Skin Sensitization to p-Phenylenediamine: The Diverging Roles of Oxidation and N-Acetylation for Dendritic Cell Activation and the Immune Response

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Skin Sensitization to *p*-Phenylenediamine: The Diverging Roles of Oxidation and *N*-Acetylation for Dendritic Cell Activation and the Immune Response

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Skin is a target of allergic reactions to aromatic amine hair dye precursors, such as *p*-phenylenediamine (PPD). As conversion of PPD on or in the skin is expected to be required for the induction of allergic contact dermatitis, we analyzed the role of oxidation and *N*-acetylation as major transformation steps. PPD and its oxidative and *N*-acetylated derivatives were tested for their sensitizing potential *in vitro* using a dendritic cell (DC) activation assay and *in vivo* using the local lymph node assay (LLNA). PPD did not induce relevant DC activation but induced a positive LLNA response. In contrast, DC activation was obtained when PPD was chemically pre-oxidized or after air oxygen exposure. Under both conditions, the potent sensitizing PPD oxidation product Bandrowski's base was identified along with other di- and trimeric species, indicating that PPD oxidation products provide an effective immune stimulation (danger signal). In contrast mono- and diacetylated PPD did not induce DC activation or a positive LLNA response. We conclude that dermal *N*-acetylation of PPD competes with the formation of oxidized PPD whereas skin exposure conditions allowing auto-oxidation, as in the LLNA, provide an effective danger signal necessary to induce skin sensitization to PPD.

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INTRODUCTION

The sensitizing potency of *p*-phenylenediamine (PPD) has been well established in animal studies, such as the local lymph node assay (LLNA; Warbrick *et al.*, 1999) and guinea pig tests (Xie *et al.*, 2000). In addition, PPD has been demonstrated to be a human allergen based on numerous studies reported in the literature (Marzulli and Maibach, 1974; Sosted *et al.*, 2006; Koopmans and Bruynzeelm, 2003; Jerschow *et al.*, 2001). In many of the animal studies (Warbrick *et al.*, 1999; Xie *et al.*, 2000) and one human study (Marzulli and Maibach, 1974), PPD is repeatedly applied topically and remains on the skin for several days. This exposure scenario would also apply to the diagnostic test procedure used to determine if patients have a skin allergy to PPD (Friedmann, 2007). In most instances, the PPD elicitation response is analyzed after a single application followed by a 48-hour exposure period (diagnostic patch test; Koopmans and Bruynzeelm, 2003; Friedmann, 2007).

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Abbreviations: AQP3, aquaporin 3; BB, Bandrowski's base, BQDI; benzoquinone diimine; DC, dendritic cell; LLNA, local lymph node assay; PPD, p-phenylenediamine; RT, reverse transcriptase

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However, despite the routine use of PPD in these assays, the mechanism of the induction or elicitation of skin sensitization by PPD is poorly understood on a chemical basis, that is, the molecular alterations of PPD responsible for its immunogenicity are not yet identified (Basketter and Goodwin, 1988; Basketter and Liden, 1992; White *et al.*, 2006).

This lack of knowledge is presumably due to the complex oxidation properties of PPD when it is exposed to air oxygen for a prolonged period on the skin or in solution according to the prehapten concept recently introduced by Lepoittevin (2006). This concept suggests that the auto-oxidation of PPD by air oxygen is pivotal for its immunogenicity. Accordingly, several (auto) oxidation products have been proposed to be responsible for skin sensitization to PPD such as the trimer Bandrowski's base (BB) and the monomer benzoquinone (Krasteva *et al.*, 1993; Lisi and Hansel, 1998), but no clear picture of the hapten-forming process is available to date. Recently, the oxidation of a PPD derivative to the reactive benzoquinone diimine (BQDI) formed by air oxygen was described as a prerequisite for its reaction with proteins (Eilstein *et al.*, 2006).

Furthermore, the limited knowledge about the relevant conditions for the conversion of a pro/prehapten such as PPD into a hapten impaired the interpretation of the PPD-specific T-cell recognition. Analysis of the primary T-cell response *in vitro* indicated that PPD is the prohapten and BB the hapten, as PPD did not induce a lymphoproliferative response whereas BB did (Krasteva *et al.*, 1996). In contrast,

when T cells from PPD- allergic donors were analyzed for their recognition of PPD itself, restimulation was successful even if fixed antigen-presenting cells were used, whereas restimulation by BB was only obtained with functional antigen-presenting cells (Sieben *et al.*, 2002). More recently, BB was shown to induce lymphocyte proliferation in PPD-allergic patients and volunteers, but only lymphocytes from patients additionally proliferated to PPD, suggesting that PPD alone or a degradation product other than BB specifically contributes to the generation of allergic contact dermatitis to PPD (Coulter *et al.*, 2008).

Apart from the limited understanding of the passive, nonenzymatic formation of immunogenic PPD, active skin metabolism of PPD is known to occur following permeation of PPD through the stratum corneum (Merk *et al.*, 2006). Under exposure conditions provided by hair dyeing with PPD, approximately 1.3% of the applied dose is considered as (bio)available in the epidermis and dermis (Hueber-Becker *et al.*, 2004). Subsequent analysis of PPD skin metabolism revealed that under these conditions *N*-acetylation is the major metabolic pathway (Kawakubo *et al.*, 2000; Nohynek *et al.*, 2006) and that epidermal keratinocytes are the responsible skin cells due to their high content of the mediating enzyme *N*-acetyltransferase 1 (Kawakubo *et al.*, 2000). When the *N*-acetylated PPD metabolites were analyzed for their capacity to restimulate T cells from PPD-allergic patients, no T-cell response was induced (Sieben *et al.*, 2001), suggesting that *N*-acetylation represents a detoxification pathway regarding PPD-induced allergic contact dermatitis (Merk *et al.*, 2006).

The role of enzymatic oxidation of PPD in the skin remains unclear, as to our knowledge no corresponding data are available to date that demonstrate oxidative metabolism of PPD in the skin. For example, no indication for the generation of oxidative PPD metabolites was found following skin exposure to PPD during hair dyeing (Nohynek *et al.*, 2004). However, enzymatic epidermal oxidation of other sensitizing low molecular weight chemicals to their immunogenic form has been reported for cinnamic alcohol (Smith *et al.*, 2000), eugenol, and isoeugenol (Bertrand *et al.*, 1997).

In the present paper, we asked which putative auto-oxidation products do occur after exposing PPD (1) to strong oxidizing conditions to monitor all possible oxidation products or (2) to air oxygen to mimic oxidative exposure conditions *in vitro* (aqueous solution) and *in vivo* as is the case in the LLNA. Furthermore, *N*-acetylated PPD derivatives, representing the main products of active skin metabolism, were synthesized. Subsequently, we investigated the sensitizing potential of PPD, its oxidation products, and its *N*-acetylated derivatives by analyzing their ability to induce *in vitro* activation of dendritic cell (DC)-like cells. Such *in vitro* DC activation protocol based on the modulation of specific cell surface markers or cytokine gene expression patterns by contact sensitizers has been already used in our laboratory (Aeby *et al.*, 2004) and by others (Bergström *et al.*, 2007) to assess the sensitizing potential of low molecular weight chemicals. Finally, we compared the DC activation patterns induced by PPD, its oxidative products, and its *N*-acetylated derivatives with their ability to induce skin sensitization *in vivo* using the LLNA.

RESULTS

Comparison of PPD auto-oxidation products after exposure to ferrocyanide or to air oxygen

PPD was exposed to ferrocyanide in aqueous media for 30 minutes and the resulting reaction products were investigated by liquid chromatography/tandem mass spectrometry (Figure 1). Among the parent compound (PPD at 0.99 minutes), other species could be identified, that is, a second monomeric structure appeared at 2.22 minutes (possibly corresponding to BQDI; Figure 1 , top), a dimeric structure at 8.2 minutes (Figure 1 , middle), and three trimeric structures at 7.3, 8.6 (corresponding to BB, as confirmed by comparison to the analytical standard), and 9.0 minutes (Figure 1, bottom).

Eight hours after the addition of PPD to an aqueous solution constantly saturated with air oxygen by an airflow, substantial amounts of a putative dimer, of BB and of a second trimer were detectable, but at smaller quantities compared to the ferrocyanide oxidation. Correspondingly, the concentration of PPD decreased to approximately 50% of the initial concentration (Figure 2).

