Characterization of the Sensitizing Potential of Chemicals by *In Vitro* Analysis of Dendritic Cell Activation and Skin Penetration

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Development of *in vitro* models to identify sensitizing chemicals receives public interest since animal testing should be avoided whenever possible. In this article we analyze two essential properties of sensitizing chemicals: skin penetration and dendritic cell (DC) activation. Activation of immature DC derived from peripheral blood monocytes was evaluated by flow cytometric analysis of CD86 positive cells and quantitative measurement of interleukin-1β and aquaporin P3 gene expression. The sensitizer 2,4,6-trinitrobenzenesulfonic acid induced a concentration-dependent response for all parameters, whereas the irritant sodium lauryl sulfate did not. When two related aromatic amines, p-toluylenediamine (PTD) and hydroxyethyl-p-phenylenediamine (HE-PPD) were tested, both induced substantial DC activation indicating their potential sensitizing properties. These findings contrasted with *in vivo* results: in murine local lymph node assays (LLNA) PTD, but not HE-PPD, was sensitizing using acetone/aqua/olive oil as vehicle. Skin penetration measurement revealed that this was due to bioavailability differences. On retesting HE-PPD in the LLNA using the penetration enhancer dimethylsulfoxide as vehicle, it induced a specific response. We conclude that *in vitro* analysis of DC activation capability of the two selected chemicals demonstrates that prediction of skin sensitization potential is possible provided that skin penetration data indicate sufficient bioavailability of the test compound.

Key words: CD86 antigen/IL-1β/aquaporins/LLNA/hair dyes
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Allergic contact dermatitis (ACD) is a delayed-type hypersensitivity reaction induced by small reactive chemicals (haptens). In pharmaceutical and cosmetic industries it is mandatory to identify chemicals that are potential ACD inducers before they become part of a new product. Currently, the sensitizing potential of chemicals is usually identified on the basis of animal studies, such as the local lymph node assay (LLNA). There is, however, an increasing public concern regarding the use of animal testing for the screening of new chemicals. The development of *in vitro* models for predicting the sensitizing potential of new chemicals is therefore receiving widespread interest.

In vitro sensitization tests are furthermore needed to identify the relevant aspects of the complex interactions of a chemical with the different compartments of the immune system (Ryan *et al*, 2001): The chemical must penetrate the skin and react with endogenous proteins. Some chemicals, termed prohaptens, require activation through skin metabolism in order to become haptens capable of binding to skin proteins. Haptenated self-proteins are internalized and processed by immature dendritic cells (DC). DC activation occurs upon skin contact with immunogenic chemicals and activated DC upregulate a set of cell surface markers (e.g., CD83 or CD86), secrete various cytokines, such as interleukin (IL)-1β, and downregulate proteins involved in antigen uptake such as aquaporins. In parallel, activated DC start to migrate from the epidermis into the draining lymph node, complete maturation and present fragments of the haptenated self-proteins to T helper cells, resulting in an antigen-specific immune response (Lepoittevin *et al*, 1998; Smith and Hotchkiss, 2001).

The early work of Enk and Katz (1992), who demonstrated that topical exposure of mice to chemical allergens induces a rapid and specific upregulation of IL-1β in skin DC, initiated the work of many research groups who attempted to correlate the sensitizing potential of a chemical with its ability to activate *in vitro* generated DC (see Ryan *et al*, 2001 or Kimber *et al*, 2001, for recent reviews). One important step towards the development of *in vitro* sensitization assays was the generation of dendritic-like cells from human peripheral blood monocytes (Lenz *et al*, 1993; Romani *et al*, 1994; Sallusto and Lanzavecchia, 1994). But due to the variability between human donors and the modest increases (usually approximately 2–3-fold) in IL-1β mRNA upregulation observed *in vitro*, these assays did not represent a robust test system for measuring the sensitizing potential of a given chemical (Reutter *et al*, 1997; Pichowski *et al*, 2000; Kimber *et al*, 2001). The capability of

Abbreviations: AAOO, acetone/aqua/olive oil 2:2:1; ACD, allergic contact dermatitis; AQP3, aquaporin P3; DC, dendritic cell; DMSO, dimethylsulfoxide; FDA, fluorescein diacetate; FITC, fluorescein isothiocyanate; HE-PPD, hydroxyethyl-*p*-phenylenediamine; IL, interleukin; LLNA, local lymph node assay; PE, phycoerythrin; PTD, *p*-toluylenediamine; SDS, sodium lauryl sulfate; TNBS, 2,4,6-trinitrobenzenesulfonic acid

chemicals to induce the activation of *in vitro* generated DC was further evaluated through measurements of HLA-DR, CD54, CD83, CD86 (Aiba *et al*, 1997; Degwert *et al*, 1997; Rougier *et al*, 2000; Tuschl and Kovac, 2001; Hulette *et al*, 2002), the production of various cytokines or chemokines, for example, TNF-α, IL-1α, IL-6, IL-8, IL-10, and IL-12 (Loré *et al*, 1998; Aiba *et al*, 1999), or the phosphorylation of protein kinases and tyrosine (Arrighi *et al*, 2001; Becker *et al*, 2003). Nevertheless, a reliable prediction of the sensitizing potential based on DC activation *in vitro* was not achieved because the *in vitro* results did not reflect the actual ability to induce contact hypersensitivity *in vivo* (Ryan *et al*, 2001).

We assume that the lack of the skin barrier *in vitro* and the difficulties in obtaining relevant DC activation data are responsible for this discrepancy. Therefore, we propose to integrate *in vitro* skin penetration analysis using pig skin, the most suitable model of human skin (Simon and Maibach, 2000; Schmook *et al*, 2001), and to use pooled immature DC obtained from different donors to reduce inter-individual variability that limit the interpretation of DC activation data. The suitability of this approach was tested with two aromatic amines, a class of chemicals comprising several known sensitizers (Malkowski *et al*, 1983). The *in vitro* findings for the two compounds were compared with their potential to induce sensitization *in vivo* as measured by the LLNA in mice.

Results

Human monocytes differentiate into dendritic-like cells in the presence of IL-4 and GM-CSF Human monocytes obtained from peripheral blood and cultured in presence of IL-4 and GM-CSF resulted in cells displaying markers typical of DC phenotype ($CD14^-$, $CD1a^+$, $HLA\text{-}DR^+$, and $CD86^+$, see Figs 1 and 2). After 4 d in culture, these cells were considered as immature DC-like cells, expressing a low amount of CD86, and were used for the *in vitro* stimulation test.

