

p-Phenylenediamine allergy: the role of Bandrowski's base

J. M. L. White*, P. Kullavanijaya[†], I. Duangdeeden[†], R. Zazzeroni[‡], N. J. Gilmour[‡], D. A. Basketter[‡] and J. P. McFadden*

*St John's Institute of Dermatology, St Thomas' Hospital London, UK, [†]Institute of Dermatology, Bangkok, Thailand and [‡]Safety and Environmental Assurance Centre, Unilever, Bedfordshire, UK

Clinical and Experimental Allergy

Summary

p-Phenylenediamine (PPD) is a commonly used hair-dye and a potent skin allergen. The mechanism of sensitization is unknown, as PPD is protein unreactive. We studied Bandrowski's base (BB), a PPD trimer, as well as 1,4-benzoquinone (BQ), a PPD hapten. PPD patch-test positive patients were patch-tested to BB and BQ. All tests were negative to 0.01% BQ and 0.01% BB. Five of 14 (35.7%) tested had true positive reactions to 0.1% BQ. One percent BQ was found to be irritant. Seven of 43 tested (16%) were positive to either 0.1% or 1% BB. The positive reactions to BB were weak, even when PPD reactions were strong. Mice lymph node assay gave EC₃ values of 0.14% for PPD compared with 0.03% for BB. Therefore, BB is approximately 10 times more potent than PPD, taking into account the molarity. We suggest that while PPD may act as a prohaptent, there is probably a spectrum of antigenic determinants *in vivo*. BB may be bound or metabolized by keratinocytes before it reacts with Langerhans cells.

Keywords Bandrowski's Base, benzoquinone, elicitation, hair dye allergy, para-phenylenediamine, prohaptent

Submitted 19 October 2005; revised 25 May 2006; accepted 22 June 2006

Correspondence:

J. M. L. White, Contact Clinic, St. John's Institute of Dermatology, St. Thomas' Hospital, Lambeth Palace Road, London SE1 7EH, UK.

E-mail: jonathanmlwhite@hotmail.com

Introduction

It has been estimated that approximately 40% of women dye their hair [1] and hair dye allergy has been identified as a common problem in most populations studied [2–4]. The commonest cause of this allergy, and therefore the standard chemical used for diagnosis, is *p*-phenylenediamine (PPD), which is present in approximately 70% of all hair dyes world-wide [5]. PPD is the most important hair dye allergen [2] and is a potent sensitizer [6].

Alternative dyes, such as *p*-aminophenol and *o*-aminophenol, have a similar chemical structure and may be able to cross-react in PPD-sensitive subjects [7].

PPD is a prohaptent, so the main driver of the allergic reaction must be due to one or more of its oxidation products and/or metabolites [7]. Bandrowski's base (BB) a trimer which forms quickly upon storage of PPD, has been proposed as the main allergen in patients reacting to PPD [8]. The basis for this has been described: *in vitro* reactive lymphocytes from subjects who are patch test positive to PPD generally respond *in vitro* to BB [8].

There is conflicting previous data. One study showed T cell clones recognizing both PPD and BB (although BB needed antigen processing whereas PPD did not) [9].

Another study demonstrated poor *in vivo* correlation of skin readings to PPD and BB [10]. We therefore studied the incidence of positivity to BB in patients attending contact dermatitis clinics and healthy volunteers in Thailand. We also investigated whether subjects from both the United Kingdom and Thailand already known to be patch test positive to PPD also react to BB on patch testing. In addition, we also examined again the extent to which PPD allergic subjects would react to another putative *in vivo* hapten formed from PPD: 1,4-benzoquinone (BQ) [11–13]. Finally, the relative allergenic potential of BB in the local lymph node assay (LLNA) was compared with that of PPD.

Materials and methods

Materials and analysis

Ammonium formate (99.995%) was supplied from Aldrich (Milwaukee, WI, USA); HPLC quality acetonitrile (ACN) was supplied by Rathburn Chemicals Ltd. (Walkerburn, Scotland), formic acid, Analar 98/100% was supplied from BDH (Poole, UK). The Ultrapure water used in the preparation of mobile phase and standard solutions was Milli Q Plus (Millipore, Watford, UK). BB was supplied by Apollo Scientific Limited

(Stockport, UK) and stored at -20°C under nitrogen head space. PPD was supplied by Sigma (Gillingham, UK) and stored at ambient temperature under nitrogen head space.