We further assessed the pH dependency of BB formation in aqueous solution (Figure 3) to check if the skin surface pH milieu (pH 5.4–5.9; Schmid-Wendtner and Korting, 2006) was consistent with the auto-oxidative generation of BB. Following exposure of PPD to ferrocyanide for 1 hour at pH 4, no BB could be detected whereas at a pH range between 5 and 7 favorable conditions for BB formation were observed, reaching an optimum at pH 6.

Concentration-dependent activation of DC-like cells by PPD, oxidized PPD, and BB

In a preliminary experiment, the optimal exposure period was determined based on the measurement of the proportion of CD86 bright cells in a population of DC-like cells exposed to 73 and 180 μM fresh PPD, 73 μM ferrocyanide-oxidized PPD, 31.4 μM BB, 243 μM monoacetyl PPD, and 190 μM diacetyl PPD. The optimal exposure times were 24 or 30 hours (data available as online-only Figure S1). Subsequently, DC-like cells obtained from a pool of four donors were exposed for 24 hours to 30–243 μM fresh PPD, 30–91 μM ferrocyanide-oxidized PPD, and 0.5–47 μM BB. Higher concentrations could not be analyzed due to cell toxicity as determined by flow cytometry measurements of fluorescein diacetate incorporation. As negative controls, the same volume of vehicles (25–50 μl H_2O for PPD, 25–50 μl H_2O containing a concentration of K4[Fe(CN)6] $3H_2O$ (reduced form of the oxidizing agent) identical to that of K3[Fe(CN)6]) used for oxidizing the PPD for the oxidized PPD or 10 μl of DMSO for BB was tested in parallel.

As activation markers, the proportion of CD86 bright cells, the upregulation of IL-1β and IL-8 genes, and the down-regulation of aquaporin 3 (*AQP3*), a gene known to be downregulated during DC maturation (de Baey and Lanzavecchia, 2000), was measured (Figure 4). BB was the most

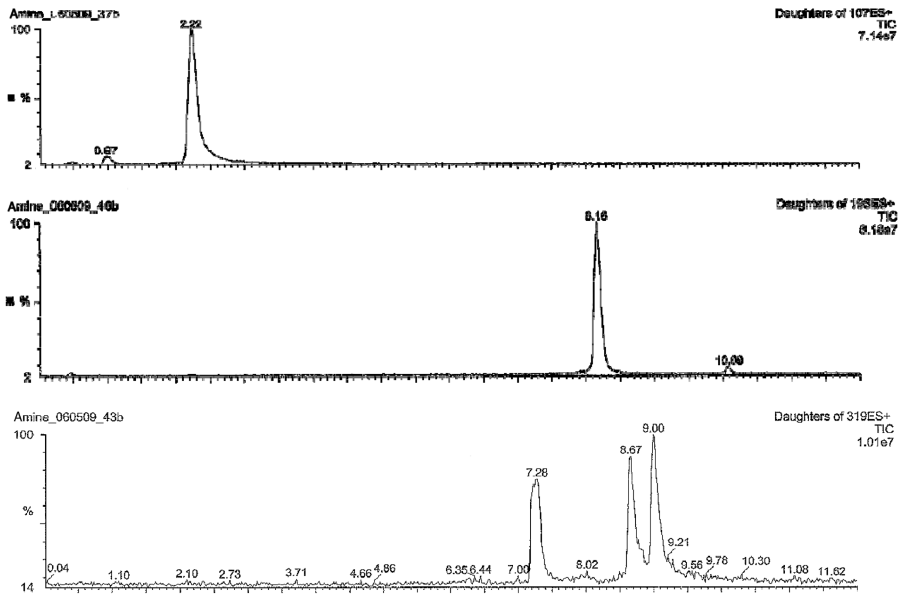

Figure 1. Typical daughter scan chromatograms obtained of a reaction mixture of PPD in the presence of potassium ferrocyanide after 30 minutes of reaction time. Top chromatogram represents a potential monomeric species (peak at 2.2 minutes, $m/z = 107$, typical fragments at $m/z = 80$, 53), possibly corresponding to benzoquinone diimine. Middle chromatogram represents a potential dimeric species ($m/z = 198$). Bottom chromatogram represents potential trimeric species ($m/z = 319$, peak at 7.3 and 9.0 minutes), the peak at 8.7 minutes was identified as BB.

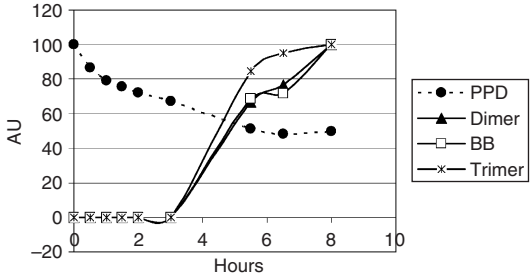

Figure 2. Air oxidation of PPD leads to the formation of dimeric and trimeric auto-oxidation products including BB. The presence of auto-oxidation products was analyzed by qualitative HPLC at the indicated time after addition of PPD to an aqueous solution constantly saturated with air oxygen by a flow. Peak area values were transformed to arbitrary units (AU).

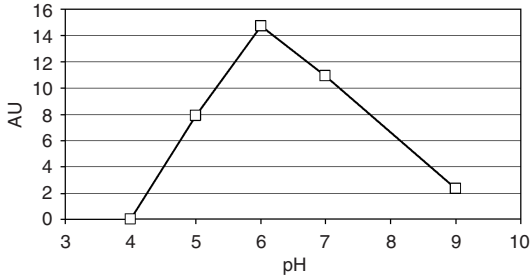

Figure 3. PPD oxidation to BB is pH dependent. The presence of BB was analyzed by qualitative HPLC 1 hour after oxidation of PPD was initiated by the addition of potassium ferrocyanide at the indicated pH. Peak area values were transformed to arbitrary units (AU).

potent inducer of CD86 bright cell population (see Figure 4a), reaching 528% of the negative control at a concentration of 30 μM. Oxidized PPD was less potent, inducing 209% of the negative control at a concentration of 61 μM. Fresh PPD was a comparatively much weaker inducer of the CD86 bright population, reaching a maximum of 127% of the negative control at 182 μM (down to 118% at 243 μM). Very similar patterns were observed with the other activation markers (IL-1β, IL-8, and AQP3; see Figure 4b–d): BB was always the most potent chemical, inducing 432% of negative control at 15.7 μM (IL-1β), 584% at 47 μM (IL-8), and 37.5% at 30 μM (AQP3). Oxidized PPD (268% at 61 μM for IL-1β, 285% at 73 μM for IL-8, and 50% at 73 μM for AQP3) was less potent than BB but nevertheless induced relevant effects at concentrations much lower than PPD (318% at 243 μM for IL-1β, 443% at 122 μM for IL-8, and 74% at 243 μM for AQP3).

Concentration-dependent activation of DC-like cells by mono- and diacetyl PPD

DC-like cells obtained from a pool of four donors were exposed for 24 hours to 30–243 μM monoacetyl PPD and 65–260 μM diacetyl PPD. Higher concentrations were not evaluated due to cytotoxic effects as determined by flow cytometry measurements of fluorescein diacetate incorporation. As a negative control, 25–100 μl of RPMI, the vehicle used for preparing the test items, was tested in parallel.

