access of the chemical into the epidermis (Fig. 13). The penetration of DNTB at higher doses correlates with our in vivo work in which we demonstrated that, despite being a poor sensitizer in comparison with DNCB, presensitization with high doses of

![FIGURE 7](image_url) Both DNCB and DNTB cause an extensive dinitrophenylation of intracellular proteins in keratinocytes. Primary human keratinocytes were exposed to vehicle (A), DNCB (B), and DNTB (C; 5 μM) for 10 min. Intracellular dinitrophenylation was detected using a polyclonal anti-DNP Ab (shown in green). Nuclear staining was conducted using To-Pro-3 (shown in blue), and the scale bar represents 300 μm. Figures show a representative stain of four independent experiments.

![FIGURE 8](image_url) Differential binding of DNCB and DNTB to the free thiol GSH. The initial absorption spectrum was taken from equimolar mixtures (200 μM) of DNCB (●) or DNTB (▲) with GSH in PBS at room temperature. GST was added (5 U/ml), and after 1 h the absorption spectrum was taken again: DNCB, ▼; DNTB, ◆.

![FIGURE 9](image_url) DNTB causes a greater depletion of intracellular glutathione. Human monocytes, isolated from PBMC, were incubated with 5 μM DNCB (■) and DNTB (▲) for up to 30 min. Intracellular glutathione levels were detected by flow cytometry using MBB. The glutathione content of the cells is expressed as the percentage of mean fluorescent intensity of the untreated cells. The graph shows the mean of three experiments with the error bars indicating the SEM. *p < 0.05 (Student’s t test).
DNB (125 µg/cm²) induced weak sensitization in 2 of the 11 individuals (group 2; Fig. 2B).

Discussion

The results of the in vivo sensitization experiments reconfirm that DNBCB is a potent inducer of contact hypersensitivity (CHS) in humans and establish T cell-mediated immunological memory. However, DNTB was shown to be a weak immunogen, inducing low-level sensitization only at high exposure concentrations and with repeated exposure. Importantly, DNTB failed to induce immunological tolerance, or to impair subsequent sensitization by DNBCB. Rather, it was able to induce subclinical priming for subsequent sensitization by DNBCB. Thus, DNBCB is potently able to activate a primary immune response, whereas DNTB is barely able to do so. Our observations help to distinguish between possible explanations for the apparent difference in sensitizing potency; these include the failure of DNTB to form an immunogen, an immunizing Ag; differences between the two compounds in their capacity to activate DCs via danger signals; and differences between the compounds in their capacity to penetrate into skin.

DNTB appears to form Ag(s) that are similar, if not identical with those of DNBCB because they are recognized by DNBCB-induced immunological memory. Thus, TCC generated by stimulation with DNBCB recognized both molecules and responded equally. This suggests the dinitrophenyl group is the hapten, which may differ in this respect is that DNBCB is clearly an irritant, and application of as little as 12.5 µg to nonsensitized individuals can produce a red mark in the skin after 24–48 h. DNTB is virtually nonirritant, and quantities as high as 125 µg/cm² never generated any redness or irritation. It is notable that Kimber et al. (7) observed an irritant response in a single human volunteer who received 100 µg of DNTB as the sensitizing dose. We observed that epidermal LCs responded differently to topical application of similar molar concentrations of DNBCB and DNTB. Thus, after in vivo exposure to DNBCB, there was a significant depletion of LCs from the epidermis; however, there was no tendency for LCs to have emigrated following DNTB exposure. This is consistent with two independent studies performed in mice in which significant LC migration was observed following topical application of DNBCB, but not DNTB (10, 11); the accumulation of DCs in the regional lymph nodes in one of those studies suggested the possibility that other DCs in mice, such as dermal dendrocytes, are activated to migrate by DNTB. Consequently, it might be expected that the key cytokines involved in emigration of LCs from the epidermis, TNF-α and IL-1β, would be generated by exposure to DNBCB, but not by DNTB. We therefore investigated whether the provision of an irritant danger signal could make DNTB a more effective sensitizer. The addition of 3% croton oil to the sensitizing dose of DNTB did indeed result in sensitization of those individuals, although to a lesser degree than after sensitization with DNBCB. Both chemicals induced similar up-regulation of cytokine mRNA expression in keratinocytes (although we did not quantify the protein levels of the cytokines and it is conceivable there could be differences at the protein level). Nevertheless, the apparent lack of difference in the cytokine responses induced by the two chemicals prompted us to explore

![FIGURE 10.](Image) DNBCB causes greater depletion of glutathione in the epidermis than DNTB. Normal human breast skin mounted on Franz’s diffusion chambers was treated with vehicle alone (A), DNBCB (B), and DNTB (C), and incubated for 12 h at 37°C. Cryosections were obtained, and glutathione levels were determined using MBB (shown in blue). Nuclear staining was carried using To-Pro-3 (shown in red), and the scale bar represents 50 µm. Figure shows representative stain from three separate experiments.

![FIGURE 11.](Image) The stratum corneum contains a thiol-rich layer. Human breast skin (n = 3) was treated epicutaneously with diluent control (A) and MBB (B; 90 µM) for 1 h at room temperature. Cryosections were obtained, and MBB binding to free thiols was detected by fluorescent microscopy (blue). Nuclei were counterstained with To-Pro-3 (red).

![FIGURE 12.](Image) DNTB penetration is enhanced by saturation or removal of the stratum corneum thiol-rich layer. Skin was pretreated with either vehicle alone (A) or MBB (B) for 1 h, or repeatedly tape stripped (×20) to remove the stratum corneum (C), before the application of DNTB (50 µg/cm²). Protein binding was determined with an anti-DNP Ab (green). Nuclear staining was carried using To-Pro-3 (shown in blue), and the scale bar represents 50 µm.
the nature of their penetration and their chemical interaction with cells and tissues.

The question of how xenobiotic chemicals such as these might signal danger to DCs, activating them so they become capable of stimulating naive T cells, is not clear. There are likely to be at least two components to the process, as follows: one is a stress to the cell such as an oxidative stress; the other may be the result of the formation of the activating immunogen through interaction with and haptenation of cellular proteins. When a xenobiotic such as DNCB enters a cell, it would normally undergo detoxification by conjugation with scavengers such as glutathione. In the case of DNCB, this involves enzymatic conjugation by GST. The glutathionyl conjugate is then exported from the cell, with a net depletion of intracellular glutathione. However, DNTB can react directly with glutathione (or other thiol groups) without the need for enzymatic activity (20). 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epidermis (Fig. 7), it was clear that croton oil was also augmenting the penetration of DNTB; the increased sensitizing potency of DNTB with croton oil may have been due to either the croton oil-induced activation of DCs (either LCs or dermal dendrocytes) and/or the increased exposure quantity of DNTB, or a combination of both.

It was previously known that the outer layers of the stratum corneum are sulfur rich (18), but it was generally presumed that this was a reflection of the structural properties of the area. However, we have shown for the first time that the sulfur-rich layer of the stratum corneum performs a biochemical buffering function that makes an important contribution to the overall barrier function of the skin and thus affects the downstream immunological responses to a chemical sensitizer. It will now be important to investigate whether this biochemical buffer contributes to individual susceptibility to other contact sensitizers, irritants, and environmental xenobiotics.

Disclosures
The authors have no financial conflict of interest.

References