

***Para*-phenylenediamine-specific lymphocyte activation test: a sensitive *in vitro* assay to detect *para*-phenylenediamine sensitization in patients with severe allergic reactions**

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Abstract: Patients sensitized to *para*-phenylenediamine (PPD) by semi-permanent tattoos increasingly develop threatening allergic reactions in response to black hair dye. The gold standard to diagnose allergic contact dermatitis is to perform epicutaneous patch tests, however, iatrogenic sensitizations and severe patch test reactions to PPD have been described, the latter especially in patients with severe allergic reactions. We examined nine patients with severe allergic reactions in response to permanent hair dyes. Patch tests using the standard concentration of 1% or 0.5% PPD resulted in severe and sometimes even bullous reactions in all patients responsive to PPD. Titration revealed that at 1% of the standard concentration (0.01% PPD), patch test sensitivity decreased and only 50% of patients responded. Consequently, we established an *in vitro* assay to diagnose PPD allergy. Freshly isolated peripheral blood mononuclear cells (PBMC) were cultured with titrated concentrations of PPD with or without IL-2 supplementation, and cell proliferation was determined by

[3H]-thymidine incorporation. Lymphocyte activation test (LAT) detected PBMC cell proliferation specific to PPD, with at least 3.5-fold increase in [3H]-thymidine uptake in all PPD allergic patients. Most importantly, PPD-LAT without IL-2 supplementation remained negative in three out of eight PPD allergic patients. Thus, PPD-LAT with IL-2 supplementation demonstrated a sensitivity of 100%, remained unresponsive in controls not sensitized to PPD, and in one patient sensitive to other *p*-amino compounds. These data demonstrate that LAT with PPD can be used to detect PPD sensitization as a possible alternative to patch testing at least in patients with severe allergic reactions to PPD.

Key words: contact allergy – skin inflammation – PPD – lymphocyte activation test – patch test – cross reactivity – PBMC – lymphocytes – T cells – black hair dyes

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Introduction

Permanent hair dyes can induce severe adverse skin reactions of the delayed type that include lymph node hyperplasia and massive oedema of the head and neck (1–3). Adverse skin reactions to hair dyes were reported in about 5% of individuals who have ever used hair dye and many of the hair dyes contain potent haptens such as *para*-phenylenediamine (PPD) and related substances (2,4). These haptens are responsible for most allergic responses to hair dyes and up to 24% of hair stylists were shown to be allergic to PPD (5). The North American Contact Dermatitis Group studied

positive patch test results in more than 34 000 patients and found about 5% PPD responsive patients in 2002 (6). Recent data from Scandinavian, central, and southern European patch test centres between 2003 and 2007 revealed among 21 515 patients an weighted average prevalence for PPD sensitization of 4.6% with PPD sensitization being more frequent in southern Europe (7). PPD use in humans in 'rinse off' products such as black hair dyes is approved for the US by the Food and Drug Administration irrespective of the concentration (8), in the EU up to a maximum concentration of 6%, and in Australia, a warning label is required for PPD concentrations higher than 5%. In contrast, PPD

supplementation is prohibited in 'leave on' products such as cosmetics or semi-permanent tattoos in many countries. Despite these regulations, PPD is illegally used in semi-permanent tattoos, often referred to as 'black henna tattoos'. The PPD content in these illegal 'henna tattoo inks' lies between 0.43 and 15.7% and is considered to be a major source of PPD sensitizations (2,9–11).

Allergic contact dermatitis is mostly caused by skin contact to low molecular weight haptens such as PPD, and may lead to severe reactions causing sick leave and increased national health-care expenses (12). A prerequisite for antigen-specific hypersensitivity in allergic contact dermatitis is a sensitization phase where hapten-carrier complexes lead to T-cell activation (13–16). Subsequent recognition of the antigen by T cells in the skin leads to skin inflammation with predominantly epidermal changes such as spongiosis leading to scales, vesicles or pustules (17).

