

Consumer available permanent hair dye products cause major allergic immune activation in an animal model

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Summary

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Background p-Phenylenediamine (PPD) and related substances are ingredients of more than two-thirds of oxidative (permanent) hair dyes currently used. Although PPD is a potent skin sensitizer in predictive assays, the extent to which permanent hair dyes sensitize humans has been questioned due to the in-use conditions, e.g. the presence of couplers in the hair dye gel and rapid oxidation using a developer.

Objectives To study the skin sensitizing potential of permanent hair dyes in mice.

Methods Two different permanent hair dye products containing PPD were studied in CBA mice using a modified version of the local lymph node assay. The colour gel and developer (oxidant) were tested separately and in combination. Response was measured by ear swelling and cytokine production in ear tissue and serum by enzyme-linked immunosorbent assay. The immune cellular response in the draining lymph nodes was analysed by flow cytometry.

Results Application of the colour gel both alone and mixed with the developer induced skin production of interleukin (IL)-1 β , tumour necrosis factor- α and IL-6 as well as systemic IL-6 release. Both treatments induced B- and T-cell infiltration as well as T-cell proliferation within the draining lymph nodes. Treatment with the mixture induced at least 20% more skin inflammation, cytokine production and CD4+ T-cell activation compared with the colour gel alone.

Conclusions Consumer available PPD-containing permanent hair dyes can be potent and rapid immune activators. Mixing the colour gel and developer (oxidant) increased the induction of skin inflammation compared with application of the colour gel alone.

During the past century advancement in the field of chemistry has allowed synthesis of the more than 100 000 chemicals currently used world wide. Today, approximately 500 new chemicals are introduced annually.¹ Some of these chemicals are used to improve and develop new products to meet the needs of consumers. However, exposure to certain chemicals can be hazardous to humans. Apart from acute toxicity, long-term health impacts following chemical exposure include cancer, infertility, neurotoxicity and allergy. Thus, appropriate risk assessment and regulation of chemicals used in consumer products are of general public health interest.

Cosmetics and haircare products are a major source of life-style-related chemical exposure in the general population of the developed world. In 2007 approximately 16 billion euros were spent on haircare products within the EU,² with 75% of

women and 18% of men having used a hair dye product at least once during their lifetime.³ The known skin sensitizer p-phenylenediamine (PPD), or PPD-related substances, are used in more than two-thirds of permanent (oxidative) hair dyes.⁴ Some consumers experience adverse allergic skin reactions following hair dyeing. These patients may present with severe facial oedema/dermatitis and general symptoms of malaise.^{3,5} Most of the patients have a positive patch test reaction to PPD. PPD-containing hair dyes would be expected to elicit allergic reactions in sensitized individuals. Yet the skin sensitization potential of hair dye products is controversial as it has been claimed that in-use hair dyes contain a large quantity of couplers that bind free PPD and thereby inhibit the sensitization potential of the product. Further, in the in-use situation the mixture of colour gel and developer (oxi-

dant) results in rapid polymerization of the hair dye chemicals and washing the hair after 30 min reduces exposure time.⁶

In the present study, we investigated the sensitization potential of hair dye products available to the consumer using a modified version of the local lymph node assay (LLNA).

Materials and methods

Mice

Female CBA/Ca mice were purchased from Harlan (Horst, the Netherlands) and housed in the specific pathogen-free animal facility at the University of Copenhagen in accordance with national animal protection guidelines (licence number 2007/561-1357). In each experiment groups of four mice were used for each chemical tested. Each chemical was tested in at least two separate experiments.

Contact sensitization

For testing the sensitizing potential of the test substances we used a modified version of the LLNA as previously described.⁷ Mice were sensitized by painting test substance on the dorsal side of both ears for three consecutive days. The following substances were tested: 25 µL of 0.1% PPD (Fluka, Copenhagen, Denmark), 0.25% PPD or 1% PPD in acetone/olive oil (4 : 1), 25 µL of hair dye mixture consisting of colour gel and oxidizer (1 : 1), and 12.5 µL of the oxidizer or colour gel alone. For testing the effect of washing away the mixture after 30 min, the mixture was applied on the ears and after 30 min these were washed with cotton wool soaked in water until no more colour could be removed from the ears. The commercial hair dye products tested were from two leading international brands obtained (June 2008) from the retail market in the U.K. Both products were of a dark shade and contained PPD according to the list of ingredients on the label. Product names, manufacturers and outlets from where purchased have been disclosed to the BJD Editor.