The percentage of CD86 bright population and IL-1β, IL-8, and AQP3 gene expressions were measured after the 24-hour exposure period (see Figure 5). Monoacetyl PPD had no relevant influence on the modulation of the four selected DC activation markers. Diacetyl PPD had slight effects on the

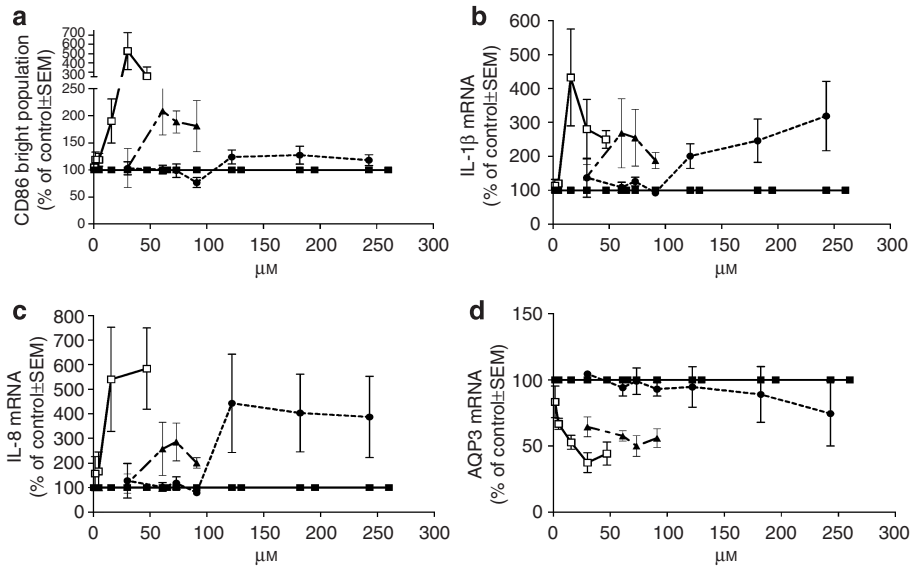

Figure 4. Concentration-dependent activation of DC-like cells by PPD, oxidized PPD, and BB. DC-like cells were exposed for 24 hours to vehicle alone (■), to 30–243 μM fresh PPD (●), 30–91 μM oxidized PPD (▲), and 0.5–47 μM BB (□). (**a**) Flow cytometry analysis of the CD86 bright population, expressed as the percent ratio of the negative control. The mean and standard error of the mean are represented; $n=6$ independent experiments for PPD and BB and $n=3$ for oxidized PPD. (**b**) Corresponding results for IL-1β gene expression measured by RT–PCR. (**c**) Corresponding results for IL-8 gene expression measured by RT–PCR ($n=4$ for BB). (**d**) Corresponding results for *AQP3* gene expression ($n=5$ for BB).

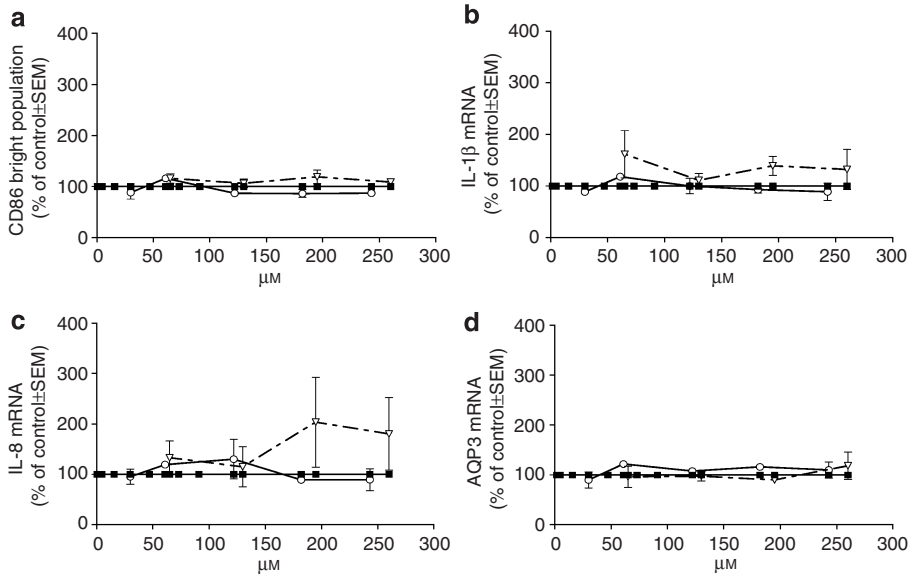

Figure 5. Concentration-dependent activation of DC-like cells by mono- and diacetyl PPD. DC-like cells were exposed for 24 hours to vehicle alone (■), to 30–243 μM monoacetyl PPD (○) and 65–260 μM diacetyl PPD (▽). (**a**) Flow cytometry analysis of the CD86 bright population, expressed as the percent ratio of the negative control. The mean and standard error of the mean are represented; $n=3$ independent experiments. (**b**) Corresponding results for IL-1β gene expression measured by RT–PCR. (**c**) Corresponding results for IL-8 gene expression measured by RT–PCR. (**d**) Corresponding results for *AQP3* gene expression.

mRNA expression of IL-1β (up to 162% of negative control at 65 μM, without dose–effect relationship) and IL-8 (up to 203% of negative control at 195 μM). On the other hand, it had no relevant effect on the percentage of the CD86 bright population or on AQP3 gene expression. Compared to PPD, oxidized PPD, and BB, the acetylated forms of PPD had much less or no effect on the expression of the selected activation markers.

Evaluation of PPD, BB, mono- and diacetyl PPD in the LLNA

The sensitizing properties of PPD (as free base), mono- and diacetyl PPD, and BB were evaluated in a standard LLNA (according to the OECD 429 guideline) by measurement of cell proliferation in the draining lymph nodes after topical application on the ears of mice (see Figure 6). The animals received 25 μl of the test item formulations on each ear on days 0–2 and were killed on day 5 for assessment of cell

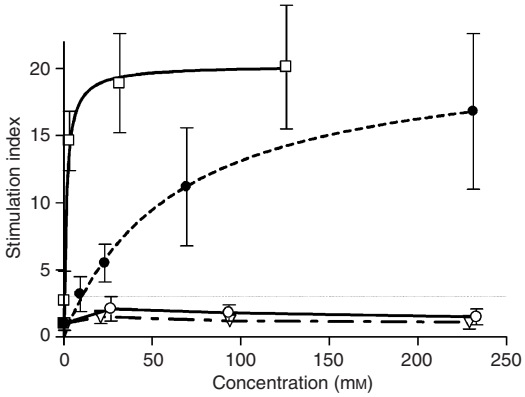

Figure 6. LLNA dose–response of PPD, BB, mono-, and diacetyl PPD. Groups of mice ($n=5$) received topical applications on the dorsum of both ears of 25 µl of PPD (●), BB (□), monoacetyl PPD (○), or diacetyl PPD (▽) in DMSO at the indicated concentrations for 3 consecutive days. On day 5 following the 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.

proliferation in the draining auricular lymph nodes. Concentrations of 0.1% (~9 mM), 0.25% (~23 mM), 0.75% (~70 mM), and 2.50% (~230 mM) of PPD; 0.35% (~27 mM), 1.4% (~93 mM), and 3.5% (~233 mM) of monoacetyl PPD; 0.44% (~21 mM), 1.78% (~94 mM), and 4.44% (~229 mM) of diacetyl PPD; and 0.01% (~0.3 mM), 0.10% (~3 mM), 1.0% (~31 mM), and 4.0% (~126 mM) of BB in DMSO were assessed. PPD and BB were able to induce dose-dependent increases in the LLNA response with EC3 values (calculated by nonlinear regression) of 0.09% (~10 mM) and 0.008% (~0.3 mM), respectively. In contrast, diacetyl PPD tested up its solubility limit in DMSO and monoacetyl PPD applied approximately at the same molar concentrations did not induce a stimulation index above 3 (see Figure 6).

DISCUSSION

In this paper, we investigated the modifications of PPD following its application onto the skin and its subsequent penetration through the stratum corneum until reaching the living epidermis. Specifically, we analyzed the role of oxidation and *N*-acetylation as the major PPD transformation steps, and their significance for activation of the skin immune system that may result in allergic contact dermatitis.