Standard solutions of PPD and BB were prepared at 1 mg/mL in ACN:ultrapure water (1:1) and analysed by LC-UV-MS. The samples were analysed on an Agilent 1100 LC/MS G1946B version MSD with Electrospray interface (Agilent Technologies Ltd. Stockport, UK). Chromatographic separation was carried out on a Luna C₁₈ (2) analytical column (150 × 2 mm i.d., 3 µm particle size) with a 2 mm i.d. C₁₈ (2) guard cartridge, both from Phenomenex (Macclesfield, UK). The flow rate was maintained at 0.20 mL/min and 5 µL of the standard solutions were injected onto the column thermostatically held at 30 °C. The samples were analysed under gradient elution conditions with a mobile phase of A, 10 mM ammonium formate pH 3.5 and B, ACN. The mobile phase composition was 5% B for the first 2 min of the gradient and then linearly increased to 95% B in 20 min. It was then held for a further 6 min before re-equilibrating at the initial conditions for 12 min. The Ultraviolet detector (Multi-Wavelength Detector, MWD) was set to measure the absorbance at 240 nm with a 40 nm bandwidth. The MS was operated in positive ion mode electrospray with gas temperature 300 °C, drying gas 7.0 L/min, nebulizer gas pressure 35 psi, and capillary voltage 3000 V. The MS was operated in full scan mode over the range m/z 5–700 with a fragmentor voltage of 70 V.

Survey

To assess if BB may be a useful screening tool for hair dye allergy in addition to PPD, 1% BB was used as a patch test screen in 159 patients attending a contact dermatitis clinic and 642 healthy volunteers, both groups from Bangkok. All subjects from the two centres in these studies gave informed, written consent and the studies were approved by local ethics committees. Agents were applied in 5 mm Finn[®] (Epitest Oy, Tuusula, Finland) chambers using Scanpor[®] tape (Norgesplaster, Vennesla, Norway).

Elicitation studies

Fifteen subjects who had previously attended the Contact Dermatitis Clinic in London, and who were found to be patch test positive to PPD, were enrolled into the study, as well as 29 similar PPD-positive patients from Bangkok. In London, they were tested (using the method described above) to PPD, BB and BQ in gradually increasing concentrations of 0.01%, 0.1% and 1.0% in petroleum (pet.) according to the following timetable:

Day 0 PPD, BQ, BB all 0.01% pet.
 Day 2 PPD, BQ, BB all 0.1% pet. and reading of any reactions
 Day 4 PPD, BQ, BB all 1.0% and reading of any reactions
 Day 7 Reading of any reactions only

Reactions were read according to the standard criteria [8]. However, it became apparent that 1% BQ was causing many irritant reactions and therefore a true allergic reaction to BQ was adjudged to occur only if there was a reaction at 0.1%. Of the 15 recruited, their original diagnostic patch test reactions were + ($n=6$), ++ ($n=7$), +++ ($n=2$).

In Bangkok, the 29 PPD-positive patients were tested according to the above schedule, however, testing was only with PPD and 0.1% BB.

Local Lymph Node Assay

The assay was performed essentially as described elsewhere [14]. Briefly, groups of four CBA/Ca mice (7–12 weeks of age) (RCC Basel, Itingen, Switzerland) were treated topically on the dorsum of both ears with 25 µL of test material, or with an equal volume of the vehicle (A00) alone. Dosing concentrations ranged from 0.01% to 0.25% (six groups), with a sixth vehicle control group. Treatment was performed daily for 3 consecutive days. Five days following the initiation of exposure, all mice were injected via the tail vein with 250 µL of phosphate-buffered saline (PBS) containing 20 µCi of tritiated thymidine (2 Ci/mmol; Amersham International, Amersham, UK). Mice were sacrificed 5 h later and the draining lymph nodes excised and pooled for each experimental group. The LNC suspension was washed twice in an excess of PBS and then precipitated with 5% trichloroacetic acid (TCA) at 4 °C for 18 h. Pellets were re-suspended in TCA and the incorporation of tritiated thymidine measured by β-scintillation counting. The concentration of the chemical required to produce a stimulation of proliferation of three compared with the vehicle treated control, the EC3 value, was determined to provide a measure of relative skin-sensitizing potential. EC3 values were derived from consideration of LLNA dose responses using a method described previously [15], that can be summarized briefly as follows. The EC3 value was calculated by interpolating between two points on the SI axis, one immediately above, and one immediately below, the stimulation value of 3. Where the data points lying immediately above and below the SI value of 3 have the co-ordinates (a, b) and (c, d) respectively, then the EC3 value may be calculated using the equation