Patch testing is an *in vivo* test system considered as the gold standard for identifying haptens responsible for contact allergic reactions such as PPD (18,19). In rare cases, patients respond adversely to patch testing with bullous reactions that carry the risk of scar formation (20). Dilution series are used to estimate individual sensitivity and to determine the clinical relevance for the actual 'use situations'. Patch tests provide high sensitivity and high specificity, and generally carry a low risk of iatrogenic sensitization. However, this is a current issue of debate for PPD (7,21–24,28). Patch testing with 1% PPD has been demonstrated to have a sensitivity of 87% (12), but has been found to cause iatrogenic sensitizations (21). Therefore, PPD was removed from the German patch test standard series in 2005. However these data were questioned by others (7,21–24,28). In our study with patients who developed severe skin and lymph node reactions in response to hair dyes, we systematically compared the use of *in vivo* and *in vitro* PPD test systems. PPD patch test titrations demonstrated severe and partly bullous patch test responses with 1.0–0.1% PPD, and no response with 0.01% PPD in 50% of the patients. Based on these observations, an *in vitro* test system, the lymphocyte activation test (LAT) with and without IL-2 supplementation, was established to diagnose PPD allergy. After IL-2 supplementation PPD-LAT was responsive in all PPD allergic patients but remained negative in all controls. Thus, the risk of severe patch test reactions to PPD in patients with severe allergic reactions could be avoided by the use of a possible alternative *in vitro* test system, the PPD-specific LAT.

Materials and methods

Patients

Nine patients with a history of severe skin and lymph node reactions of the head and neck following a recent use of

hair dyes were included into the study. In addition, seven volunteers with use of hair dyes without any clinical sign of allergy, and 10 volunteers without a history of using permanent black hair dye were examined. The Ethical Committee of the University of Tübingen, Germany, approved the protocol; informed consents were obtained from all participants before inclusion into the study.

Patch testing

According to the current diagnostic measures carried out in patients with allergic reaction to hair dyes, the standard series of the German Contact Dermatitis Group (DKG) and titrated concentrations of PPD were applied on the upper back. In most patients, a test series containing a number of *p*-amino compounds was also applied. The patch test was carried out with commercial patch test preparations supplied by Trolab (Almirall Hermal GmbH, Reinbeck, Germany) and Finn Chambers® (Epitest Ltd. Oy, Tuusula, Finland). The two initial patients were still tested with the standard PPD concentration only used at that time (1% PPD in pet.); the two following patients were tested with titrated PPD concentrations from 1% to 0.01%, whereas all subsequent patients were only tested with PPD in pet. <1%. Patch test substances were applied onto the back skin under occlusion for 48 h and were read on day 2–3. The standard read out of the patch test was defined by morphology, and graded as 0 to +++ reaction between days 2 and 3 after application of the patch test. The descriptive analysis of our data followed the current guidelines established by the European Surveillance System and Contact Allergies (ESSCA) (25).

LAT with PPD

Fresh PBMC of 2.5×10^5 from 9 patients, as well as 17 controls with or without a history of using permanent black hair dye were cultured with titrated concentrations of PPD ($50\text{--}10^4$ ng/ml PPD) from Fluka (Biochrom, Berlin, Germany) in RPMI (Biochrom) containing 10% autologous patient serum and 1% penicillin/streptomycin (Biochrom). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂, with or without 100 U/ml IL-2 (Chiron, Emeryville, CA, USA) in a total volume of 100 µl of medium, in 96-well round bottom plates (Greiner, Frickenhausen, Germany). As positive controls, cells were stimulated with 2 mg/ml Concanavalin A (ConA; Sigma, Steinheim, Germany) or 1.5 mg/ml Phytohaemagglutinin (PHA; Sigma). In half of the patients, blood samples were taken before patch testing and in the other half blood samples were taken 1, 5, 6 and 8 months after patch testing. After 6 days, cells were pulsed with [³H]-thymidine for the final 18 h. The [³H]-thymidine uptake by PBMC was used to calculate a stimulation index (SI) as follows: cpm of stimulated cells/cpm of unstimulated cells.

Statistical data analysis

The Dunnett's test was used to compare differences in SI between PPD allergic patients and non-PPD allergic patients with and without use of black hair dyes. A *P* value below 0.05 was regarded as significant. Confidence intervals for sensitivity and specificity were given as exact 95% confidence limits (26).