In a first step, we asked what happens to PPD when applied to human or rodent skin surface in skin sensitization studies such as in the guinea pig or LLNA or in a human diagnostic patch test. The skin surface environment is expected to provide excellent conditions for the auto-oxidation of PPD due to its large surface area exposed to air oxygen. In addition, optimal conditions for skin permeation are provided as skin penetration-enhancing vehicles (for example, acetone olive oil in the LLNA; see Trommer and Neubert, 2006) or occlusive conditions (with white petrolatum as vehicle for diagnostic patch testing; see Marzulli and Maibach, 1974; Kim *et al.*, 1987; Basketter *et al.*, 2006; Bruze *et al.*, 2007) are recommended. The recommended exposure times of >48 hours and/or repeated applications support further auto-oxidation and penetration of PPD. To analyze what happens to PPD under auto-oxidation conditions that are expected to occur on the skin surface, we measured PPD oxidation in aqueous solution (1) by air oxygen and (2) under exaggerated conditions using a strong oxidizer (ferrocyanide). In the presence of air oxygen, we found that PPD itself is initially stable, but already after 30 minutes a peak of a second monomer was detected. After 30-minute exposure to strong oxidizing conditions, relatively high amounts of that monomer (Figure 1) possibly corresponding to BQDI were detectable, and polymerization to dimeric and trimeric auto-oxidation products, including BB occurred (Figure 1). A similar spectrum of auto-oxidation products was detected after 8-hour exposure to air oxygen (Figure 2). Similar observations concerning BB formation in aqueous solution (cell culture medium) containing PPD were made by Coulter *et al.* (2007, 2008). Moreover, in a recent work with PPD and *p*-toluenediamine (Goux *et al.*, 2007), similar species were detected. Thus, PPD oxidized by ferrocyanide was used as a source of auto-oxidation products that are expected to occur on the skin under conditions similar to those of *in vivo* skin sensitization tests.

Assuming that, in addition to PPD, its auto-oxidation products are able to overcome the skin barrier and penetrate into the epidermis, activation of skin DC (Langerhans cells) will be required for the induction of skin sensitization. Therefore, we tested fresh, non-oxidized and oxidized PPD (that is, the mixture of PPD with ferrocyanide after 30-minute incubation) as well as BB for their potential to induce *in vitro* activation of human immature DC derived from peripheral blood monocytes (see Figure 4).

BB was the most potent DC activator, inducing a strong modulation of the four chosen markers at relatively low concentrations (<30 µM). Pre-oxidized PPD solution, which contained PPD, BB, BQDI, and other oxidation products (see Figure 1), was less potent than pure BB, that is, higher concentrations were needed to induce a relatively weaker modulation of all tested markers (maximal effects were observed after exposure to 61 µM of initial PPD). On the other hand, the fresh PPD solution was a comparatively much weaker inducer of DC activation. Consequently, the observed DC activation may not be due to PPD itself but to its oxidation products formed during the 24-hour incubation period under the reducing conditions of the culture medium in presence of living cells. It had no relevant effect on the CD86 bright population up to the highest subtoxic concentration tested (243 µM). Interestingly, relevant effects were observed with the other three markers with a sharp increase at concentration >91 µM (see Figure 4). This may be due to a threshold effect when exceeding the limited reducing capacity of the culture medium or to the need of a threshold PPD concentration for forming sufficient auto-oxidation products of PPD. Interestingly, similar results were obtained by Toebak *et al.* (2006) who observed an upregulation of activation markers such as CD86 and IL-8 at a concentration range of 50–200 µM and by Hulette *et al.* (2005) who

measured a limited CD86 expression after exposure to 2,500 μM PPD whereas exposure to BB induced a robust increase at a much lower concentration (100 μM). On the basis of these results and on their experiments using a similar test system, Coulter *et al.* (2007) proposed concentration-dependent effects of PPD on DC function, that is, low concentrations (<50 μM) induce CD40 and major histocompatibility complex class II expression, medium concentrations (⩽200 μM) initiate CXCL8 secretion, whereas concentrations of 2,500 μM and above lead to CD86 expression and strong cytotoxicity.

Our *in vitro* results for BB were fully confirmed by LLNA experiments. As expected, BB induced a vigorous dose-dependent LLNA response (see Figure 6). The apparent discrepancy between the weak *in vitro* DC activation potential of fresh PPD in the cell culture and its strong sensitizing potency in the LLNA following application on the mouse ear (see Figures 4 and 6) is likely explained by the presumed auto-oxidation conditions prevalent on the skin surface. Consequently, the strong dose-dependent LLNA response to PPD is considered equivalent to the relevant DC activation potential of pre-oxidized PPD.

So far, our data indicate that oxidation is important during sensitization to PPD as indicated by the strong DC activation potential of oxidized PPD solution. Non-oxidized PPD has only a limited potential to activate DCs, probably due to an initial absence of auto-oxidation products (as present in oxidized PPD). Over time and with increasing concentrations, DC activation starts to occur in line with the formation of auto-oxidation products during the 24-hour *in vitro* incubation period (Figure 4b and c). This is in agreement with results from human testing, showing that successful induction and elicitation of sensitization to PPD is dependent on dose, duration, and frequency of exposure, that is, induction of sensitization to 1% PPD in the human repeated insult patch test decreased from 54 to 3% when the exposure duration was reduced from 48 hours to 5 minutes (Basketter *et al.*, 2006). Similarly, elicitation of sensitization to 1% PPD during patch testing decreased from 69 to 16%, when the exposure time was reduced from 120 to 5 minutes (Hextall *et al.*, 2002 and similar in McFadden *et al.*, 1998 and White *et al.*, 2007). On the basis of our results (Figure 2), an exposure period of 5 minutes is too short for the formation of sufficient amounts of auto-oxidation products to cause DC activation. This is also consistent with the considerable exposure time-dependent differences in the absorbed concentrations of PPD equivalents: after non-rinse exposure for 48 hours to 1% PPD, approximately 20–50% of the applied patch test dose became absorbed (Kim *et al.*, 1987), whereas exposure to 2% for 30 minutes and rinse off (hair dyeing conditions) led to approximately 1.1% absorption and 1.3% in the epidermis and dermis (Hueber-Becker *et al.*, 2004).

The observation that a spectrum of PPD auto-oxidation products is probably responsible for the induction of PPD sensitization is further supported by the finding that human Langerhans cells exposed to BB, but not PPD, were able to induce a primary T-cell response *in vitro* (Krasteva *et al.*, 1996). Furthermore, T cells from a number of PPD-allergic patients did not react to PPD, but to BB (Krasteva *et al.*, 1993; Sieben *et al.*, 2002), and T-cell clones from mice immunized with a model peptide actively haptenated with PPD (in the presence of air oxygen) reacted to the same peptide when haptenated with BB and *vice versa* (Wulferink *et al.*, 2005) indicating that a common oxidation product of PPD is responsible for T-cell recognition. However, in some studies only a limited number of PPD-allergic patients developed an elicitation response to BB under patch test conditions (Sieben *et al.*, 2002; White *et al.*, 2006), probably due to the limited skin-penetrating properties of BB compared to PPD (Smith Pease *et al.*, 2005). In more recent experiments (Coulter *et al.*, 2008), BB formation (prevented by addition of glutathione) was not required to induce additional proliferation of lymphocytes from the allergic patients, indicating that BB might not be necessarily present during elicitation responses, that is, might not directly be involved in T-cell receptor-mediated recognition. However, successful induction of a primary immune response to PPD is dependent on PPD oxidation to BB (Krasteva *et al.*, 1996), which, based on our findings is required to provide efficient DC activation and thus for costimulatory (danger) signaling, that is, the difference in the magnitude of the danger signal provided by PPD (low) versus BB/oxidized PPD (high, see Figure 4) is considered as predominately relevant during the induction phase of contact sensitization.

BQDI was described to be the first oxidation derivative of allergenic *p*-amino aromatic compounds that can directly react with nucleophilic amino acids and was seriously considered as a potential candidate for the formation of antigenic determinants (Eilstein *et al.*, 2006). Our findings support the view that the formation of a monomer corresponding to BQDI is the starting event to render PPD immunogenic either by a direct reaction with proteins or by autoreaction to further haptens such as BB (Figures 1 and 2) that themselves are protein reactive. For BB, strong protein reactivity has been already confirmed by Gerberick *et al.* (2007) using a peptide reactivity assay. Consequently, these PPD auto-oxidation products were able to induce DC activation (Figure 4), a decisive step to initiate an immune response. The concept with BQDI as the activated monomer giving rise to common antigenic determinants is also in line with the frequently observed cross reactivity with other *p*-amino aromatic compounds (Uter *et al.*, 2002).