On day 5 the mice were killed and the draining auricular lymph nodes removed for further analyses. Skin inflammation was quantified by the increase in ear thickness as measured in a blinded manner using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Ears were removed and prepared for immunohistochemistry or cytokine analysis as described below.

Immunohistochemistry

Mouse ears were snap frozen in liquid nitrogen or fixed in 4% formalin, and cut in 5-µm sections. Paraffin-embedded, formalin-fixed sections were deparaffinized and stained with haematoxylin and eosin. Frozen sections were air dried and immunostained using a two-step immunofluorescence labelling. In brief, sections were blocked with 10% rabbit serum and incubated at 4 °C with anti-CD3ε biotinylated (145-2C11; BD Biosciences, Brøndby, Denmark), washed and incubated for 1 h with fluorescein isothiocyanate-conjugated streptavidin,

washed and mounted. All labelled T cells were counted on a Zeiss (Jena, Germany) immunofluorescence microscope.

Cytokine measurements in tissue and serum

Measurements of cytokine production in skin tissue were performed as previously described.⁸ Briefly, frozen ear tissue was pulverized and homogenized in phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20 and protease inhibitors (1 mg mL⁻¹ Pefabloc SC, 10 mg mL⁻¹ leupeptin and 10 mg mL⁻¹ pepstatin from Boehringer Mannheim, Mannheim, Germany) using a mortar and pestle. Tissue homogenates were frozen in liquid nitrogen, thawed at 37 °C, sonicated for 30 s and centrifuged to remove debris. Supernatants were harvested and stored at -80 °C before cytokine measurements. Serum was collected from the mice just before killing and stored at -20 °C before cytokine measurements. Sample tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 levels were determined by enzyme-linked immunosorbent assay according to the manufacturer's protocol (Ready-Set-Go; eBioscience, San Diego, CA, U.S.A.).

Flow cytometry

To determine the expression of various cell surface markers, lymph node cells were incubated with fluorochrome-conjugated anti-CD4 (RM4-5), anti-CD8a (53-6.7) or anti-CD19 (ID3) monoclonal antibodies all from BD Biosciences. Cells were incubated with the indicated monoclonal antibody diluted in ice-cold PBS containing 2% fetal calf serum and 0.1% NaN₃ for 30 min on ice. Finally, the cells were analysed on a FACSCalibur (BD Biosciences) with CELLQUEST software. CD4+ and CD8+ proliferating cells were identified by intracellular bromodeoxyuridine (BrdU) staining using the BrdU flow kit from BD Pharmingen (Brøndby, Denmark) according to the manufacturer's instructions.

Results

Using a modified version of the LLNA we confirmed that pure PPD is a potent inducer of skin inflammation as measured by ear swelling (Fig. 1a). Permanent hair dye products consist of a PPD-containing colour component and an oxidizer component, which when mixed become ready to use. We found that the oxidizer induced minor ear swelling and no CD3+ T-cell infiltration to the exposed ears (Fig. 1a,b). In contrast, treatment with either the colour gel or the final mixture of colour gel and oxidizer induced major ear swelling and CD3+ T-cell infiltration to the exposed ears (Fig. 1a,b). Comparing the responses induced by the colour gel compared with the mixture, a 20% and 50% increase, respectively, on ear swelling and CD3+ T-cell infiltration was seen with the mixture. Only a minor reduction on ear swelling was seen by washing the ears 30 min after application of the mixture compared with ears that were not washed (Fig. 1a). Washing the ears 30 min after application of the mixture reduced the CD3+ T-cell