Results

Severe skin and lymph node reactions after application of *p*-phenylenediamine

Five female (15–43 years old; mean age 31.4 ± 11.3 years) and four male (14–19 years old; mean age 16.8 ± 2.2 years) patients were admitted to the outpatient clinic of our department for severe reactions upon recent use of black hair dyes. In all patients, these reactions occurred within 2–3 days after black hair dye application. Patients presented with marked oedema of the head, face and neck, and severely enlarged preauricular, jugular and nuchal lymph nodes. Many of these patients were severely disfigured. Six patients suffered from dyspnea because of lymph node swelling, or were visually disabled because of severe blepharal oedema and needed hospitalization (Table 1).

Seven out of nine patients reported obtaining temporary black 'henna' tattoos 3–7 years prior to the hair staining, which resulted in a strong inflammation at the site of the tattoo: three patients reported a disseminated rash. One patient was a former hairdresser who had changed profession because of occupational contact dermatitis.

PPD patch test results

The patients' histories suggested contact allergic reactions to PPD, the most prominent contact allergen in black hair dyes. Therefore, patch tests including PPD were performed in all patients within 1 year after allergic contact dermatitis.

Eight out of nine patients responded strongly to PPD. One patient did not respond to PPD, but responded to *p*-toluylendiamine (PTD) and disperse orange 3 (DO3) only (Table 1). Initial patch tests using 1.0% PPD induced severe bullous reactions (Fig. 1a). Therefore, PPD was titrated from 0.5% to 0.1% and 0.01% (Fig. 1b). Patch testing caused similarly severe reactions in all patients at 0.5% and at 0.1% PPD. In contrast, at 0.01% PPD, 50% of the patients did not react, despite severe responses to 0.1% PPD (Fig. 1b). Thus, with concentrations of PPD $\leq 0.1\%$, patch test sensitivity rapidly declined with only 50% of the patients responding.

Patch testing confirmed cross reactivity to azo dyes and other *para*-amino compounds in these patients. Further analysis of our results revealed that cross reactivity to PPD was dependent on the number of aromatic rings, amino groups and hydroxyl groups of the compounds. Consequently, all patients reacting to PPD showed cross reactivity to PTD, DO3 and *p*-aminoazobenzole, and 75–86% showed cross reactivity to *p*-aminophenol, 3-aminophenol, 4,4'-diaminodiphenylmethane and hydroquinone (Fig. 2).

Results of lymphocyte activation test with PPD

We aimed to establish an *in vitro* assay to detect PPD sensitization. The LAT was performed between 10 days and 1.5 years after allergic contact dermatitis caused by the permanent black hair dyes. Freshly isolated PBMC from all patients and 17 controls (with no contact allergies or with positive patch test results to compounds other than PPD) were co-incubated with titrated concentrations of PPD, with or without IL-2 supplementation. [^3H]-thymidine was added 6 days later, and T-cell proliferation was determined 18 h later. In all patients with positive patch test reactions to PPD, LAT resulted in a 3.5- to 53.7-fold increase in [^3H]-thymidine uptake (Figs 3a,b and 4a,b,e,f; Table 1). T-cell proliferation was detected at concentrations between

Table 1. History of black hair dye allergic patients

Patient no.	Sex	Age	Henna-tattoo (prior to reaction)	Reaction to 'Henna-Tattoo'	Inpatient treatment	Patch test (time after allergy)	LAT (time after allergy)	LAT (SI) no IL-2	LAT (SI) + IL-2
1	♂	14	Yes (3 a)	Local	Yes	Positive (2 months)	Positive (10 months)	53.7 (5×10^3)	7.8 (5×10^3)
2	♂	16	Yes (6 a)	Local and systemic	No	Positive (12 months)	Positive (18 months)	9.6 (5×10^3)	25.5 (5×10^3)
3	♀	41	unknown	n.d.	No	Positive (4 months)	Positive (5 months)	4.9 (2.5×10^3)	6.6 (2.5×10^3)
4	♂	18	Yes (8 a)	Local	Yes	Positive (3 months)	Positive (1, 3, 8 months)	8.7 (2.5×10^3)	9.3 (2.5×10^3)
5	♀	30	Yes (4 a)	n.d.	No	Positive (10 days)	Positive (10 days)	3.2 (2.5×10^3)	5.5 (2.5×10^3)
6	♂	19	Yes (6 a)	n.d.	Yes	Positive (2 months)	Positive (2 months)	0.4 (2.5×10^3)	4.3 (2.5×10^3)
7	♀	15	Yes (6 a)	Local and systemic	Yes	Positive (2 months)	Positive (2 months)	0.1 (1×10^4)	3.5 (1×10^4)
8	♀	28	Yes (7 a)	Local	No	Positive (3 months)	Positive (8 months)	11.3 (2.5×10^3)	13.5 (2.5×10^3)
9	♀	43	No	–	Yes	Negative	Negative	1.1 (2.5×10^3)	0.7 (2.5×10^3)