Modulation of the CD86 bright cell population and IL-1β or AQP3 gene expression by exposure to irritant or sensitizer DC-like cells obtained from a pool of four donors were exposed to 2.5 and 5 μg per mL SDS as a model irritant or to 200 μg per mL TNBS as a model sensitizer. Due to the high cytotoxicity of SDS, concentrations higher than 5 μg per mL could not be tested. Since the effect of TNBS was obvious, we wanted to check that the chosen markers were not modulated by cell stress (5 μg per mL of SDS is at the limit of cytotoxicity) and therefore tested two different concentrations of SDS. Deionized water, the solvent for SDS and TNBS was used as negative control. The CD86 positive population can be subdivided into two distinct subpopulations (see Fig 2). The maturation of the DC-like

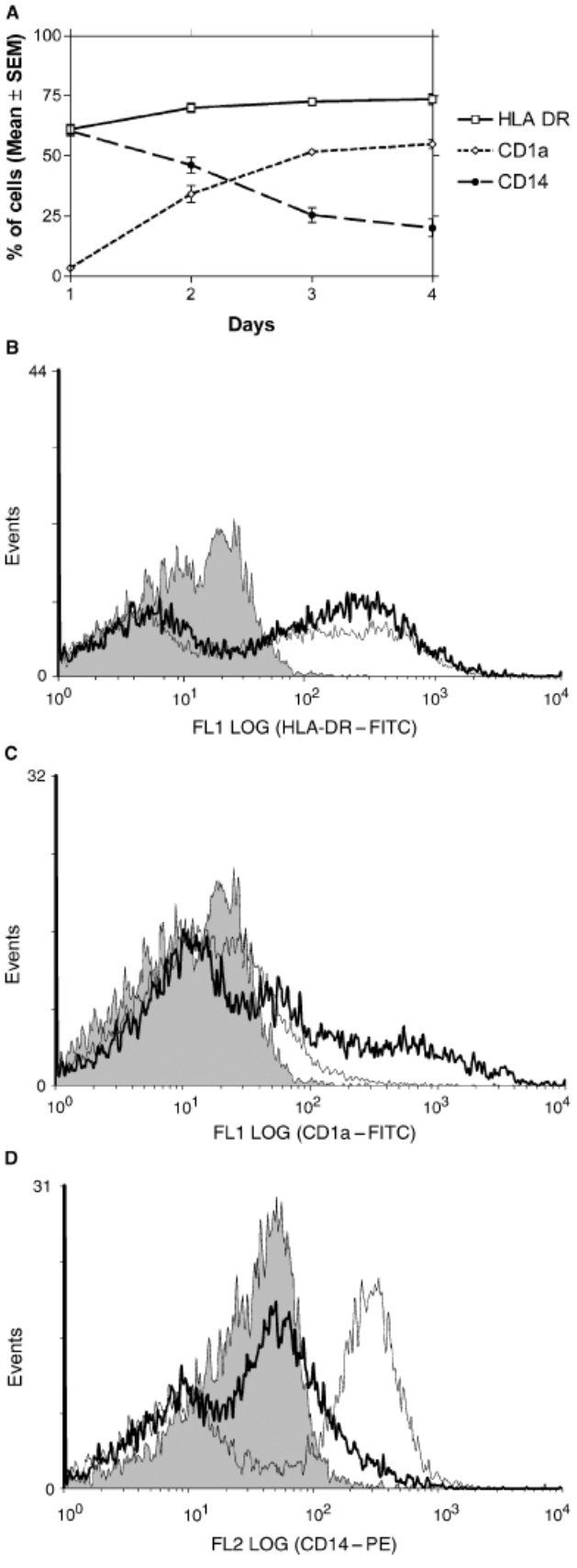

Figure 1
Flow cytometry analysis during the generation of DC-like cells. (*A*) Freshly isolated peripheral blood monocytes obtained from four different donors were thawed at day 0, pooled and cultured in presence of IL-4 and GM-CSF as described. The percentage of cells positive for CD1a, HLA-DR and CD14 was measured on days 1–4. The mean and the standard error of the mean of 10 independent preparations are shown. (*B–D*) Cytometry analysis of the cells during a representative experiment. Filled, gray histograms give the reactivity with the appropriate isotype-matched control. Thin line: Analysis performed at day 1; bold line: Analysis at day 4.

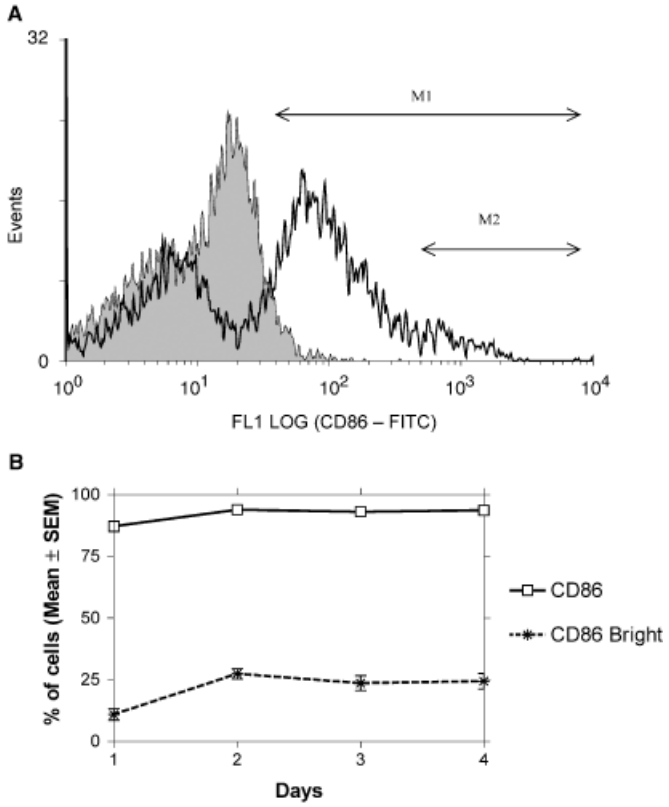

Figure 2
Flow cytometric analysis of the CD86 marker during the generation of DC-like cells. (*A*) The CD86 positive cell population can be subdivided into two distinct sub-populations: M1 indicates the CD86 positive cell population (signal higher than the corresponding isotopic control). M2 indicates the CD86 "bright" subpopulation. Data were collected during a representative routine experiment. Filled gray histogram gives the reactivity with the appropriate isotype-matched control and the line shows the profile of a typical DC population at day 4. (*B*) The expression of CD86 was measured during the generation of dendritic-like cells from day 1 to 4 of culture. The mean and standard error of the mean of the values obtained during 10 independent preparations are represented. Of these CD86 positive cells, 5%–40% expressed a comparatively large amount of CD86 (CD86 bright).