$$\text{EC3} = c + [(3 - d)/(b - d)](a - c)$$

Results

Analytical results

Bandrowski's base. The identity of BB was confirmed by the mass spectrum of the largest peak in the total ion chromatogram (TIC). The base peak ion of m/z 319

Table 1. Patch test elicitation data

Original PPD reading	0.01% PPD	0.1% PPD	1% PPD	No reaction to PPD	All BQ reactions	All BB reactions
London						
+	1/6	3/6	5/6	1/6	0/6	0/6
++	4/7	3/7	7/7	0/7	3/7	2/7
+++	1/2	1/2	2/2	0/2	2/2	0/2
Original 1% PPD reading						Reaction to 0.1% BB
Bangkok						
+						4/26
++						1/2
+++						0/1

PPD, p-Phenylenediamine; BB, Bandrowski's base.

$[M+H]^+$ was consistent with the expected molecular weight of 318 Da. The purity of the sample was calculated to be 88.8% (calculated by peaks area normalization of the LC-UV_{240 nm} chromatographic trace). The TIC contained an unknown of approximately 6% of the total peak area. The unknown was characterized by a base peak ion of m/z 213 $[M+H]^+$ consistent with the chemical formulae $C_{12}H_{12}N_4$. The impurity was tentatively identified as 4,4'-azodi-aniline. Other unknowns of higher molecular weights, likely to be the products of further oxidative polymerization reactions of PPD, were also observed in the chromatographic traces of the sample. BB was found to contain 0.9% of its precursor PPD (by LC-UV_{240 nm}). The PPD concentration remained unchanged at the end of the 7 months stability investigation.

p-Phenylenediamine

The identity of PPD was confirmed by the mass spectrum of the largest peak in the TIC. The base peak ion of m/z 109 $[M+H]^+$ was consistent with the expected molecular weight of 108 Da. The purity of the sample was calculated to be 99.9% (by LC-UV_{240 nm}). BB at 0.1% was found to be the main impurity of PPD (by LC/UV_{240 nm}). Traces of an organic impurity tentatively identified as 4,4'-azodi-aniline were evident in the m/z 213 extracted ion chromatogram. The 7 months stability analysis showed an increase of BB concentration from 0.1% to 0.5%.

Survey results. Of 159 patients attending the contact dermatitis clinic, eight patients were patch test positive to PPD. Only one patient was positive to BB and that one positive was the only patient who had a strong positive reaction to PPD (+++). Of the 642 normal volunteers, 14 were positive to PPD. Five were positive to BB, of whom three were also positive to PPD.

Patch test elicitation data. The results are summarized in Table 1. There were no positive reactions to either 0.01% BQ or 0.01% BB in any of the subjects recruited. Of the six

subjects whose original clinic patch test reaction to PPD was +, 1/6 showed a reaction to 0.01% PPD, 3/6 reacted to 0.1% PPD and 5/6 reacted to 1% PPD; 1/6 was non-reactive to any concentration of PPD. All + reactors were negative to 0.1% BQ. All + reactors were negative to all concentrations of BB. Of the seven subjects who originally had ++ patch test reactions to PPD, 4/7 reacted to 0.01% PPD and 3/7 reacted to 0.1% PPD. Three of seven reacted to 0.1% BQ. Two of seven reacted to 1% BB but none to 0.1% BB. There were only two +++ reactors in this study, one was positive to 0.01% PPD and the other to 0.1% PPD. Both were positive to 0.1% BQ. Neither subject reacted to BB. In total, therefore, of 14 patients who were repeatedly PPD positive on patch testing, 5/14 had true positive reactions to BQ and 2/14 to BB. In Bangkok, 29 PPD positive subjects were tested to 0.1% BB (see Table 1). Twenty six of 29 subjects were graded 1+ to PPD. Only 5/29 subjects displayed a reaction to BB. Even when a subject reacted strongly to PPD, the reaction to BB was relatively weak (see Fig. 1). When these two sets of data are combined, of 43 subjects who gave positive reactions to PPD, only seven (16%) responded to a patch test with BB.