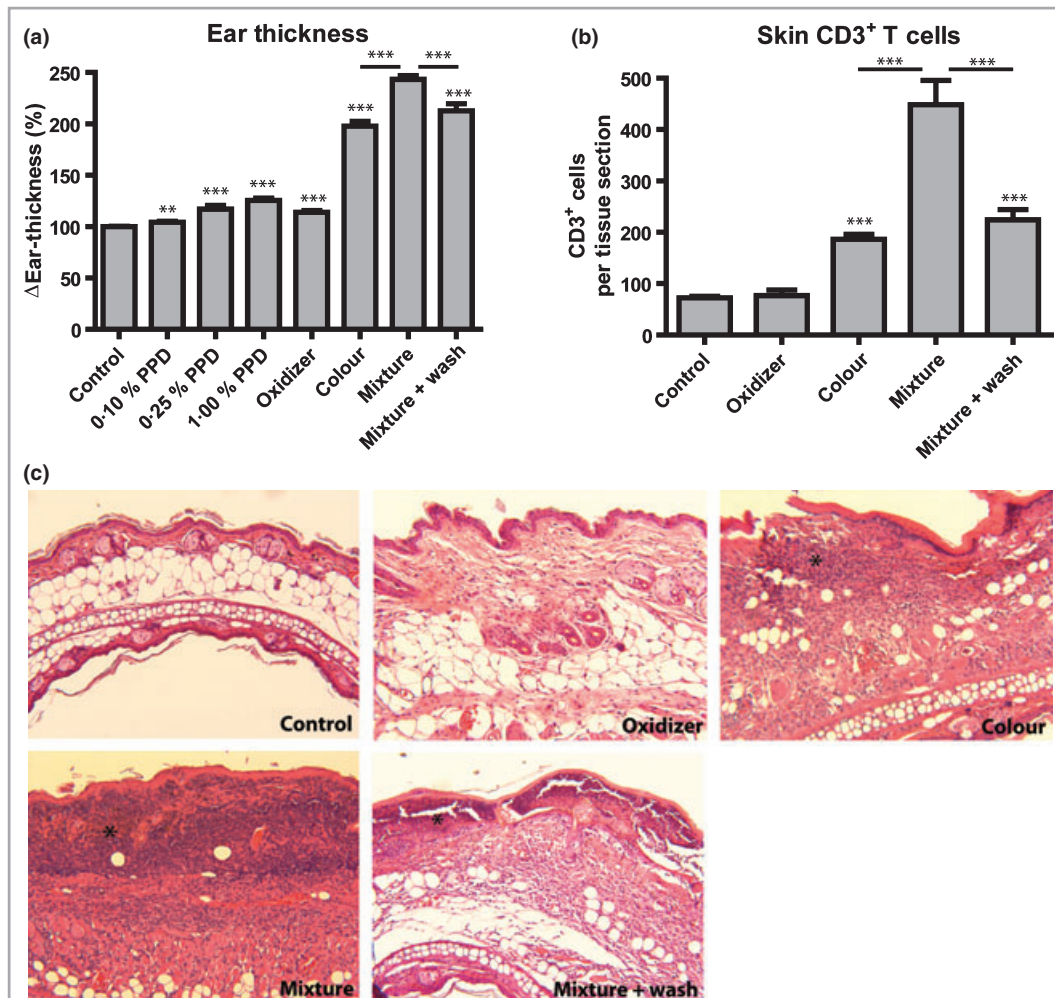


Fig 1. Hair dyes induce major skin inflammation. (a) Change in ear thickness following ear sensitization of mice. (b) T-cell counts in nontreated and treated mouse ears based on immunohistochemical staining. PPD, p-phenylenediamine. Results are presented as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$. (c) Haematoxylin and eosin staining of histological ear sections. Asterisks show massive infiltration of neutrophils and T cells. Test substances: control (acetone/olive oil 4 : 1), mixture (1 : 1 mix between oxidizer and colour), mixture + wash (treatment with the mixture for 30 min followed by washing).

infiltration of the exposed ears by 50% compared with not washing the mixture away (Fig. 1b). Histological sections of mouse ears treated with oxidizer showed little swelling with few inflammatory cells compared with the normal control sections. In contrast, ears from mice treated with either colour gel, mixture or mixture followed by washing showed profound inflammation with neutrophilic infiltration and necrotic areas (Fig. 1c).

After application of either colour or mixture, TNF- α , IL-1 β and IL-6 were present within the exposed ears whereas none of these cytokines was found after application of oxidizer (Fig. 2a–c). Exposure to the mixture led to a 15–30% higher cytokine production than applying the colour alone. Even in the setting where the mixture was washed away after 30 min we found production of IL-1 β , TNF- α and IL-6 within the skin. Both colour and mixture led to a systemic increase of serum IL-6 levels whereas IL-1 β and TNF- α were undetectable

in serum (Fig. 2d and data not shown). By washing away the mixture after 30 min the systemic effect of hair dyeing was reduced by approximately 50% as measured by IL-6 concentrations in the serum.