cells was estimated by measuring the proportion of the CD86 "bright" subpopulation in the population of CD86 positive cells. This way of expressing the data allowed a better readability of the results than a mean intensity of fluorescence measurement. This is presumably due to the fact that not all cells respond to the test substance. Additionally, the relative amount of IL-1β mRNA and the relative amount of AQP3 mRNA, a gene which is expressed in immature DC and downregulated during DC maturation (de Baey and Lanzavecchia, 2000) were measured. Time points were 1.5, 3, 6, 11, 24, and 30 h (see Fig 3). In presence of TNBS, the percentage of the CD86 bright cells was comparable or below the value obtained with the control up to 11 h incubation time and then increased up to approximately 200% of control at 30 h. On the other hand, SDS did not induce relevant modification of the CD86 bright population after 24 or 30 h (see Fig 3*A*). A similar effect was observed on the modulation of IL-1β gene expression. Incubation with TNBS for more than 6 h induced relevant increases in IL-1β gene expression and reached a maximum of approximately 800% of control after 30 h exposure. Exposure to SDS induced a maximum of 170% of control after 11 h and then went back to 80% of control after 30 h (see Fig 3*D*). Analysis of AQP3 gene expression demonstrated a pronounced decrease of AQP3 expression (down to 25% of control at 30 h) in the cells exposed to TNBS. SDS did not affect AQP3 gene expression at any of the tested time points (see Fig 3*E*). Based on these results, the accumulation of CD86 bright cells and the analysis of IL-1β and AQP3 gene expression by real-time RT-PCR were chosen as parameters for further *in vitro* sensitization studies. Due to the high variability of the test system at short exposure times, 30 h was chosen as the optimal time point for discriminating irritants from sensitizers.

The pooling of cell preparations containing DC and T cells may lead to an allogeneic mixed lymphocyte reaction (MLR). Control experiments (data not shown) were thus performed with DC obtained from four single donors and with DC obtained from a pool of the same donors. Upon exposure to SDS and TNBS, similar and specific DC activation patterns were observed with cells from single or pooled donors. Moreover, the amount of contaminating T cells (flow cytometric measurement of the CD3 positive population) decreased sharply (9%–18% at day 1 to $<3\%$ at day 4) during the culture of DC obtained from the single or pooled donors. The predominance of an MLR in our test system can thus be excluded.

Comparison of the dose response effects of PTD, HE-PPD, and TNBS on cultured DC-like cells DC-like cells were exposed to water as a negative control, 57–454 μM PTD, 48–2500 μM HE-PPD, and 85–850 μM TNBS. Higher doses showed a strong response inhibition or could not be analyzed because of cell toxicity as determined by Trypan blue exclusion or flow cytometry measurement of FDA incorporation (data not shown). IL-1β and AQP3 gene expression and the proportion of CD86 bright cells were measured after 30 h incubation (see Fig 4). PTD, HE-PPD, and TNBS were able to induce relevant increases in the CD86 bright cell population (see Fig 4*A*). The concentrations required to induce comparable effects were four to eight times higher for HE-PPD and for TNBS than for PTD. The three test substances induced also significant increases in IL-1β gene expression (see Fig 4*B*). Again, the concentrations required for a similar induction of IL-1β gene expression were five to eight times higher for HE-PPD than for PTD. TNBS showed intermediate induction potency. The relatively stronger effect of PTD compared with HE-PPD was confirmed by the AQP3 measurements. Again, PTD was four to six times more potent than HE-PPD in inducing a decrease in AQP3 gene expression and TNBS had an intermediate effect (see Fig 4*C*).

Induction of contact sensitization in the LLNA PTD was able to induce a concentration-dependent increase in the LLNA response in the vehicle AAOO. A concentration increase of 10 mg per mL (from 5 to 15 mg per mL) induced an increase in the stimulation index from 4.4 to 10.4. The highest tested concentration of 28 mg per mL (limit of solubility in AAOO) induced a stimulation index of 19.4 (Fig 5). The EC3, that is, the concentration that induced a stimulation index of 3 was derived by linear regression