This auto-oxidation scenario of PPD is believed to occur under most skin sensitization induction and elicitation protocols and thus helps to explain the strong sensitizing potency of PPD. Consequently, the DC activation properties and the sensitizing potency of BB are higher than that of PPD, as BB is one stable end product of the PPD auto-oxidation process (Figure 2). This concept is also in line with the comparatively low EC3 value of BB (0.01%, equivalent to 0.3 mM; see Figure 6) and the described ability of BB to induce primary T-cell responses *in vitro* (Krasteva *et al.*, 1996). In patients, the strong linear association between PPD containing temporary black henna tattoos and strong patch test reactions (grade + + +) (Ho *et al.*, 2005) supports our hypothesis that uncontrolled air oxidation of PPD on the skin

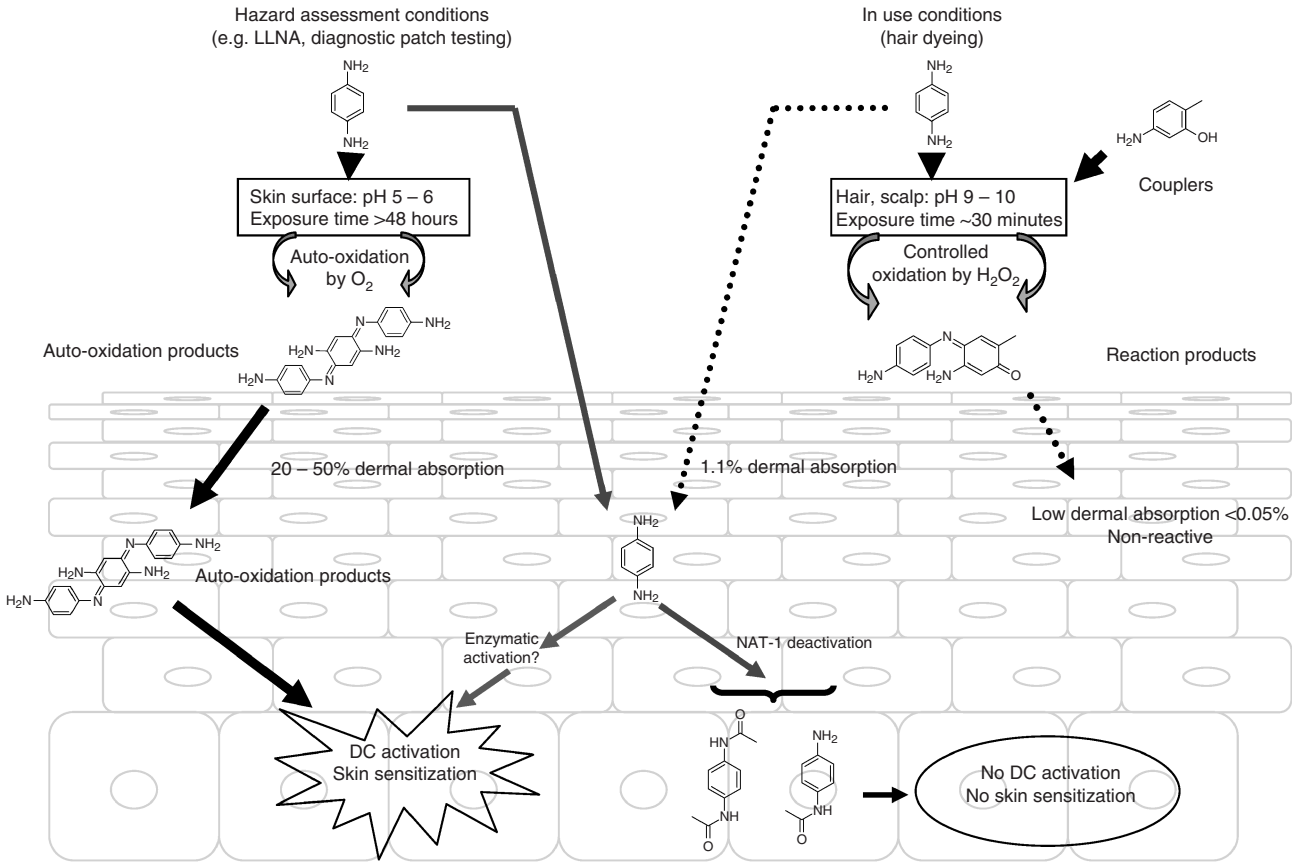

Figure 7. Comparison of hazard assessing conditions and in-use conditions (hair dyeing) of PPD. In hazard assessment, the skin is exposed to PPD and air oxygen for >48 hours at pH 5–6, that is, optimal conditions for the auto-oxidation of PPD resulting in the formation of BB and further dimeric and trimeric oxidation products. The long exposure period further enhances the dermal absorption of PPD and its auto-oxidation products (Kim *et al.*, 1987) that may induce DC activation and finally allergic contact dermatitis. Under in-use conditions of hair dyeing, the scalp is exposed to PPD for a limited period at high pH, and PPD auto-oxidation is blocked by an excess of couplers, leading to the controlled generation of the hair coloring, nonreactive reaction products. The short exposure time leads to a comparatively low dermal absorption of PPD (Hueber-Becker *et al.*, 2004) and of the reaction products (SCCP, 2006). Non-oxidized PPD that reaches the epidermis is subjected to *N*-acetylation in keratinocytes, due to their high content of the mediating enzyme *N*-acetyltransferase 1. The resulting mono- and diacetylated PPD metabolites have no DC activating potential and are nonsensitizers. For quantities of PPD exceeding the saturation threshold of *N*-acetylation, PPD activation by oxidation to protein reactive auto-oxidation products may occur by enzymes such as peroxidase, cyclooxygenase, or cytochromes P450 (Strohm and Kulkarni, 1986; Sieben *et al.*, 2002; Merk *et al.*, 2006). Consequently, in situations of disturbed barrier function (Proksch and Brasch, 1997), or deficiencies in cutaneous *N*-acetylation (Kawakubo *et al.*, 1997) activation of the innate immune system by PPD oxidation products becomes more likely.

can lead to very strong allergic reactions, as temporary tattoo users are exposed to PPD concentrations as high as 15.7% for several weeks (Brancaccio *et al.*, 2002).

In contrast, when PPD is applied to human skin under oxidative hair dyeing conditions, PPD is activated by hydrogen peroxide to BQDI, but the formation of higher molecular PPD auto-oxidation products is blocked (SCCP, 2005). Instead, the intended reaction to higher molecular, nonreactive hair coloring molecules is chemically preferred due to the presence of an excess of couplers (that is, resorcinols, *m*-aminophenols, *m*-phenylenediamines; Brody and Burns, 1968). When the formation of the final coloring molecules is completed, up to 1.1% of unconsumed PPD was found (Rastogi *et al.*, 2006). The recent finding that exposure to complete PPD containing shades under realistic conditions for 30 minutes and 1 hour was insufficient to elicit a positive patch test reaction in PPD-allergic patients that reacted to the standard diagnostic procedure (48-hour exposure to 1% PPD) with grade (+) or (++) (Jowsey *et al* 2006) is in line with our hypothesis that unmodified PPD is less immunogenic than PPD auto-oxidation products.

Unconsumed PPD that arrives in the living epidermis (Hueber-Becker *et al.*, 2004; White *et al.*, 2007) is considered to predominantly undergo dermal *N*-acetylation as the majority is diacetylated when reaching the plasma (Dressler and Appelqvist, 2006) and more than 80% are mono- or diacetylated when excreted in the urine following hair dyeing (Nohynek *et al.*, 2004). This is due to the high capacity of keratinocytes to *N*-acetylate PPD and aromatic amines in general by the mediating enzyme *N*-acetyltransferase 1 (Kawakubo *et al.*, 1990, 2000; Nohynek *et al.*, 2006). Both, mono- and diacetylated PPD, were unable to induce DC activation *in vitro* (Figure 5) and *in vivo* (Figure 6) consistent with the described inability of lymphocytes from PPD-allergic

patients to recognize acetylated metabolites of PPD (Sieben *et al.*, 2001).