Local lymph node assay. Full details of the results are contained in Table 2. The EC3 value for PPD in this study was 0.14% compared with a value of 0.03% for BB. On a molar basis, this indicates that BB is, at least in the LLNA, a little over 10-times more sensitizing than PPD.

Discussion

Despite the many years in which allergy to PPD has been recognized, the chemical mechanism by which it induces skin sensitization has remained elusive, PPD itself being protein unreactive. Dupuis and Benezra [16] promoted the concept that it was a prohaptens which became reactive via oxidative metabolism to BQ. However, evidence for such a process has proven singularly lacking in either guinea-pig models of sensitization [17] or in humans sensitized to



Fig. 1. Reactions to *p*-phenylenediamine (PPD) at the top and Bandrowski's base (BB) at the bottom of the figure.

PPD [7]. These latter observations are further confirmed in the present study, with only a minority of the PPD allergic subjects giving a convincing positive response to BQ at 0.1% with no reactions at concentrations lower than this.

An alternative mechanism of PPD sensitization has been proposed with the suggestion that the PPD trimer, BB, which is always found as a contaminant of PPD preparations could be the culprit. In support of this, previous *in vitro* work using lymphocytes from PPD-allergic subjects, found that all (7/7) proliferated to BB but none to PPD, suggesting that PPD only acts as a prohaptens [8, 18]. This study demonstrates the highly allergenic potential of BB through LLNA assessment, but a lack of reactivity in PPD-allergic subjects.

In the present study, the average PPD EC₃ value of 0.14% was well within the reported range of 0.06–0.18%

[11] and is consistent with the view that it is a highly potent chemical [3]. The ability of PPD to induce other pro-inflammatory events such as oxidative stress [12] may be important in its ability to induce sensitization. This study supports previous guinea-pig work that shows a limited cross-reactivity, in PPD-sensitized subjects, to agents such as BQ [13]. Previous human studies on PPD-sensitized subjects also show reactivity to BQ in 4/22 subjects [13], and in another 3/10 [7]. These works taken together suggest that, while aromatic amines such as PPD may act as prohaptens, rather than giving rise to a single common hapten, there is more likely to be a spectrum of antigenic determinants produced *in vivo*.

We have demonstrated from the survey that BB is not a useful screening agent for hair dye allergy. We found only 2/14 responded to BB on patch testing, but then only at 1%, whereas several reacted to concentrations as low as 0.01% PPD. In Bangkok, the results were very similar, with just 5/29 positive to BB. With only 16% of PPD-allergic subjects reacting to BB, even though BB is clearly a powerful skin sensitizer in its own right (according to the LLNA), then it is evident that BB cannot be an important haptenic species involved in the development of sensitization to the prohaptens PPD.

There is some experimental data from human keratinocyte culture that PPD may cause lipoperoxidative damage and a modification of the intracellular anti-oxidant pool, which may be important in the pre-immunological phase of contact sensitization [19]. It is therefore possible if BB is applied to the skin, it may be bound or metabolized by keratinocytes before it can react with Langerhans cells. BB itself does not cause significant functional maturation of dendritic cells *in vitro*, though another PPD oxidation product may effect this [Coulter *et al.*, submitted].

In conclusion, we have demonstrated that BB is an even more powerful skin sensitizer than PPD. However, despite its potency, the very limited extent to which skin reactions can be demonstrated in PPD-allergic subjects leads to the conclusion that it does not play an important role in the development of PPD sensitization and is not the *in vivo* hapten.