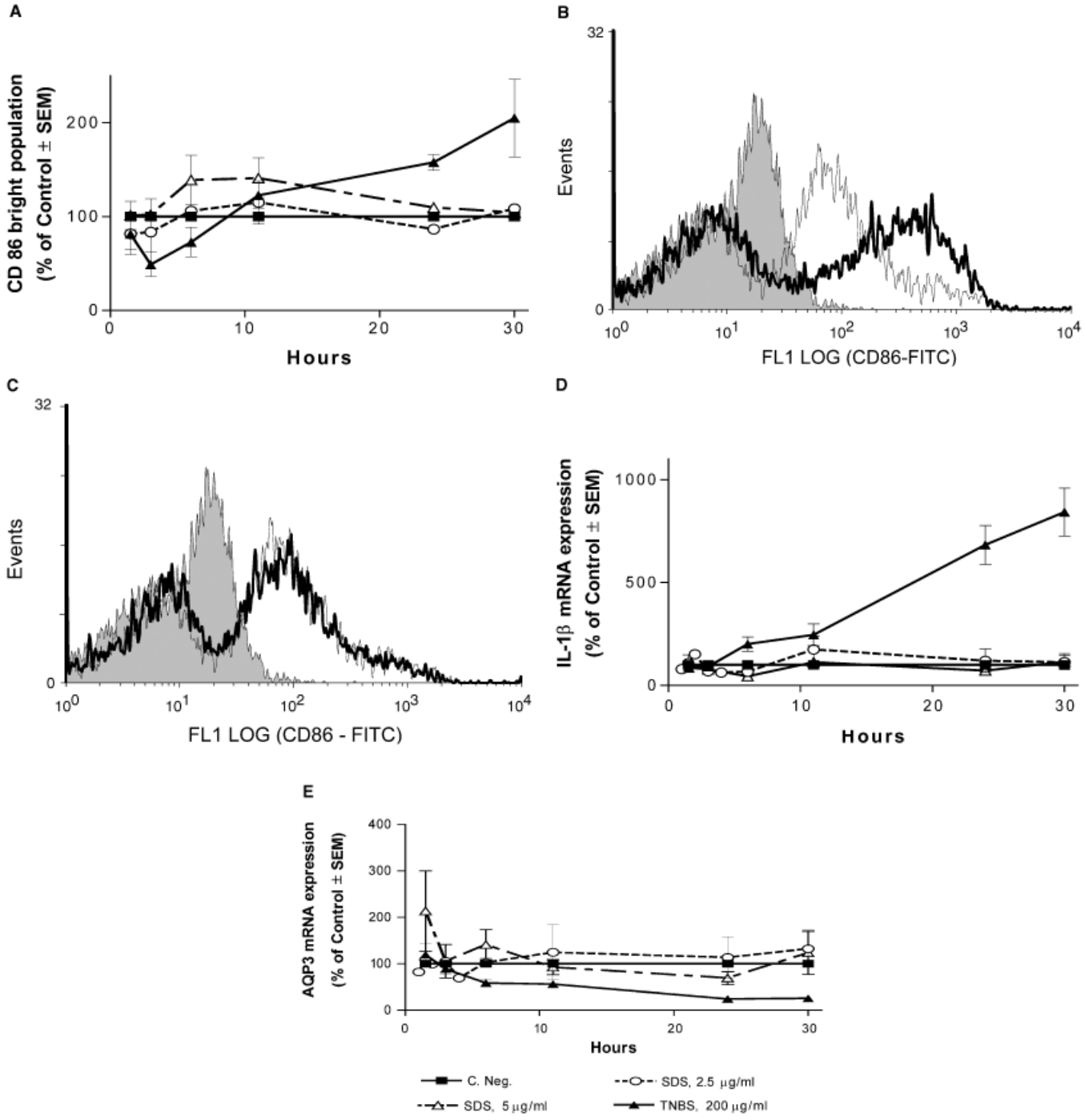

Figure 3
Kinetic analysis of CD86 surface expression and IL-1β and AQP3 gene expression on DC-like cells exposed to TNBS or SDS. DC-like cells were exposed to vehicle alone (■) or to 200 μg per mL TNBS (▲), 2.5 μg per mL (○) or 5 μg per mL (△) SDS. (*A*) Flow cytometry analysis of the CD86 bright population. The mean and standard error of the mean are indicated for TNBS (n = 5 independent experiments), for SDS at 2.5 μg per mL (n = 2) and SDS at 5 μg per mL (n = 3). (*B*, *C*) Cytometry analysis of the cells exposed to TNBS (*B*) or SDS (*C*) during a representative experiment. Filled, gray histograms give the reactivity with the appropriate isotype-matched control. Thin line: vehicle treated cells; bold line: cells treated with 200 μg per mL TNBS (*B*) or 5 μg per mL SDS (*C*). (*D*) Real-time RT-PCR measurements of IL-1β gene expression. The mean and standard error of the mean of the value obtained with TNBS (n = 6) and the two concentrations of SDS (n = 3) are indicated. (*E*) Corresponding results for AQP3 gene expression.

($r^2 > 0.99$). The EC3 of 0.31% indicated a moderate sensitizing potency for PTD in the vehicle AAOO (according to Basketter *et al*, 2000). In contrast, HE-PPD did not induce a stimulation index above 3 in AAOO at concentrations of up to 28 mg per mL (limit of solubility in AAOO), that is, HE-PPD would not be regarded as a sensitizer under these conditions.

When DMSO was used instead of AAOO as vehicle for HE-PPD, a concentration-dependent increase in the LLNA response was observed. A concentration increase from 5 to

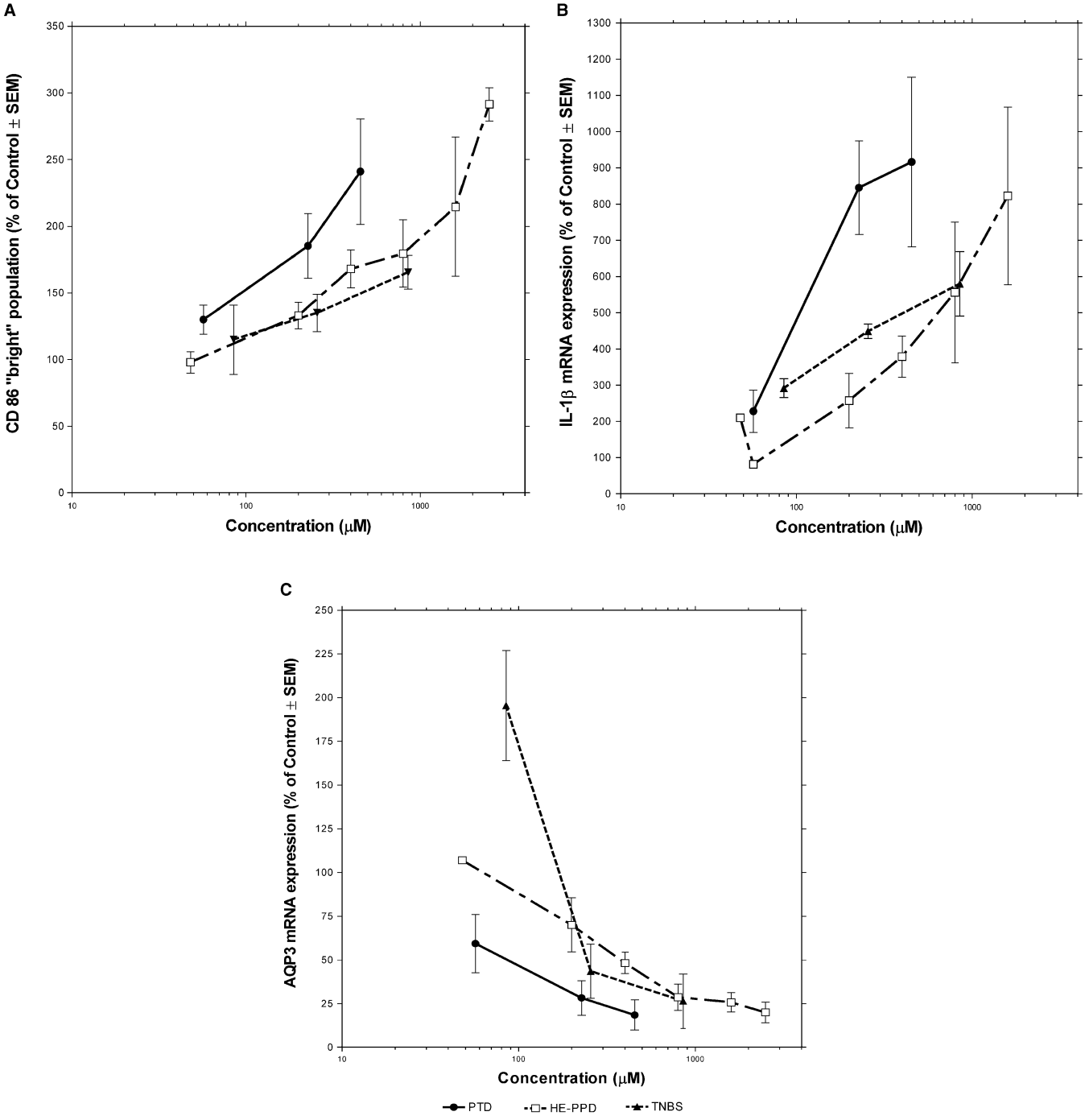

Figure 4
Dose response effects of PTD, HE-PPD and TNBS on cultured DC-like cells. DC-like cells were exposed to vehicle alone, 57–454 μM PTD (●), 48–2500 μM HE-PPD (□) and 85–850 μM TNBS (▲) for 30 h. (*A*) Flow cytometry analysis of the CD86 bright population, expressed as the percentage ratio of the negative control. The mean and standard error of the mean of n = 4 independent experiments are represented (or n = 2 for TNBS). (*B*) Measurements of IL-1β gene expression. The mean and standard error of the mean of 4 (PTD and HE-PPD) or 2 (TNBS) independent experiments are shown. (*C*) Corresponding results for AQP3 gene expression.

10 mg per mL increased the stimulation index from 2.7 to 4.5 (Fig 5). The respective EC3, calculated by linear interpolation using these data points, was 0.58% (according to Basketter *et al*, 2000). When the EC3 was derived by linear regression from all data points ($r^2 > 0.99$), a nearly identical value of 0.62% was found. The maximum solubility of HE-PPD in DMSO was 20 mg per mL, which caused a stimulation index of 7. PTD could not be tested in this vehicle because it is nearly insoluble in DMSO.