We found B-cell infiltration within draining lymph nodes following sensitization with 1% PPD, colour gel and the mixture (colour gel and oxidizer), whereas none was seen following application of the oxidizer alone (Fig. 3a). More than a twofold increase in infiltrating B cells was induced by the colour gel and the mixture compared with 1% PPD. Both the colour gel and the mixture induced CD4⁺ and CD8⁺ T-cell infiltration and proliferation (Fig. 3). CD4⁺ T-cell proliferation induced by application of the mixture was increased by approximately 30% compared with CD4⁺ T-cell proliferation following application of oxidizer and colour alone (Fig. 3b). Washing the mixture away after 30 min led to an approximately 25% reduction in both B-cell infiltration and

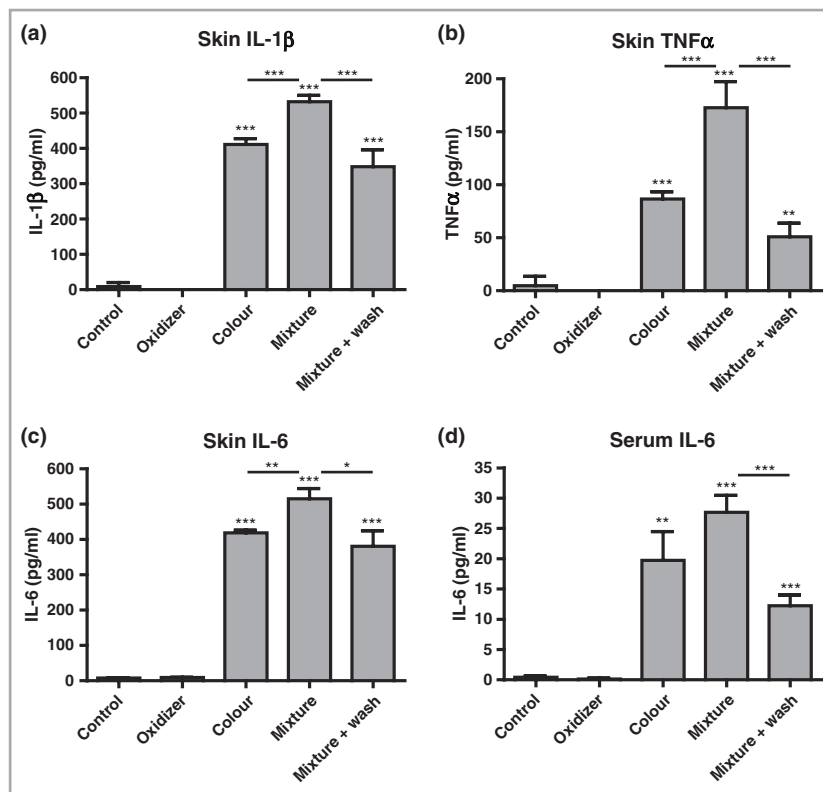


Fig 2. Cytokine release following hair dyeing. Cytokines in hair dye-sensitized mice measured by enzyme-linked immunosorbent assay. (a–c) Cytokines in ear tissue homogenates. IL, interleukin; TNF, tumour necrosis factor. (d) IL-6 in serum, day 5. Test substances: control (acetone/olive oil 4 : 1), mixture (1 : 1 mix between oxidizer and colour), mixture + wash (treatment with the mixture for 30 min followed by washing). Results are presented as mean $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001 (Student's *t*-test).

proliferation of CD4 $^{+}$ T cells compared with leaving the mixture on the ears. In contrast, washing the mixture away after 30 min had no effect on CD4 $^{+}$ and CD8 $^{+}$ T-cell infiltration or on proliferation of CD8 $^{+}$ T cells (Fig. 3).

Discussion

It has recently been suggested by Aebly *et al.* that PPD-containing hair dyes are weak sensitizers due to excess of couplers, controlled oxidation and short exposure time.⁶ In order to challenge this suggestion, we used a modified version of the LLNA to investigate the skin sensitization potential of PPD-containing consumer available hair dyes.⁷ We have shown that these are potent skin sensitizers as demonstrated by profound skin inflammation, induction of the proinflammatory cytokines IL-1 β , TNF- α and IL-6 within the skin as well as systemic release of IL-6. Finally, exposure to PPD-containing hair dyes induced B- and T-cell infiltration as well as T-cell proliferation within the draining lymph nodes. Our data suggest that the allergenic potential of hair dyes is greater under in-use conditions compared with the separate colour gel and oxidizer components making up the product. These findings contradict recent suggestions by Aebly *et al.*⁶