In half of the patients blood samples for PPD–LAT analysis were taken before patch testing and in the other half 1, 5, 6, and 8 months after patch testing.

n.d., Not done; LAT, lymphocyte activation test; SI, stimulation index.

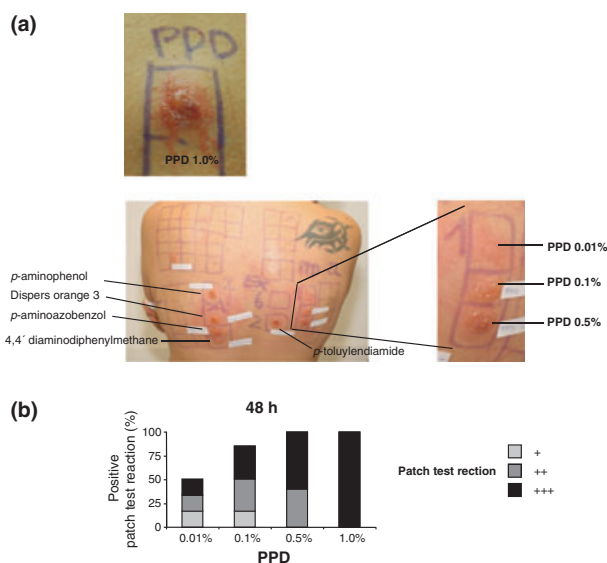


Figure 1. Severe PPD patch test reactions to high PPD concentrations. Patch test reaction to 1.0% (a, upper, right), 0.5%, 0.1% and 0.01% PPD, and cross-reactivity to azo dyes and other *para*-amino compounds (a, lower, left). (b) A graphic representation of patch test reactions to titrated PPD-concentrations.

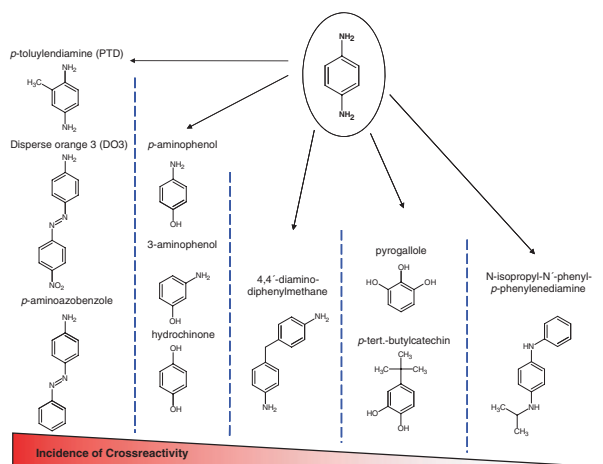


Figure 2. Incidence of cross reactivity to PPD was dependent on the number of aromatic rings, amino groups and hydroxyl groups. 100% of our PPD-sensitized patients with severe allergic contact dermatitis to PPD showed cross reactivity to PTD, DO3 and *p*-aminoazobenzol; 75–86% to *p*-aminophenole, 3-aminophenole, 4,4'-diaminodiphenylmethane and hydroquinone; 57–50% to pyrogallol and *p*-tert-butylcatechin; and 25% to *N*-isopropyl-*N'*-phenyl-*p*-phenylenediamine.