Vehicle-dependent penetration of PTD and HE-PPD An amount of about 4% PTD of the applied dose was found to penetrate through pig skin after topical exposure for 30 min, when AAOO was used as vehicle (Fig 6). Under

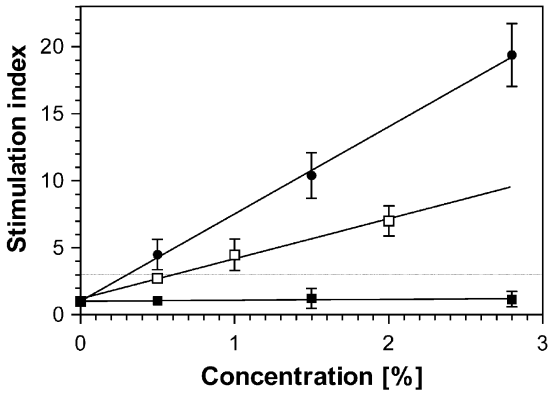

Figure 5
Concentration- and vehicle-dependent differences in LLNA response. Groups of mice (n = 5) received topical applications on the dorsum of both ears of 25 μL of PTD (●) or HE-PPD (■) in the vehicle acetone/aqua/olive oil (AAOO) in doses of 2.8%, 1.5%, and 0.5% for 3 consecutive days. Additionally, groups of mice received applications of HE-PPD in DMSO in doses of 2.0%, 1.0%, and 0.5% (□) for the same period. On day 5 following the initiation of the treatment all mice were injected intravenously with radioactive thymidine and its respective incorporation into the auricular lymph nodes was measured as described. The arithmetic mean of the stimulation index and the standard deviation relative to the concurrent vehicle-treated control is shown for each concentration tested.

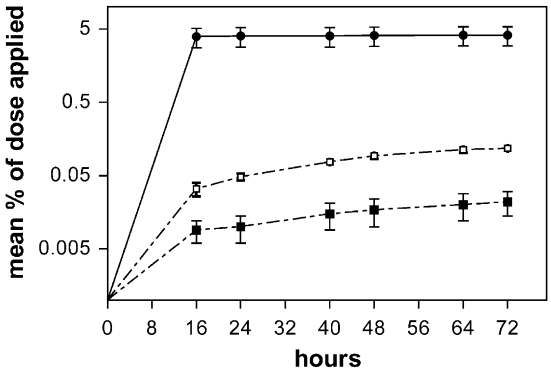

Figure 6
Percutaneous penetration rates of HE-PPD and PTD in different vehicles. The surface of pig skin samples was incubated with 100μL per cm^2 of acetone/aqua/olive oil (AAOO) containing 0.6% PTD (●) (n = 5) or 1.6% HE-PPD (■) (n = 6) for 30 min. Additionally, skin samples (n = 6) were treated with 1.6% HE-PPD in DMSO (□) for the same period. Remaining amounts of the test substances were removed after 30 min by extensive washing using a shampoo. At the points in time indicated the cumulative cutaneous absorption was determined in the receptor fluid as described. The mean of the respective amount absorbed and the standard deviation is shown for each time point.

the same conditions, the penetration of HE-PPD was about 200-fold lower (Fig 6). The penetration rate of HE-PPD could be increased 5-fold to 0.1%, when DMSO was used as vehicle instead of AAOO (Fig 6). Lower penetration rates were measured for PTD and HE-PPD from commercial hair dye formulations (Table I). The recovery of PTD and HE-PPD was greater than 97% from AAOO or DMSO and between 85% and 97% from formulations (Table I).

Discussion

The establishment of *in vitro* sensitization methods for the screening of new industrial chemicals is of major importance for the reduction of animal testing and the maintenance of product safety. The aim of this study was to develop a test system based on two essential properties of a skin sensitizing chemical that can be quantitatively determined *in vitro*, namely, skin penetration and DC activation. We evaluated whether these parameters are suitable to explain the different sensitizing potential of the two related aromatic amines, PTD and HE-PPD that are used as oxidative hair dye precursors. Despite their structural similarity, only PTD is known to induce positive patch test reactions in hairdressers (Uter *et al*, 2000). The different sensitizing potentials of PTD and HE-PPD in combination with their similar coloring abilities pointed to these molecules as excellent examples of the class of monocyclic aromatic amines that should be distinguishable in our *in vitro* test system.

The various *in vitro* approaches already published have been used with limited success for the detection of the sensitizing potential of chemicals (Kimber *et al*, 2001; Ryan *et al*, 2001). These studies, however, demonstrated that exposure to sensitizers modulates the expression of some DC activation markers *in vitro*. We thus decided to set up an optimized *in vitro* test system for measuring the activation of *in vitro* generated immature DC-like cells by chemicals through quantitative analysis of DC activation markers. We based our approach on immature DC generated by a short 5 d culture. DC obtained with similar protocols have been shown to be functionally active in mixed leukocyte reactions (Reutter and Jaeger, 1997; Ebner *et al*, 2001) or after additional stimulation with TNF-α were shown to be able to prime T cells following exposure to sensitizers (Guironnet *et al*, 2000). A possible unspecific induction of activation markers was limited by coating the bottom of the culture wells with an agarose matrix in order to avoid direct contact with the plastic material (Degwert *et al*, 1997; Aeby and Bracher, 1999). DC differentiation state was monitored by HLA-DR, CD14, and CD86 measurements (see Fig 1). The influence of the apparent variations between donors used as a source of DC progenitors observed by Reutter and Jaeger (1997) and Pichowski *et al* (2000) was minimized by the use of cell pools obtained from four different donors (Aeby and Bracher, 1999).

Analysis of DC activation was performed using CD86 and IL-1β as significant markers, since both were shown to be upregulated by murine and human Langerhans' cells during the initiation of contact sensitization in the skin (Enk *et al*, 1993; Ozawa *et al*, 1996; Rambukkana *et al*, 1996; Katayama *et al*, 1997; Kimber *et al*, 1998; Kermani *et al*, 2000; Mellman and Steinman, 2001; Stoitzner *et al*, 2003). Moreover, CD86 and IL-1β are generally considered suitable markers for developing an *in vitro* test system for contact sensitizers and allow a widespread comparison with already published data as far as *in vitro* activation of DC by contact sensitizers is concerned (Aiba *et al*, 1997; Reutter and Jaeger, 1997; Pichowski *et al*, 2000, 2001; Tuschl and Kovac, 2001; De Smedt *et al*, 2002). Other specific markers of DC activation, such as tyrosine phosphorylation or CD83/CD208

Table I. Mean % of cumulative cutaneous absorption[a]