Active skin metabolism forms nonsensitizing N-acetylated derivatives of PPD following its penetration through the stratum corneum.⁶ This has been suggested as a detoxification pathway in humans with regard to reducing PPD sensitization potential.⁹ As this detoxification pathway is also present in the strain of mice used in our study,^{10,11} an absence of this

mechanism cannot explain the profound immune activation seen in our study. Reducing the exposure time to the hair dye by washing the ears of the mice 30 min after exposure had a beneficial effect on the response as skin inflammation, cytokine production, B-cell infiltration and T-cell proliferation within the draining lymph nodes were reduced by at least 20% compared with ears where the mixture was not washed away. A concentration of up to 1.1% of unconsumed PPD has been found after mixing the oxidizer and colour for 30 min.¹² We found that the inflammatory response was at least 50% higher when hair dye was washed away according to the manufacturer's protocol compared with mice exposed to 1% PPD that was not washed away. Thus, the chemical composition within final mixtures is a more potent immune activator than 1% pure PPD; this is probably caused by a cocktail effect mediated by irritants and allergens within the mixture. Taken together, our finding indicates that hair dyes available to the consumer are potent and rapid immune activators and that the effect is not inhibited by the oxidation or by short exposure time.

The severity of the skin inflammation illustrated in Figure 1 could be caused by the experimental setting in which the mice were treated for three consecutive days with hair dyes. Furthermore, the immune cellular composition in noninflamed mouse and human skin differs, e.g. 40–60% of the T cells are $\gamma\delta$ T cells in noninflamed mouse epidermis compared with only 18–29% in human epidermis.^{13,14} The $\gamma\delta$ T cells can be activated directly by proinflammatory cytokines independently of T-cell receptor stimulation which leads to production of