In summary, based on the results obtained with DCs and the chemical analysis of the auto-oxidation process of PPD in aqueous solution, we could provide evidence that under *in vivo* conditions of prolonged skin exposure (for example, temporary henna tattoos, LLNA, human diagnostic patch testing) a substantial quantity of PPD will be air-oxidized on the skin surface and produce sensitizing auto-oxidation products possibly with BQDI as initial short-lived intermediate of the auto-oxidation process (Brody and Burns, 1968; Goux *et al.*, 2007) that itself is considered as too reactive to pass the skin barrier (see Figure 7, upper left part). However, formation of the strong sensitizer BB as one example for a PPD auto-oxidative end product is very likely, as the normal skin pH of about 5.7 (Schmid-Wendtner and Korting, 2006) provides optimal conditions for the stability of BB (Figure 3). During oxidative hair dyeing, the scalp skin is exposed to formulations containing PPD and couplers for a short period ($\sim$30 minutes) and the chemistry (pH $>$ 9) is designed to favor coupling with couplers, and therefore the formation of di- and trimeric PPD auto-oxidation products such as BB is prevented (Brody and Burns, 1968; Bracher *et al.*, 1990; SCCP, 2005; see Figure 7, upper right part). Furthermore, there is no exposure to BQDI due to its high reactivity with couplers to form the hair coloring reaction products (Goux *et al.*, 2007, Goux *et al.*, follow-up manuscript in preparation). When non-consumed PPD reaches the epidermis, it will be metabolized to mono- and diacetylated PPD. For PPD quantities exceeding the individual capacity for *N*-acetylation, we speculate that PPD oxidation to protein reactive auto-oxidation products may occur by active metabolism (see Figure 7, bottom). We conclude that the equilibrium between enzymatic deactivation and activation of PPD is critical for obtaining an epidermal concentration of activated PPD that provides effective DC activation needed to induce skin sensitization.

This paper emphasizes that *in vitro* DC activation testing allowed a precise analysis of different sensitizing properties of chemicals, that is, identifying that PPD itself is nonreactive *in vitro*, but needs activation by air oxygen to become immunogenic.

MATERIALS AND METHODS

Materials

PPD sulfate (CAS no. 16245-77-5) was purchased from Acros Organics through Chemie Brunschwig AG, Basel, Switzerland. 4-Aminoacetanilide (monoacetyl PPD; CAS no. 122-80-5), 2,4,6-trinitrobenzenesulfonic acid (CAS no. 2508-19-2) 5% (w/v) solution in water and potassium hexacyanoferrate(II) trihydrate ($K4[Fe(CN)6] \times 3H_2O$; CAS no. 14459-95-1) were purchased from Sigma-Aldrich, Buchs, Switzerland. Potassium hexacyanoferrate(III) ($K3[Fe(CN)6]$; CAS no. 13746-66-2) was purchased from Merck (Schweiz) AG Chemicals, Dietikon, Switzerland. *N,N′*-Diacetyl-PPD (diacetyl PPD; CAS no. 140-50-1) and BB (CAS no. 20048-27-5) of documented purity (approximately 97% by HPLC) were obtained through our in-house synthesis department.

Culture medium

The culture medium was RPMI 1640 without phenol red (Sigma-Aldrich) supplemented with 10% fetal calf serum (Amimed, Allschwil, Switzerland), 2 mM L-glutamine (Biochrom KG, Berlin, Germany), 800 U ml^{-1} of rhGM-CSF (Leucomax, Essex Chemie AG, Luzern, Switzerland) and 1,000 U ml^{-1} of IL-4 (Strathmann Biotech GmbH, Hannover, Germany) referred below as complete culture medium.

Purification of human monocytes and generation of dendritic-like cells. Dendritic-like cells were prepared as described (Aeby *et al.*, 2004). Briefly, fresh buffy coats were obtained from the 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 centrifugations and 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 days in complete culture medium. On the fourth day, half of the culture medium was replaced by fresh medium. The CD1a, CD45, 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 mixing 1 volume of cells (approximately 0.4×10^6 cells) with 1 volume of Isoton (Coulter Isoton II diluent, Beckmann Coulter, Zürich, Switzerland) followed by a 20-minute incubation at 2–8 °C with the FITC or phycoerythrin-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). Nonspecific 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 phycoerythrin-labeled antibodies. Cell viability was determined by measuring the percentage of cells positive in the FL1 channel (living cells) after 20-minute incubation with 50 ng ml^{-1} fluorescein diacetate (Sigma-Aldrich). The data analysis was performed with the System II, v. 3.0 software (Beckman Coulter).

In vitro DC activation test protocol. The tests were performed as described (Aeby *et al.*, 2004). Briefly, the test items and the control dissolved in 100 µl of vehicle (10 µl for DMSO) are added at day 4 to 2 ml wells containing 1–2 $\times$ 10^6 cells. After 6 hours, half of the medium is replaced by fresh complete medium. At the indicated time points, cells are harvested for reverse transcriptase (RT)–PCR and flow cytometry analysis as described. In the concentration-dependent activation experiments, the highest nontoxic concentration was selected as the upper limit of the dose range (a test item concentration was considered as nontoxic when the relative cell

viability reached at least 85% of the respective negative control). Cell viability is determined by measuring the percentage of cells positive in the FL1 channel (living cells) after 20-minute incubation with 50 ng ml^{-1} fluorescein diacetate (Sigma-Aldrich).

Three negative controls with an appropriate volume of the solvent used for the test item are included in each assay for each time point. In each experiment, one well was treated with 682 μM 2,4,6-trinitrobenzenesulfonic acid as a positive control.

Oxidation of PPD. For the generation of pre-oxidized PPD by ferrocyanide exposure, 1 ml of a 67 μM PPD (13.8 μg ml^{-1}) aqueous solution was mixed with 1 ml of a 134 μM K3[Fe(CN)6] (44.02. μg ml^{-1}) solution for 30 minutes at room temperature and was analyzed. For the experiments with exposure of PPD to air oxygen, an aqueous solution containing 67 μM PPD was constantly saturated by sparging air through a fritted gas dispersion tube and analysis was performed at several time points between 30 minutes and 8 hours after addition of PPD. For HPLC analysis, a mobile phase consisting of methanol (Biosolve, Valkenswaard, The Netherlands), ammonium acetate (Fluka, Buchs, Switzerland, puriss p.a.), and formic acid (Fluka, HPLC grade) was used. A triple quadrupole mass spectrometer (Micromass, LC-Quattro) controlled by the Masslynx software and equipped with an Agilent HPLC (1,100 Series) was used for ESI-MS^2 measurements; these were performed in positive ion mode. Typical instrumental setup used was: 3 kV capillary gas, 25 V cone voltage, 2 V extractor cone voltage, and 0.2 V radio frequency lens voltage. The source temperature was set to 120 °C and the desolvation temperature was 600 °C. In the case of the MS^2 experiments, argon was used as collision gas and the pressure and collision cell parameters were optimized for optimal intensity of fragments. MS^2 spectra were recorded in Daughter ion mode and total ion current-MS spectra were recorded in full-scan mode.

PH-dependent oxidation of PPD to BB. PPD was dissolved at 1.5 mg ml^{-1} in the following standard buffer solutions at the indicated pH (potassium hydrogen phtalate/NaOH for pH 4, 5, and 6, potassium dihydrophosphate/sodium hydrophosphate for pH 7 and sodium tetraborate/HCl for pH 9) at room temperature. Then, an equimolar amount of potassium ferrocyanide was added and 1 hour later the samples were analyzed for the presence of BB by qualitative HPLC analysis as follows: supernatants obtained after centrifugation were filtered, diluted in HPLC eluent and injected on an Atlantis column (Waters AG, Baden-Dättwil, Basel, Switzerland) under isocratic conditions using 0.06 M ammonium formiate at pH 7.5 in 25% acetonitrile at a flow rate of 0.8 ml min^{-1}. A diode array detector (Waters AG) was used and the BB peak area was measured at 480 nm at 25–26 minutes.

RNA extraction and reverse transcription. Total RNA was isolated from cell cultures using the TRIzol method (Invitrogen AG, Basel, Switzerland) according to the manufacturer's instructions. Briefly, the cells were pelleted by centrifugation at 600 *g* for 5 minutes at 2–8 °C. Subsequent reactions took place at 2–8 °C. The pellet was resuspended in the TRIzol reagent (400 μl per 10^6 cells), homogenized by pipetting vigorously, and briefly vortexed. After addition of chloroform (Fluka; 80 μl per 10^6 cells) the sample was vortexed, incubated for 5 minutes and centrifuged at 12,000 *g* for 30 minutes. The aqueous phase containing RNA was transferred to a new tube and RNA was precipitated with 1 volume isopropanol for at least 45 minutes (Fluka). The pellet was washed with 75% ethanol (Fluka) and resuspended in 50 μl TE buffer (Amresco, BioConcept, Allschwil, Switzerland). The RNA was quantified by OD_{260} measurement and adjusted to 50 μg ml^{-1}. Purified total RNA (450 ng) was reverse transcribed using random hexamers (50 ng per reaction) using the ThermoScript RT–PCR system (Invitrogen), according to the instruction manual.