	Test substance				
	PTD		HE-PPD		
Vehicle	AAOO	Commercial formulation[b]	AAOO	DMSO	Commercial formulation[c]
Receptor fluid	4.1 ± 1.2	0.28 ± 0.12	0.022 ± 0.008	0.118 ± 0.01	0.017 ± 0.005
Rinsing solution	100.8 ± 3.9	97.3 ± 11.1	105.9 ± 4.2	97.4 ± 2.6	84.9 ± 8.2
Total balance[d]	107.7 ± 4.5	97.8 ± 11.0	107.0 ± 4.1	98.4 ± 2.6	85.0 ± 8.1

[a]Mean % of cumulative cutaneous absorption ± SD is given.
[b]Formulation contains: water, cetearyl alcohol, glyceryl stearate SE, sodium laureth sulfate, glycol distearate, ammonia, lanolin alcohol, sodium lauryl sulfate, isopropyl alcohol, sodium sulfite, ascorbic acid, benzoic acid. Experiment was terminated after 24 h.
[c]Formulation contains: water, cetearyl alcohol, 2-amino-4-hydroxyethylaminoanisole sulfate, sodium laureth sulfate, ammonia, sodium sulfite, benzoic acid.
[d]Values given include amount found in the skin.

modulation, were not included, since their expression after exposure to contact sensitizers need a very short exposure period (15 min, Becker *et al*, 2003) not compatible with our test system, or have been described to be either weak (Aiba *et al*, 2000; De Smedt *et al*, 2002), or yield very similar information as provided by CD86 measurement (de Saint-Vis *et al*, 1998).

In general, the modulation of the activation markers through sensitizers was expected to be rather weak. Reutter and Jaeger (1997) and Pichowski *et al* (2000) observed a maximum of 2–3-fold increase in IL-1β gene expression using semi-quantitative PCR measurements. Such increases are barely detectable with semi-quantitative PCR approaches and we decided to perform IL-1β gene expression measurements with a quantitative methodology and opted for real-time PCR using the LightCycler system. To exclude unspecific signals due to experimental artifacts that may lead to the overall unspecific upregulation of the measured parameters, we wanted to include a down-regulated DC maturation marker in our test battery. Recently, the role of aquaporins that regulate the flow of water during macropinocytosis in immature DC has been analyzed (de Baey and Lanzavecchia, 2000). These authors have shown that two members of the aquaporin family, AQP3 and AQP7, are downregulated during DC activation. A protocol for AQP3 mRNA measurement by real-time RT-PCR was developed and this marker was included in our test battery. Taken together, our strategy was to show that exposure to a contact sensitizer induces modifications of three functionally different markers, that is, DC switch from a state with low costimulation capacity (CD86 +/−), high antigen uptake (AQP3 +), and low migration signal (IL-1β−) to a state with a high costimulation (CD86 + +), low antigen uptake (AQP3−), and high migration signal (IL-1β +). That test strategy is not limited to the markers used and additional markers might be included to optimize the analysis of contact sensitizers of different chemical classes. But the three currently used markers represent a valid subset of the known DC activation markers linked to contact sensitization, are compatible with the current test protocol, and allow comparison with already published data.

Next we analyzed the expression kinetics of the selected endpoints (AQP3, CD86, and IL-1β) on *in vitro* generated DC exposed to the model irritant SDS and the model sensitizer TNBS (see Fig 3). The response of the test system differed significantly after 30 h of exposure to the two substances: DC were activated by the sensitizer but not by the irritant. Longer exposure periods (e.g., 48 h) affected the cell viability and were not analyzed. Exposure periods shorter than 11 h did not produce useful signals. Thirty-hour exposure was thus chosen as the optimum exposure time for the differential expression of the selected activation markers. This exposure time is comparable with those published by other groups who measured protein markers (Aiba *et al*, 1997; Degwert *et al*, 1997; Tuschl and Kovac, 2001). It is, however, much longer than the exposure period (30 min) used by Reutter and Jaeger (1997) or Pichowski *et al* (2000) who measured IL-1β gene expression at the mRNA level.

This optimized test protocol was then applied for comparing the dose response curves of PTD, HE-PPD and TNBS (see Fig 4). The concentrations required to induce similar effects on the selected activation markers were approximately four to eight times higher for HE-PPD than for PTD. We concluded that PTD is approximately four to eight times more potent for inducing DC activation *in vitro* than HE-PPD. The dose response curve of TNBS was in the same range as those of the aromatic amines. In order to check the relevance of these *in vitro* findings, PTD and HE-PPD were evaluated *in vivo* in the murine LLNA.

The LLNA results obtained with PTD in AAOO were in line with its comparatively potent biological activity in the DC maturation assay. The EC3 of 0.31% indicates that topical application of relatively low concentrations of PTD suffice to induce an LLNA response above 3 indicating the induction of a specific immune response (see Fig 5). Under the same conditions, HE-PPD did not induce a positive response. This lack of an LLNA response was unexpected since the results of the *in vitro* DC activation assay did indicate a clear activity of HE-PPD for all three markers tested, although it was four to eight times less potent than PTD (see Fig 4).

Could differences in the bioavailability of PTD and HE-PPD be responsible for their different ability to induce a LLNA response? *In vitro* assessment of their skin penetration properties using the pig skin model revealed that the bioavailability of PTD was approximately 200 times higher than that of HE-PPD when applied in AAOO (see Fig 6).

When applied in DMSO, a vehicle known to increase dermal bioavailability (Priborsky and Muhlbachova, 1990; Kim *et al*, 1999; Ryan *et al*, 2002), the penetrating properties of HE-PPD were enhanced 5-fold. Besides the enhancement of skin penetration, DMSO probably also affected the sensitizing potential of HE-PPD *in vivo* by additional mechanisms such as the modulation of cytokines necessary for DC maturation and/or induction of dermal irritation. These effects that were reported for DMSO (Depraetere *et al*, 1995; Sjogren and Anderson 2000) and for other vehicles (Lea *et al*, 1999; Warbrick *et al*, 1999; Wright *et al*, 2001, reviewed in Basketter *et al*, 2001) possibly led to an overestimation of the relative sensitizing potency of HE-PPD in the corresponding LLNA performed in DMSO with respect to an EC3 of 0.62%. Notwithstanding, the LLNA response showed a concentration-dependent increase in the stimulation index (see Fig 5) as expected from the concentration-dependent biological activity of HE-PPD observed in the *in vitro* DC activation assay (see Fig 4).

We concluded that *in vitro* assessment of DC activation combined with skin penetration measurements allowed the correct prediction of the sensitizing properties of the two selected aromatic amines: The higher sensitizing potential of PTD *versus* HE-PPD can be correctly predicted due to a relatively strong induction of DC activation and a high skin penetration capacity. The relatively lower sensitizing potential of HE-PPD can be correctly predicted since it was less effective than PTD in inducing DC maturation and had much lower skin penetrating properties using either AAOO or DMSO as vehicles.

Interestingly, TNCB (trinitrochlorobenzene), a chemical that forms the immunologically identical hapten as does TNBS (Robinson, 1989), induced an EC3 of 0.26% in dimethylformamide (Smith and Hotchkiss, 2001), which is in the same order of magnitude as the EC3 values of 0.31% for PTD and of 0.62% for HE-PPD. This finding also supports the good predictivity of our *in vitro* test system since TNBS, PTD, and HE-PPD induced comparable activation of DC (see Fig 4).