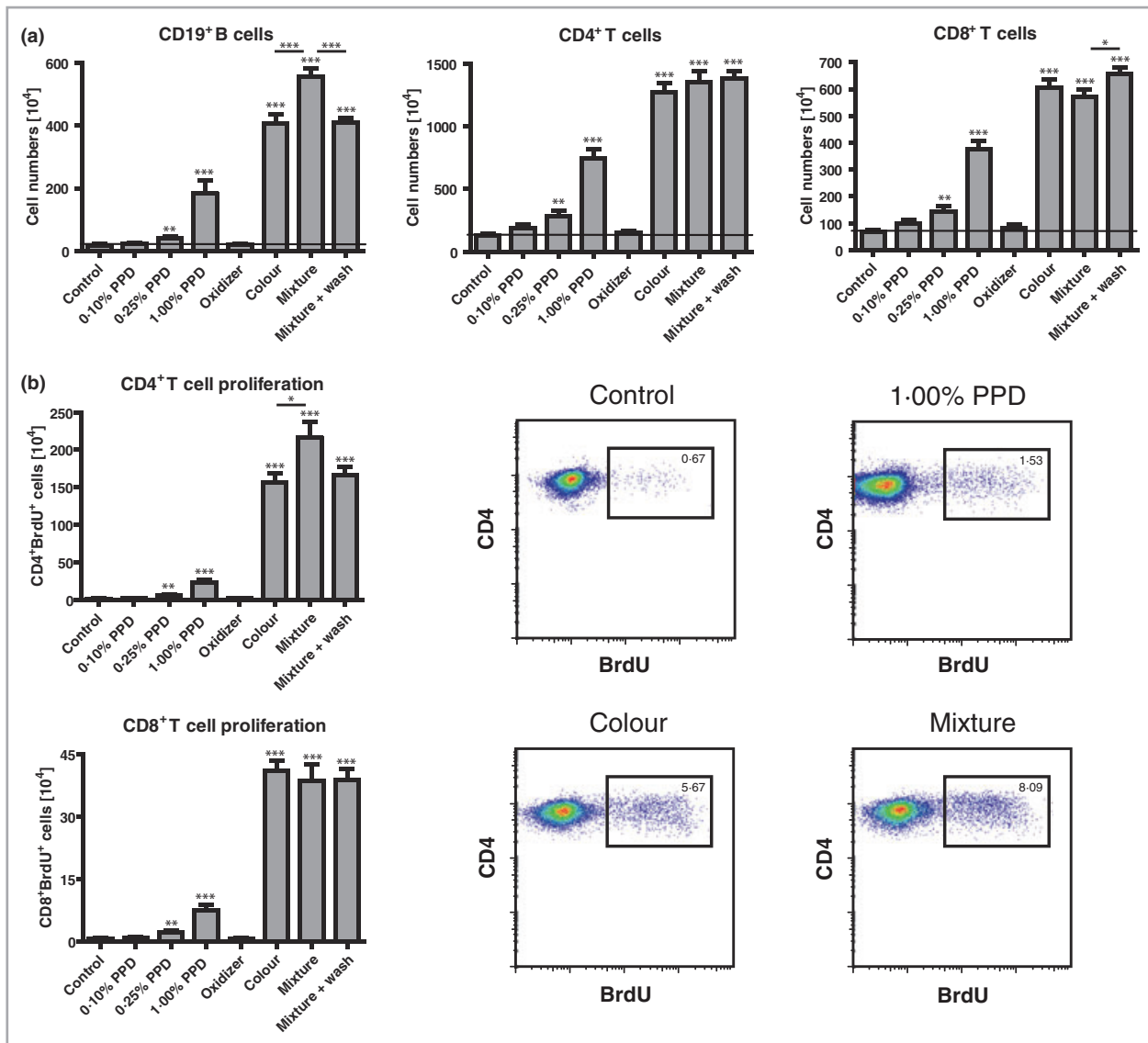


Fig 3. Hair dye-induced cellular infiltration and proliferation. Cellular response in draining lymph nodes of *p*-phenylenediamine (PPD)- and hair dye-sensitized mice analysed by flow cytometry. (a) Number of B cells and T cells. (b) T-cell proliferation. Test substances: control (acetone/olive oil 4 : 1), mixture (1 : 1 mix between oxidizer and colour), mixture + wash (treatment with the mixture for 30 min followed by washing). Results are presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. In FACS plots, numbers represent percentage of gated cells within the gate of living cells. Data shown are representative of two independent experiments, with $n = 4$ per group. BrdU, bromodeoxyuridine.

more cytokines and chemokines.¹⁵ The skin of mice might therefore be more susceptible to severe skin inflammation than human skin. However, as 1% PPD did not induce these severe reactions, and as washing the mixture away after 30 min had only a minor effect on ear swelling compared with leaving the mixture on the ears, we find it unlikely that the duration of exposure or differences in skin between humans and mice can explain our results.

Rapidly after skin exposure to contact allergens keratinocytes and Langerhans cells start to produce various proinflammatory cytokines.^{16–19} Among these are TNF- α , IL-1 β and IL-6, mediators of acute-phase responses including vascular

leak, recruitment of neutrophils, and production of acute-phase proteins. In agreement with this we found that both colour and mixture induced TNF- α , IL-1 β and IL-6 within the skin of treated mice. However, to our surprise, 3 days after the last treatment we were able to measure IL-6 in serum of mice treated with colour or mixture. Th17 cells, a newly identified CD4⁺ T-cell subtype, are characterized by their production of the proinflammatory cytokines IL-17 and IL-22.²⁰ Th17 cells are involved in the pathogenesis of various allergic and autoimmune diseases, e.g. nickel contact allergy, atopic dermatitis and psoriasis.^{21–23} IL-6 seems to be an important cytokine for the differentiation of naïve CD4⁺ T

cells into Th17 cells.²⁰ It may be speculated that regular exposure to IL-6, which our data indicated to follow regular hair dyeing, could have an impact on the development of allergies and autoimmunity.

In conclusion, our data show that permanent hair dyes available to the consumer are potent skin sensitizers. The sensitizing potential was greater than expected and demonstrates the inadequate measures taken to prevent the sensitization potential of the allergens present in these products. The severity and nature of the allergic reactions observed in the mice are similar to the clinical presentation of hair dye contact allergy in consumers due to the delayed hypersensitivity reaction, which may include systemic cytokine release. Current safety assessments of commercial products and regulatory actions are based on data from testing individual chemical substances. However, our results demonstrate that the sensitizing potential of in-use hair dyes is greater than that of the separate components. This may also apply to other toxicological endpoints. Thus in the interests of public health, regulation of products should be based on the toxicological analysis of the single ingredients as well as the chemical composition to which the consumer is exposed.