Analysis of gene expression by quantitative PCR (real-time PCR). The 28S ribosomal RNA (28S rRNA) cDNA was quantified through real-time PCR on the LightCycler with the kit ''LightCycler—FastStart DNA Master SYBR Green I'' (Roche Diagnostics, Rotkreuz, Switzerland). The oligonucleotide primers for 28S rRNA (GenBank accession no. M11167) used were 5′-CGGTACACCTGTCAAACGG TAAC-3′ and 5′-TTAGAGGCGTTCAGTCATAATCCC-3′ (Microsynth GmbH, Balgach, Switzerland). The PCR was carried out in glass capillaries for 40 cycles according to the manufacturer's instructions at a final $MgCl_2$ concentration of 4 mM. Briefly, a cycle consisted of a denaturation for 15 seconds at 95 °C, an annealing for 5 seconds at 65 °C, and an elongation at 72 °C for 21 seconds. The green fluorescence was detected at the end of each elongation cycle. The amplification specificity of the PCR products was checked by the melting curve analysis. The quantification data were then analyzed with the LightCycler analysis software using the second derivative maximum method.

The IL-1β, IL-8, and AQP3 cDNAs were quantified with the kit ''Premix Ex Taq (Perfect Real Time)'' (TaKaRa, AxonLab, Le Mont-sur-Lausanne, Switzerland), using TaqMan probes purchased from Applied Biosystems (Rotkreuz, Switzerland) through the website http://www.appliedbiosystems.com. The probes (TaqMan Gene Expression Assays) used were IL-1β (assay ID: Hs00174097_m1), IL-8 (assay ID: Hs00174103_m1), and AQP3 (assay ID: Hs00185020_m1). The PCR was carried out in glass capillaries at a final $MgCl_2$ concentration of 3 mM for 50 cycles. Briefly, a cycle consisted of a denaturation for 10 seconds at 95 °C, an annealing/elongation for 30 seconds at 60 °C. The fluorescence measurement was detected at the end of each elongation cycle. The quantification data were then analyzed with the LightCycler analysis software using the second derivative maximum method. The expression levels of IL-1β and IL-8 cDNAs were finally normalized to the expression level of 28S rRNA cDNA by computing the ratio amount of IL-1β and IL-8 cDNA/amount of 28S rRNA cDNA.

Local lymph node assay. Murine LLNAs 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, PPD free base (CAS no. 106-50-3; Sigma-Aldrich), mono- and diacetyl PPD and BB were dissolved in DMSO on each treatment day and used within 5 hours of preparation. Five days following the initiation of the treatment, all mice were injected intravenously via the tail vein with 250 μl of phosphate-buffered saline containing approximately 20 μCi ^{3}H-methyl-thymidine. After 5 hours mice were killed by carbon dioxide inhalation and draining auricular lymph nodes were excised

and pooled for each animal. Cells were washed twice with phosphate-buffered saline and precipitated in 5% trichloroacetic acid at 4 °C overnight. Pellets were then resuspended in 1 ml of 5% trichloroacetic acid and transferred into scintillation vials. Incorporation of ^{3}H-methyl-thymidine 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. For calculating the EC3 values for BB and PPD, respectively, nonlinear regression with curve fit (GraphPad Prism 3.0) was used. All procedures were performed in AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International accredited facilities and conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Time course of the modulation of the CD86 bright cell population after exposure to PPD, its oxidation products, and *N*-acetylated derivatives.

REFERENCES

Aeby P, Wyss C, Beck H, Griem P, Scheffler H, Goebel C (2004) Characterization of the sensitizing potential of chemicals by *in vitro* analysis of dendritic cell activation and skin penetration. *J Invest Dermatol* 122:1154–64

Basketter DA, Goodwin BFJ (1988) Investigation of the prohapten concept cross reactions between 1,4-substituted benzene derivatives in the guinea pig. *Contact Dermatitis* 19:248–53

Basketter DA, Jefferies D, Safford BJ, Gilmor NJ, Jowsey IR, McFadden J *et al.* (2006) The impact of exposure variables on the induction of skin sensitization. *Contact Dermatitis* 55:178–85

Basketter DA, Liden C (1992) Further investigation of the prohapten concept: reactions to benzene derivatives in man. *Contact Dermatitis* 27:90–2

Bracher M, Faller C, Grötsch W, Marshall R, Spengler J (1990) Studies on the potential mutagenicity of *p*-phenylenediamine in oxidative hair dye mixtures. *Mutat Res* 241:313–23

Bergström MA, Ott H, Carlsson A, Neis M, Zwadlo-Klarwasser G, Jonsson CAM *et al.* (2007) A skin-like cytochrome P450 cocktail activates prohaptens to contact allergenic metabolites. *J Invest Dermatol* 127: 1145–53

Bertrand F, Basketter DA, Roberts DW, Lepoittevin JP (1997) Skin sensitization to eugenol and isoeugenol in mice: possible metabolic pathways involving ortho-quinone and quinone methide intermediates. *Chem Res Toxicol* 10:335–43

Brancaccio RR, Brown LH, Chang YT, Fogelman JP, Mafong EA, Cohen DE (2002) Identification and quantification of para-phenylenediamine in a temporary black henna tattoo. *Am J Cont Derm* 13:15–8

Brody F, Burns MS (1968) Studies concerning the reactions of oxidation dye intermediates. *J Soc Cosmet Chem* 19:361–79

Bruze M, Frick-Engfeldt M, Gruvberger B, Isaksson M (2007) Variation in the amount of petrolatum preparation applied at patch testing. *Contact Dermatitis* 56:38–42

Coulter EM, Farrell J, Mathews KL, Maggs JL, Pease CK, Lockley DJ *et al.* (2007) Activation of human dendritic cells by *p*-phenylenediamine. *J Pharmacol Exp Ther* 320:885–92

Coulter EM, Jenkinson C, Wu Y, Farrell J, Foster B, Smith A *et al.* (2008) Activation of T-cells from allergic patients and volunteers by *p*-phenylenediamine and Bandrowski's base. *J Invest Dermatol* 128: 897–905.

de Baey A, Lanzavecchia A (2000) The role of aquaporins in dendric cell macropinocytosis. *J Exp Med* 191:743–7

Dressler WE, Appelqvist T (2006) Plasma/blood pharmacokinetics and metabolism after dermal exposure to para-aminophenol or para-phenylenediamine. *Fd Chem Toxicol* 44:371–9

Eilstein J, Geménez-Arnau E, Duché D, Rousset F, Lepoittevin JP (2006) Synthesis and reactivity toward nucleophilic amino acids of 2,5-[^{13}C]-dimethyl-*p*-benzoquinonediimine. *Chem Res Toxicol* 19:1248–56

Friedmann PS (2007) The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. *Br J Dermatol* 157:1093–102

Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP (2007) Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. *Toxicol Sci* 97:417–27

Goux A, Pratt D, Dunsch L (2007) The reaction mechanism of *p*-toluenediamine anodic oxidation: an *in situ* ESR-UV/Vis/NIR spectroelectrochemical study. *Chemphyschem* 8:2101–6

Hextall JM, Alagraratnam NJ, Glendinning AK, Holloway DB, Blaikie L, Basketter DA *et al.* (2002) Dose–time relationships for elicitation of contact allergy to para-phenylenediamine. *Contact Dermatitis* 47:96–9

Ho SGY, Basketter DA, Rycroft RJG, White IR, Mcfadden JP (2005) Analysis of para-phenylenediamine allergic patients in relation to strength of patch test reaction. *Br J Dermatol* 153:364–7

Hueber-Becker F, Nohynek GJ, Meuling WJ, Benech-Kieffer F, Toutain H (2004) Human systemic exposure to a [14C]-para-phenylenediamine-containing oxidative hair dye and correlation with in vitro percutaneous absorption in human or pig skin. *Food Chem Toxicol* 42:1227–36