Taken together, the quantitative evaluation of DC activation through upregulation of IL-1β and CD86 as well as downregulation of AQP3 has provided a correct estimation of the skin sensitizing potential of the tested chemicals when bioavailability is not limited by dermal penetration. To establish a robust prediction model more chemicals have to be tested and corrective factors for *in vitro* metabolism and *in vitro* protein binding have to be addressed. On the basis of these future refinements the analysis of DC activation will become a useful tool to screen new industrial chemicals and cosmetic ingredients for their sensitizing potential without increasing the number of animal experiments.

Materials and Methods

Animals Female CBA/ca01aHsd mice were obtained from Harlan Winkelmann GmbH, Germany. A standard diet (1324 Altromin, Germany) and tap water were offered *ad libitum*. Mice were 8–12 wk old at the onset of the experiments.

Materials *p*-Toluylenediamine sulfate (PTD; CAS-no. 6369-59-1) and hydroxyethyl-*p*-phenylenediamine sulfate (HE-PPD; CAS-no. 93841-25-9) of documented purity were purchased from Wella production, Hünfeld, Germany. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) (CAS-no. 2508-19-2) 5% (wt/vol) solution in water and sodium lauryl sulfate (SDS) (CAS-no. 151-21-3) were purchased from Sigma, Buchs, Switzerland. 3H-Methylthymidine (TRK 300, 25 Ci per mmol) from Amersham Pharmacia Biotech, Roosendaal, NL was diluted to a working concentration of 80 μCi per mL. Trichloroacetic acid (TCA) and olive oil (OO), highly refined, were purchased from Sigma GmbH, Munich, Germany, phosphate-buffered saline (PBS) was from BSL Bioservice, Planegg, Germany, and acetone was purchased from Biesterfeld/Bender and Hobein, Hamburg, Germany. Dimethylsulfoxide (DMSO) was from Fluka, Munich, Germany.

Culture medium The culture medium was RPMI 1640 without phenol red (Sigma, Buchs, Switzerland) supplemented with 10% fetal calf serum (Amimed, Allschwill, Switzerland), 2 mM L-glutamine (Biochrom KG, Berlin, Germany), 800 U per mL of rhGM-CSF (Leucomax, Essex Chemie AG, Luzern, Switzerland), and 1000 U per mL of IL-4 (Strathmann Biotech GmbH, Hannover, Germany) referred below as complete culture medium.

Purification of human monocytes and generation of dendritic-like cells Fresh buffy coats were obtained from Blutspendedienst SRK Bern AG, Bern, Switzerland according to a written informed consent agreement signed by the transfusion center and the first author. An enriched monocytic cell fraction was isolated by sequential density centrifugation on Ficoll-Paque PLUS and Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradients as described (Degwert *et al*, 1997). The enriched monocytes were stored in liquid nitrogen. Monocyte-derived dendritic-like (DC-like) cells were generated by thawing and pooling the enriched monocytes from four different donors and growing them up to 5 d in complete culture medium at 37°C, 5% CO_2 in 12-well-culture plates (Falcon Becton Dickinson, Franklin Lakes, New Jersey) plating 1–2 $\times$ 10^6 cells in 2 mL per well. The wells were pre-coated with 0.5 mL of a 2% agarose matrix (Agarose gel, insect cell culture tested, Gibco, Basel, Switzerland) and equilibrated with complete culture medium before use. Every second day, half of the culture medium was replaced by fresh medium. The CD1a, CD14, CD86, and HLA-DR phenotypes were regularly monitored as markers of differentiation into DC-like cells. At day 4, the DC-like cells were used for the *in vitro* sensitization test.

Flow cytometry analysis Cells were prepared for flow cytometry analysis by washing 1–2 $\times$ 10^6 cells in PBS followed by a 15 min incubation at 4°C with the indicated fluorescein isothiocyanate (FITC) or phycoerythrin (PE) labeled antibodies at concentrations recommended by the manufacturer. The following antibodies were used: anti-CD1a-FITC, clone HI149, anti-CD86-FITC, clone 2331(FUN-1) (BD Biosciences, Basel, Switzerland), anti-CD45-FITC/anti-CD14-PE, clones IMMU19.2 and RMO52 and anti-HLA-DR-FITC, clone B8.12.2 (Beckman Coulter, Zürich, Switzerland). Non-specific staining was determined using relevant isotypic controls in parallel. Gates based on forward and side scatter signals were set to exclude most T lymphocytes, dead cells, and debris. Approximately 10,000 cells were analyzed during each measurement. The flow cytometry was performed on a Coulter EPICS XL (Beckman Coulter) analyzer in the FL1 channel at 525 nm for FITC labeled and in the FL2 channel at 575 nm for PE labeled antibodies. Cell viability was determined by measuring the percentage of cells positive in the FL1 channel (living cells) after 20 min incubation with 50 ng per mL fluorescein diacetate (FDA, Sigma, Buchs, Switzerland). The data analysis was performed with the SYSTEM II, v.3.0 software (Beckman Coulter). Graphs were prepared for publication using WinMDI Version 2.8 (Joseph Trotter, http://facs.scripps.edu/).

***In vitro* sensitization test protocol** At day 4, 100 μL of test items or of negative control (deionized water) were added to 2 mL wells containing 1–2 $\times$ 10^6 DC-like cells. After 6 h, half of the medium was replaced by fresh complete medium. At the indicated time

points, cells were harvested for RT-PCR and/or flow cytometry analysis.

RNA extraction and cDNA synthesis Total RNA was isolated from each 2 mL well containing 1–2 $\times$ 10^6 DC-like cells using RNAzol B (Ams biotechnology Europe Ltd, Bioggo, Switzerland) according to the manufacturer's instructions. RNA was pelleted by centrifugation at 10,000 $\times$ *g* for 15 min, washed with 75% ethanol and suspended in 50 μL of TE buffer at pH 8.0 (Amresco, BioConcept, Allschwil, Switzerland). The RNA concentration was determined by measurement of the OD_{260} and adjusted to 50 μg per mL. Four hundred and fifty nanograms of purified total RNA (9 μL) was used for cDNA synthesis using the ThermoScript RT-PCR system (Invitrogen AG, Basel, Switzerland) using the provided random hexamers (50 ng per reaction) according to the user manual instructions.