1.5×10^2 and 1×10^4 ng/ml PPD (Figs 3a,b and 4a,b,e,f; Table 1). In three patients, PPD-specific LAT was responsive (SI > 3.5) only with IL-2 supplementation (Figs 3b and 4b,e,f; Table 1). In all other patients, PPD-specific LAT was reactive with and without IL-2 (Figs 3a and 4a,e,f; Table 1). The PPD-specific SI in PPD-responsive

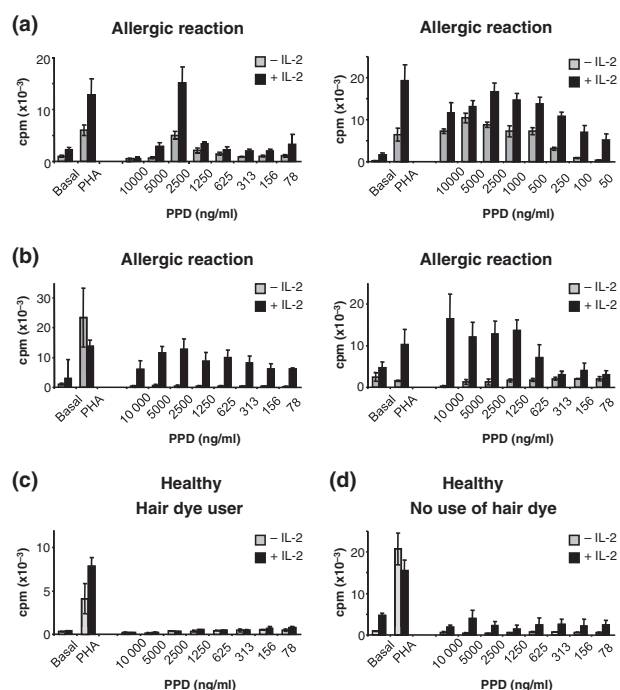


Figure 3. Lymphocyte activation test with PPD. PBMC proliferation from PPD allergic patients (a, b), healthy hair dye users (c) and healthy controls without use of hair dyes (d). PBMCs were co-incubated with titrated concentrations of PPD with or without IL-2 supplementation. (a, b) As a positive control, PBMC were also co-incubated with phytohemagglutinin (PHA); At day 6, [3H]-thymidine was added for the final 18 h of culture. [3H]-thymidine incorporation in proliferating lymphocytes was visualized by counts per minute (cpm).

patients was between 0.1 and 53.7 without IL-2 supplementation, and between 3.5 and 25.5 with IL-2 supplementation at concentrations between 2.5×10^3 and 1.0×10^4 ng PPD/ml. The SI in controls was between 0.6 and 1.5 without IL-2 supplementation (except one patient), and between 0.7 and 1.7 with IL-2 supplementation, regardless of exposure to PPD (Figs 3c,d and 4c,d,e,f). In one out of seven individuals with regular contact to PPD and no signs of PPD allergy, we measured a 3.0-fold increase in [3H]-thymidine uptake. Importantly, reactivity of this LAT to PPD was not a result of crossreactivity to amino compounds: In one patient reactive to PTD and DO3, but not to PPD, LAT with PPD remained unresponsive (data not shown). Consequently, the specificity with and without IL-2 supplementation was 100% (confidence interval 81–100%). Taken together, LAT for PPD demonstrated a sensitivity of 62.5% (confidence interval 24–91%) without IL-2, and 100% (confidence interval 63–100%) with IL-2 supplementation.

Lymphocyte activation test for PPD was repeated 1, 3 and 9 months after contact dermatitis to PPD in one patient. We found identical LAT results independent of the time after allergic contact dermatitis (Fig. 5), indicating