Hulette BC, Ryan CA, Gildea LA, Gerberick GF (2005) Relationship of CD86 surface marker expression and cytotoxicity on dendritic cells exposed to chemical allergen. *Toxicol Appl Pharmacol* 209:159–66

Jerschow E, Hostynek JJ, Maibach HI (2001) Allergic contact dermatitis elicitation thresholds of potent allergens in humans. *Fd Chem Toxicol* 39:1095–108

Jowsey IR, Basketter DA, McFadden JP, Kullavanijaya P, Duangdeen I (2006) Elicitation response characteristics to permanent hair dye in paraphenylenediamine-allergic volunteers. *Contact Dermatitis* 55:330–4

Kawakubo Y, Merk HF, Al Masaoudi T, Sieben S, Blömeke B (2000) *N*-Acetylation of paraphenylenediamine in human skin and keratinocytes. *J Pharmacol Exp Ther* 292:150–5

Kawakubo Y, Nakamori M, Schopf E, Ohkido M (1997) Acetylator phenotype in patients with *p*-phenylenediamine allergy. *Dermatology* 195:43–5

Kawakubo Y, Yamazoe Y, Kato R, Nishikawa T (1990) High capacity of human skin for *N*-acetylation of arylamines. *Skin Pharmacol* 3:180–5

Kim HO, Wester RC, McMaster JA, Bucks DA, Maibach HI (1987) Skin absorption from patch test systems. *Contact Dermatitis* 17:178–80

Kimber I, Basketter DA (1992) The murine local lymph node assay: a commentary on collaborative studies and new directions. *Fd Chem Toxicol* 30:165–9

Koopmans AK, Bruynzeel DP (2003) Is PPD a useful screening agent? *Contact Dermatitis* 48:89–92

Krasteva M, Moulon C, Peguet-Navarro J, Courtellemont P, Redziniak G, Schmitt D (1996) *In vitro* sensitization of human T cells with hapten-treated Langerhans cells: a screening test for the identification of contact allergens. *Curr Probl Dermatol* 25:28–36

Krasteva M, Nicolas JF, Chabeau G, Garrigue JL, Bour H, Thivolet J *et al.* (1993) Dissociation of allergenic and immunogenic functions in contact sensitivity to *p*-phenylenediamine. *Int Arch Allergy Immunol* 102:200–4

Lepoittevin JP (2006) Metabolism versus chemical transformation or pro- versus prehaptens? *Contact Dermatitis* 54:73–4

Lisi P, Hansel K (1998) Is benzoquinone the prohapten in cross-sensitivity among aminobenzene compounds? *Contact Dermatitis* 39:304–6

Marzulli FN, Maibach HI (1974) The use of graded concentrations in studying skin sensitizers: experimental contact sensitization in man. *Fd Chem Toxicol* 12:219–27

McFadden JP, Wakelin SH, Holloway DB, Basketter DA (1998) The effect of patch duration on the elicitation of para-phenylenediamine contact allergy. *Contact Dermatitis* 39:79–81

Merk HF, Baron JM, Heise R, Fritsche E, Schroeder P, Abel J *et al.* (2006) Concepts in molecular dermatotoxicology. *Exp Dermatol* 15:692–704

Nohynek GJ, Duche D, Garrigues A, Meunier PA, Toutain H, Leclaire J (2006) Under the skin: biotransformation of para-aminophenol and para-phenylenediamine in reconstructed human epidermis and human hepatocytes. *Toxicol Lett* 158:196–212

Nohynek GJ, Skare JA, Meuling WJ, Hein DW, De Bie AT, Toutain H (2004) Urinary acetylated metabolites and *N*-acetyltransferase-2 genotype in human subjects treated with a para-phenylenediamine-containing oxidative hair dye. *Fd Chem Toxicol* 42:1885–91

Proksch E, Brasch J (1997) Influence of epidermal permeability barrier disruption and Langerhans' cell density on allergic contact dermatitis. *Acta Derm Venereol* 77:102–4

Rastogi SC, Søsted H, Johansen JD, Menné T, Bossi R (2006) Unconsumed precursors and couplers after formation of oxidative hair dyes. *Contact Dermatitis* 55:95–100

SCCP (EU Scientific Committee on Consumer Products) (2005) (13.12.2005) Opinion on exposure to reactants and reaction products of oxidative hair dye formulations, pp 1–34

SCCP (EU Scientific Committee on Consumer Products) (2006) (20.06.2006) Skin penetration of oxidative hair dyes formed by the coupling of precursors and couplers under simulated conditions of hair dyeing. Update of the annex to the opinion on exposure to reactants and reaction products of oxidative hair dye formulations, pp 1–18

Schmid-Wendtner MH, Korting HC (2006) The pH of the skin surface and its impact on the barrier function. *Skin Pharmacol Physiol* 19:296–302

Sieben S, Kawakubo Y, Al Masaoudi T, Merk HF, Blomeke B (2002) Delayed-type hypersensitivity reaction to paraphenylenediamine is mediated by 2 different pathways of antigen recognition by specific alphabeta human T-cell clones. *J Allergy Clin Immunol* 109:1005–11

Sieben S, Kawakubo Y, Sachw B, Al Masaoudi T, Merk HF, Blömeke B (2001) T cell responses to paraphenylenediamine and to its metabolites mono- and diacetyl-paraphenylenediamine. *Int Arch Allergy Immunol* 124:356–8

Smith CK, Moore CA, Elahi EN, Smart ATS, Hotchkiss SAM (2000) Human skin absorption and metabolism of the contact allergens, cinnamic aldehyde, and cinnamic alcohol. *Toxicol Appl Pharmacol* 168:189–99

Smith Pease CK, Pendlington RU, Sanders D, Minter H, Basketter DA (2005) Skin absorption and metabolism of contact allergens: *p*-phenylenediamine. *15th International Symposium of Contact Dermatitis (ISCD), Paphos, Cyprus (abstr.)*:S169–S202

Sosted H, Menné T, Johansen JD (2006) Patch test dose–response study of *p*-phenylenediamine: thresholds and anatomical regional differences. *Contact Dermatitis* 54:145–9

Strohm BH, Kulkarni AP (1986) Peroxidase, an alternate pathway to cytochrome P-450 for xenobiotic metabolism in skin: partial purification and properties of the enzyme from neonatal rat skin. *J Biochem Toxicol* 1:83–97

Toebak MJ, Pohlmann PR, Sampat-Sardjoepersad SC, von Blomberg BM, Bruynzeel DP, Scheper RJ (2006) CXCL8 secretion by dendritic cells predicts contact allergens from irritants. *Toxicol In Vitro* 20:117–24

Trommer H, Neubert RHH (2006) Overcoming the stratum corneum: the modulation of skin penetration. *Skin Pharmacol Physiol* 19:106–21

Uter W, Lessmann H, Geier J, Becker D, Fuchs T, Richter G (2002) The spectrum of allergic (cross-)sensitivity in clinical patch testing with ''para amino'' compounds. *Allergy* 57:319–22

Warbrick EV, Dearman RJ, Lea LJ, Basketter DA, Kimber I (1999) Local lymph node assay responses to paraphenylenediamine: intra- and inter-laboratory evaluations. *J Appl Toxicol* 19:255–60

White JM, Basketter DA, Pease CK, Sanders DA, McFadden JP (2007) Intermittent exposure to low-concentration paraphenylenediamine can be equivalent to single, higher-dose exposure. *Contact Dermatitis* 56:262–5

White JM, Kullavanijaya P, Duangdeeden I, Zazzeroni R, Gilmour NJ, Basketter DA *et al.* (2006) *P*-Phenylenediamine allergy: the role of Bandrowski's base. *Clin Exp Allergy* 36:1289–93

Wulferink M, Dierkes S, Gleichmann E (2005) Cross-sensitization to haptens: formation of common haptenic metabolites, T cell recognition of cryptic peptides, and true T cell cross-reactivity. *Eur J Immunol* 32:1338–47

Xie Z, Hayakawa R, Sugiura M, Kojima H, Konishi H, Ichihara G *et al.* (2000) Experimental study on skin sensitization potencies and cross-reactivities of hair-dye-related chemicals in guinea pigs. *Contact Dermatitis* 42:270–5