PCR analysis of gene expression with the LightCycler system Quantitative PCR amplification of the 28S ribosomal RNA (28S rRNA), IL-1β, and aquaporin 3 (AQP3) cDNAs was performed in glass capillaries with the LightCycler instrument (Roche Diagnostics AG, Rotkreuz, Switzerland). The PCR reactions were set up using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 2 μL of a 1/10 diluted cDNA solution (28S rRNA: 1/50 dilution), the desired amount of $MgCl_2$ stock solution, 20 pmol each of the specific primers (Microsynth GmbH, Balgach, Switzerland) according to Table II and PCR grade water were added to the master mix up to a final volume of 20 μL. The experimental protocol included an initial denaturation step at 95°C for 10 min followed by 40 amplification cycles (denaturation: 95°C for 15 s; annealing: primer specific temperature according to Table II for 5 s; elongation: 72°C for 1s per 25 bp of amplicon length). Ramp rates were set to 20°C per s. SYBR Green I fluorescence was measured at the end of each elongation cycle. The level of primer–dimer formation and/or unspecific amplification products was monitored by melting curve analysis. The respective gene products were quantified by direct comparison with standards amplified in parallel reactions. As standards, we used serial dilutions (1/10–1/1000) of a batch of cDNA obtained from stimulated DC-like cells. The same batch of cDNA was used as standard for the negative control and all treated samples run in parallel. The same batch of cDNA, however, could not be used for all experiments due to amount and stability issues, and the results are therefore expressed as percentage of the respective negative control. All calculation steps necessary to generate a standard curve were performed with the LightCycler Software 3.5 using the second derivative maximum method. The generated standard curve was used to calculate the relative concentrations of target gene products in the unknown samples and in the negative control. In each sample, the gene expression level was further normalized to its 28S rRNA content by computing the ratio relative amount of gene cDNA/relative amount of 28S rRNA cDNA. The results are then expressed as percentage of the respective negative control.

Dermal absorption The experimental setup as well as the preparation and storage of the male porcine skin used in these experiments have been published in detail (Bracher *et al*, 1987; Noser *et al*, 1988; Beck *et al*, 1993). The cutaneous absorption of HE-PPD (in DMSO and acetone/aqua/olive oil; 2:2:1 (=AAOO)) and PTD in AAOO was investigated *in vitro*, using split thickness pig skin samples from back and flanks ("Schweizer Edelschwein", 1 mm thick; stratum corneum, stratum germinativum and part of the dermis containing blood vessels, stored at −20°C until use). The skin preparations were punched and fixed into the permeation chambers (Frantz cell type, CVO Glassware Co, Berkeley, California; 0.785 cm^2 surface) at the beginning of the experiment, kept in an incubator and continuously rinsed from underneath (dermal side) with physiological receptor fluid (flow 2.5 mL per h) at a temperature of 32°C and a constant relative humidity during the whole experiment of 72 h. Prior to the experiment the skin integrity was checked with tritiated water by scintillation counting of 1 h fractions over 4 h. Six skin samples were covered with 78 μL partially ^{14}C-labelled HE-PPD in AAOO and DMSO, respectively, containing 1.6% of HE-PPD (=1.58 mg HE-PPD per cm^2). Five skin samples were covered with 78 μL of AAOO containing 0.6% PTD (=0.59 mg PTD per cm^2), and one skin sample was covered with the vehicle only as a negative control. The solution was removed after 30 min, followed by extensive washing with deionized water and a commercially available shampoo. The receptor fluid was sampled after 16, 24, 40, 48, 64, and 72 h.

Analysis of samples from dermal absorption *Receptor fluid:* The partially ^{14}C-labelled HE-PPD was analyzed by liquid scintillation counting (Packard Tricarb 2250CA, Downers Grove, Illinois) with a dual mode $^3H/^{14}C$ program. For PTD 10 mL of the receptor fluid was reacted with 2.5 mL buffer (25 mM $K_2HPO_4 \cdot 3H_2O$; Merck 1.05099.0250, Darmstadt, Germany; p.a.; pH ≈ 8.3 adjusted with H_3PO_4; Merck 573, Darmstadt, Germany; p.a.) and 1 mL of a dansyl-chloride solution (Sigma D-2625, St Louis, Missouri; approximately 95%; to 1.5 mg per mL acetonitrile; Biosolve, Valkenswaard, The Netherlands; HPLC grade) during 2 h at 50°C, and then butanol was extracted, evaporated to dryness, redissolved in the mobile phase, and an aliquot was taken for HPLC. Control injections were prepared with permeation fluid from the control skin using the same derivatization and extraction procedures. *Rinsing solution:* For HE-PPD an aliquot of each washing solution was analyzed as described above. For PTD an aliquot was diluted in deionized water and reacted in the physiological solution buffer and dansyl-chloride solution, thereafter processed as described above. An HPLC system (Waters 996, Milford, Massachusetts) combined with a fluorescence detector (Gynkotek, RF 1002, Germering, Germany) and controlled by Millenium software

Table II. Primers, amplicons' sizes and PCR conditions

mRNA target	Primers (5′–3′)	Annealing temperature (°C)	$MgCl_2$ final concentration (mM)	Amplicon size (bp)	GenBank accession no.
28S ribosomal RNA	CGGTACACCTGTCAAACGGTAAC	65	4	507	M11167
	TTAGAGGCGTTCAGTCATAATCCC				
Interleukin-1β	AAACAGATGAAGTGCTCCTTCCAGG	60	4	391	X02532
	TGGAGAACACCACTTGTTGCTCCA				
Aquaporin 3	TCTGGCACTTTGCCGACAAC	58	3	472	AB001325
	GGCCAGCTTCACATTCTCTTC				

was used for analyses at an excitation of 340 nm and an emission of 525 nm under the following conditions: column: 4 micron C18 Nova-Pak from Waters, 3.9 × 150 mm, with an appropriate pre-column (Waters); mobile phase: 60% methanol (Biosolve; HPLC-grade) and 0.5% acetic acid (Merck 6325, Darmstadt, Germany; p.a.); flow: 0.8 mL per min.

Local lymph node assay Murine LLNA were conducted as described previously (Kimber and Basketter, 1992). Briefly, groups of mice (n = 5) were exposed topically on the dorsum of both ears to 25 μL of various concentrations of the test items, or to the same volume of the respective vehicle alone, daily for 3 consecutive days. To achieve maximum solubility, PTD and HE-PPD were dissolved in deionized water and a pH between 5 and 7.5 was adjusted with NaOH. The respective aqueous solutions were mixed with acetone 1:1 (vol/vol) to receive aqua/acetone (AA) and finally mixed 4:1 (vol/vol) with OO and will be referred to as AAOO. Additionally, DMSO served as vehicle. Five days following the initiation of the treatment, all mice were injected intravenously via the tail vein with 250 μL of PBS containing 20 μCi ^{3}H methylthymidine. Five hours later mice were euthanized and draining auricular lymph nodes were excised and pooled for each animal. Single cell suspensions were prepared by mechanical desegregation through a 200-mesh size polyamide gauze. Cells were washed twice with PBS and precipitated in 5% TCA at 4°C overnight. Pellets were then resuspended in 1 mL of 5% TCA and transferred into scintillation vials. Incorporation of ^{3}H-methylthymidine was measured by β-scintillation counting as disintegrations per minute per node for each animal. In each case a stimulation index relative to the concurrent vehicle treated control was derived.

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