References

- 1 Marcoux D, Riboulet-Deimas G. Efficacy and safety of hair-colouring agents. *Am J Contact Dermatitis* 1994; 5:123–9.
- 2 Catagliano G, Carosso A, Manzoni S *et al.* Results of routine patch testing of 834 patients in Turin. *Contact Dermatitis* 1992; 27:182–5.
- 3 Holness DL, Nethercott JR, Adams RM *et al.* Concomitant positive patch test results with standard screening tray in North America 1985–1989. *Contact Dermatitis* 1995; 32:289–92.
- 4 Katsarou A, Koufou V, Armenaka M *et al.* Patch test in children: a review of 14 years experience. *Contact Dermatitis* 1996; 34:70–1.

Table 2. Local lymph node assay results for PPD and BB

Concentration (%)	PPD		BB	
0.00	1.0	1.0	1.0	1.0
0.01	0.7	0.9	1.2	1.1
0.025	1.0	1.5	1.8	1.1
0.05	1.0	1.3	4.1	3.1
0.10	2.0	1.9	5.4	5.7
0.25	4.4	7.1	4.4	5.6
EC ₃ value (%)	0.16	0.13	0.04	0.02

PPD, *p*-phenylenediamine; BB, Bandrowski's base.

- 5 Spoor HJ. Hair coloring – a resume. *Cutis* 1977; 20:311–3.
- 6 Kligman AM. The identification of contact allergens by human assay 3. The maximisation test: a procedure for screening and rating contact sensitizers. *J Invest Dermatol* 1966; 47: 393–409.
- 7 Basketter DA, Liden C. Further investigations into the prohaptens concept reactions to benzene derivatives in man. *Contact Dermatitis* 1992; 27:90–2.
- 8 Krasteva M, Nicolas J-F, Chabeau G *et al.* Dissociation of allergenic and immunogenic functions in contact sensitivity to paraphenylenediamine. *Int Arch Allergy Immunol* 1993; 102: 200–4.
- 9 Sieben S, Kawakubo Y, Al Masaoudi T *et al.* Delayed-type hypersensitivity reaction to paraphenylenediamine is mediated by 2 different pathways of antigen recognition by specific $\alpha\beta^+$ human T-cell clones. *J. Allergy Clin Immunol* 2002; 109: 1005–11.
- 10 Hoting E, Baum C, Schulz K-H. Untersuchung zur Frage der Kreuzallergenität von amino- und nitro-substituierten aromatischen Verbindungen. *Dermatosen* 1995; 43:50–8.
- 11 Warbrick EV, Dearman RJ, Lea LJ *et al.* Local lymph node assay responses to paraphenylenediamine: inter- and intra-laboratory evaluations. *J Appl Toxicol* 1999; 19:255–60.
- 12 Picardo M, Zompetta C, Grandinetti M *et al.* Paraphenylenediamine, a contact allergen, induces oxidative stress in normal human keratinocytes in culture. *Br J Dermatol* 1996; 134: 681–5.
- 13 Lisi P, Hansel K. Is benzoquinone the prohaptens in cross-sensitivity among aminobenzene compounds? *Contact Dermatitis* 1998; 39:304–6.
- 14 Kimber I, Basketter DA. The murine local lymph node assay; collaborative studies and new directions: a commentary. *Food Chem Toxicol* 1992; 30:165–9.
- 15 Basketter DA, Lea LJ, Cooper K *et al.* A comparison of statistical approaches to derivation of EC3 values from local lymph node assay dose responses. *J Appl Toxicol* 1999; 19:261–6.
- 16 Dupuis G, Benezra C. *Allergic contact dermatitis to simple chemicals. A molecular approach*. New York: Marcel Dekker, 1982.
- 17 Basketter DA, Goodwin BF. Investigation of the prohaptens concept. Cross reactions between 1,4-substituted benzene derivatives in the guinea pig. *Contact Dermatitis* 1988; 19:248–53.
- 18 Krasteva M, Peguet-Navarro J, Moulon C *et al.* In vitro primary sensitization of hapten-specific T cells by cultured human epidermal Langerhans cells – a screening predictive assay for contact sensitizers. *Clin Exp Allergy* 1996; 26:563–70.
- 19 Picardo M, Zompetta C, Grandinetti M *et al.* Paraphenylenediamine, a contact allergen, induces oxidative stress in normal human keratinocytes in culture. *Br J Dermatol* 1996; 134: 681–5.