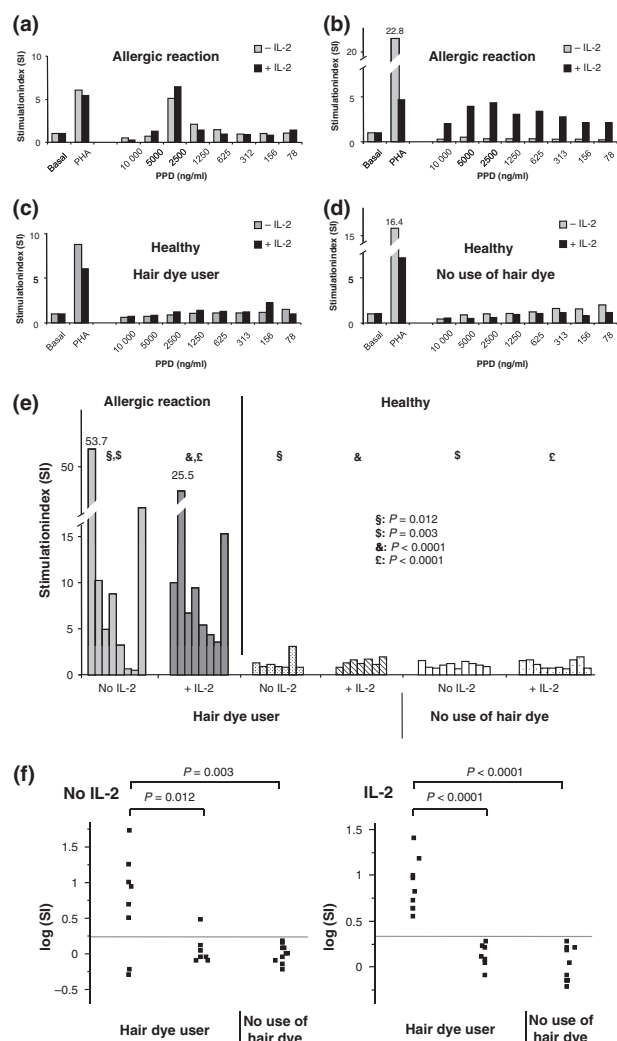


Figure 4. Analysis of lymphocyte activation tests with PPD in severely allergic patients and controls. Stimulation index (SI) of PBMC proliferation from PPD allergic patients (a, b, e, f), healthy hair dye users (c, e, f) and healthy controls without use of hair dyes (d, e, f). PBMCs were co-incubated with titrated concentrations of PPD with or without IL-2 supplementation. (a–f) As a positive control, PBMC were co-incubated with phytohemagglutinin; At day 6, [^{3} H]-thymidine was added for the final 18 h. Differences in SI were compared between PPD-allergic patients and non-PPD allergic controls with and without use of black hair dyes using Dunnett's test showing significant differences as indicated (\$, &, £, £). (e) Shows SI of analyzed PPD-allergic patients and controls and (f) log (SI) of investigated PPD-allergic patients (left in each graph) and controls either with or without IL-2 supplementation.

long-lasting presence of PPD-specific memory T cells in the peripheral blood of this patient. As the LAT for PPD already detected sensitization to PPD 10 days after the initiation of allergic contact dermatitis and remained responsive for at least 1.5 years, it constitutes a highly sensitive and specific tool for the diagnosis of allergic contact dermatitis to PPD.

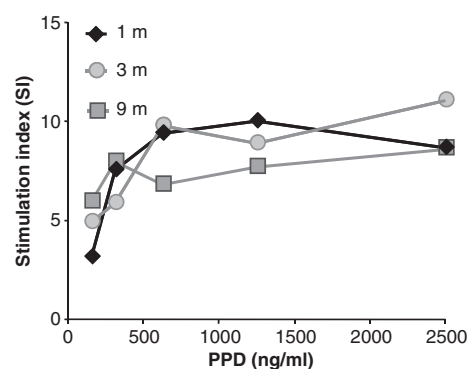


Figure 5. Long-lasting presence of PPD-specific memory T cells in peripheral blood. Stimulation index of PBMC proliferation from one PPD-allergic patient at 1 month (black diamond), 3 months (gray circle) and 9 months (grey square) after severe allergic contact dermatitis to PPD.