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References

- 1 Jackson T. *Material Concerns: Pollution, Profit and Quality of Life*. London: Routledge, 1996.
- 2 Colipa. *The European Cosmetic, Toiletry and Perfumery Market 2007*. Brussels: Colipa, 2007.
- 3 Sosted H, Hesse U, Menné T *et al.* Contact dermatitis to hair dyes in a Danish adult population: an interview-based study. *Br J Dermatol* 2005; **153**:132–5.
- 4 EU Commission. *Public Consultation Document – Regulation of Hair Dyes*. 2008. Available at: http://docs.google.com/gview?a=v&q=cache:a_CqHmNHnFYJ:ec.europa.eu/enterprise/cosmetics/doc/consultation_hair_dyes.pdf+eu+commission+regulation+of+hair+dyes%23&hl=en&gl=uk (last accessed 20 July 2009).
- 5 McFadden JP, White IR, Frosch PJ *et al.* Allergy to hair dye. *BMJ* 2007; **334**:220.
- 6 Aeby P, Sieber T, Beck H *et al.* Skin sensitization to p-phenylenediamine: the diverging roles of oxidation and N-acetylation for dendritic cell activation and the immune response. *J Invest Dermatol* 2009; **129**:99–109.
- 7 Larsen JM, Geisler C, Nielsen MW *et al.* Cellular dynamics in the draining lymph nodes during sensitization and elicitation phases of contact hypersensitivity. *Contact Dermatitis* 2007; **57**:300–8.
- 8 Maurer M, Seidel-Guyenot W, Metz M *et al.* Critical role of IL-10 in the induction of low zone tolerance to contact allergens. *J Clin Invest* 2003; **112**:432–9.
- 9 Blomeke B, Pietzsch T, Merk HF. Elicitation response characteristics to mono- and to N,N'-diacetyl-para-phenylenediamine. *Contact Dermatitis* 2008; **58**:355–8.
- 10 Boukouvala S, Price N, Sim E. Identification and functional characterization of novel polymorphisms associated with the genes for arylamine N-acetyltransferases in mice. *Pharmacogenetics* 2002; **12**:385–94.
- 11 Kawamura A, Westwood I, Wakefield L *et al.* Mouse N-acetyltransferase type 2, the homologue of human N-acetyltransferase type 1. *Biochem Pharmacol* 2008; **75**:1550–60.
- 12 Rastogi SC, Sosted H, Johansen JD *et al.* Unconsumed precursors and couplers after formation of oxidative hair dyes. *Contact Dermatitis* 2006; **55**:95–100.
- 13 Bos JD, Teunissen MB, Cairo I *et al.* T-cell receptor gamma delta bearing cells in normal human skin. *J Invest Dermatol* 1990; **94**:37–42.
- 14 Stingl G, Koning F, Yamada H *et al.* Thy-1+ dendritic epidermal cells express T3 antigen and the T-cell receptor gamma chain. *Proc Natl Acad Sci USA* 1987; **84**:4586–90.
- 15 Shibata K, Yamada H, Hara H *et al.* Resident Vdelta1+ gammadelta T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J Immunol* 2007; **178**:4466–72.
- 16 Enk AH, Katz SI. Early events in the induction phase of contact sensitivity. *J Invest Dermatol* 1992; **99**:S39–41.
- 17 Enk AH, Katz SI. Early molecular events in the induction phase of contact sensitivity. *Proc Natl Acad Sci USA* 1992; **89**:1398–402.
- 18 Enk AH, Angeloni VL, Udey MC *et al.* An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. *J Immunol* 1993; **150**:3698–704.
- 19 Hope JC, Campbell F, Hopkins SJ. Deficiency of IL-2 or IL-6 reduces lymphocyte proliferation, but only IL-6 deficiency decreases the contact hypersensitivity response. *Eur J Immunol* 2000; **30**:197–203.
- 20 Louten J, Boniface K, de Waal MR. Development and function of TH17 cells in health and disease. *J Allergy Clin Immunol* 2009; **123**:1004–11.
- 21 Di Cesare A, Di Meglio P, Nestle FO. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *J Invest Dermatol* 2009; **129**:1339–50.
- 22 Koga C, Kabashima K, Shiraishi N *et al.* Possible pathogenic role of Th17 cells for atopic dermatitis. *J Invest Dermatol* 2008; **128**:2625–30.
- 23 Larsen JM, Bonefeld CM, Poulsen SS *et al.* IL-23 and Th17-mediated inflammation in human allergic contact dermatitis. *J Allergy Clin Immunol* 2009; **123**:486–92.