Discussion

Lifestyle may have severe consequences on the health status not only with regard to cardiovascular diseases and diabetes, but also with respect to allergic diseases. When essential oils such as tea tree oil became a popular new ingredient in many over-the-counter healthcare and cosmetic products, they caused an increase in the incidence of allergic contact dermatitis (27). Temporary henna tattoos have become increasingly popular and fashionable among adolescents in recent years as they are thought to be a safe alternative to permanent tattoos. In many countries, temporary henna tattoos contain not only henna, but also other additives such as PPD that frequently induce active sensitization (10,20,21,24,28). PPD, an aromatic *para*-substituted diamino compound with potent sensitizing properties, is one of the major components of hair dyes, and a necessary intermediate in the manufacturing of several chemicals including azodyes for textiles (29). Individuals sensitized to PPD by temporary henna tattoos are at risk of developing severe contact dermatitis to hair dyes or even to black cloths (2,4,20,30).

Para-phenylenediamine is thought to be a prohaptent that is converted into reactive haptens such as benzoquinonediimin, benzochinon or *N,N'*-bis (aminophenol)-2,5-diamino-1,4-chinon-diimin, also called Bandrowski's Base (BB) (31–33). However, a recent study demonstrated that LAT with PPD, but not with BB, discriminated PPD allergic patients from non-allergic patients (34). In this study, BB-specific T cells were also detected in healthy individuals, reflecting an acquired immune response not translatable into allergic responses (34). In addition, skin reactions to *para*-substituted compounds such as PPD seem to depend on the rate of oxidation taking place on the skin and hence the amount of reactive haptens formed (29).

Complete oxidation induces polymerization of PPD and conversion into a permanent stable molecule without sensitizing effects. In contrast, incomplete oxidation of PPD results in reactive haptens responsible for the sensitization and allergic responses (31).

Population-based epidemiological studies indicate that patch testing with the recommended standard concentration of 1% PPD can lead to highly delayed patch test reactions starting on day 7 in 1.5% of patients. These delayed reactions are likely to reflect iatrogenic PPD sensitizations, a considerable adverse event (7,21–24,28)). However, the precise risk of PPD patch tests is still undefined as others have found no iatrogenic sensitization when patch testing the same cohort with PPD at intervals of 8 years (28). Thus, further studies are needed to evaluate the risk of PPD patch-testing. At present, PPD is removed from the German patch test standard series of the 'Information Network of Departments of Dermatology' (IVDK) (21). Other proposals are to use 0.3% PPD in patch tests as a safe alternative to 1% PPD (22). We did not include 0.3% PPD in our patch tests, but as both 0.5% and 0.1% PPD could still induce severe reactions in some of our patients with severe allergic reactions, further evaluations are warranted.

Because of the severity of the reactions to patch testing, we used LAT to establish an *in vitro* test system to detect PPD sensitization without patch testing for patients with severe allergic contact dermatitis. LAT was first established to investigate drug allergies and contact allergies to metals such as gold or nickel (35–37), chromium or cobalt (38). Yet, it was often argued that LAT reproducibility is low because it is investigator-dependent and difficult to standardize (34). Consequently, LAT can only be used as a diagnostic *in vitro* test system to detect delayed type sensitizations with high sensitivity and specificity when internal controls are included. In addition, external quality controls needed to be established. Most importantly, it should be emphasized that while LAT is useful for investigating sensitization, it cannot predict 'allergic reactivity' or the risk of developing allergic contact hypersensitivity reactions. To avoid misinterpretations, results from *in vitro* and *in vivo* test systems must always be interpreted according to the patients' medical history. PPD–LAT has already been reported by other investigators (7,34,39,40). In contrast to these investigations, we clearly demonstrate that IL-2 supplementation increases sensitivity of LAT with PPD without increasing unspecific background leucocyte proliferation. Importantly, the results of our study are based on patients with severe allergic responses. These results cannot be translated to all PPD responsive patients, for patients with less severe allergic responses further analyses are needed to evaluate the power of PPD-specific LAT.

In summary, LAT with PPD is able to detect sensitization to PPD in all patients with severe allergic reactions.

Reactive PPD patch tests demonstrated that LAT with PPD and IL-2 supplementation is an appropriate *in vitro* test system for diagnosing PPD sensitization. LAT for PPD without IL-2 supplementation demonstrated sensitivity of 62.5% and with IL-2 supplementation a sensitivity of 100%.

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