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Investigation of the immunogenicity of *p*-phenylenediamine and Bandrowski's base in the mouse

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ABSTRACT

p-Phenylenediamine (PPD) exposure is associated with T-cell mediated contact dermatitis. T-cells from allergic patients proliferate following exposure to PPD and the oxido-conjugation product Bandrowski's base (BB). Both compounds are classified as sensitizers in the local lymph node assay; however, because of their instability the nature of the antigenic determinant remains ill-defined. The aim of this study was to explore the immunogenic potential of PPD and BB in mice. Spleen cell proliferation and cytokine secretion was measured *ex vivo* following antigen recall with soluble PPD or BB and either irradiated or glutaraldehyde fixed, antigen pulsed dendritic cells from syngeneic mice. Glutathione was added to certain incubations. LC–MS analysis and solvent extraction were used to monitor the fate of [^{14}C]BB in culture and the extent of BB binding, respectively. Spleen cells from BB exposed, but not PPD- or vehicle-exposed, mice proliferated when stimulated with BB. Proliferating cells secreted high levels of IFN-γ, GM-CSF and IL-2. Stimulation with PPD instigated low levels of proliferation. Irradiated, but not fixed, dendritic cells pulsed with BB stimulated proliferation signifying a classical hapten mechanism involving irreversible BB binding to protein and processing. BB bound preferentially to serum protein when incubated together with cells and serum. Degradation of BB in the presence of glutathione was associated with a stronger stimulation of specific T-cells at higher BB concentrations. These data demonstrate that BB is a potent immunogen in the mouse.

1. Introduction

Exposure to *p*-phenylenediamine (PPD), a central component of most permanent hair dye formulations, is associated with the development of T-cell-mediated allergic contact dermatitis. The incidence of such reactions is on the increase, especially in young children (McFadden et al., 2007).

PPD is extremely unstable and susceptible to oxidation in aqueous solution; yielding *p*-benzoquinonediimine by two electron oxidation. 2,5-Dimethyl-*p*-benzoquinonediimine, a less reactive derivative, has recently been shown to react with nucleophilic side chains of amino acids via a complex series of conjugative and oxido-reductive mechanisms (Eilstein et al., 2006, 2007, 2008), suggesting that protein conjugates of electrophilic PPD oxidation products are antigenic determinants for immune cells. Despite this, the only compound sufficiently stable to be synthesized is a rearrangement product of the trimer, namely Bandrowski's base (BB; Fig. 1A; Coulter et al., 2007).

In dendritic cell studies assessing the ability of PPD to promote co-stimulatory signalling, activation has been shown to be dependent on PPD oxidation and the generation of unstable intermediates (Aeby et al., 2008). BB provides co-stimulatory signals to dendritic cells directly.

Topical exposure of mice to both PPD and BB leads to immune cell activation, as measured by an increased cellular infiltration of draining lymph nodes (Aeby et al., 2008; Warbrick et al., 1999; White et al., 2006). However, since auto-oxidation of PPD is likely to occur on skin, the sensitizing potential of different PPD derivatives remains unresolved.

In allergic patients, contact dermatitis is normally diagnosed by patch testing using 1% PPD (Ho et al., 2005), which has a high positive predictive value. Patch testing positivity with PPD is time-dependent (Basketter et al., 2006) indicating that oxidation on skin might be important for sensitization. Recently, only 16% of PPD patch test positive patients were shown to be responsive to BB, and in these cases the response was weak (White et al., 2006). These data suggest that a compound other than BB may be the ultimate antigenic determinant in most human subjects.

Lymphocytes isolated from PPD allergic patients proliferate vigorously following *in vitro* exposure to both PPD and BB (Coulter et al., 2008; Sieben et al., 2002). Somewhat surprisingly, lymphocytes,

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

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but not cord bloods, from tolerant individuals are also stimulated to proliferate, but only in the presence of BB (Coulter et al., 2008). The presence of BB-specific T-cells in both allergic patients and tolerant individuals suggests that the acquired immune response to BB is not translated invariably into an allergic reaction.

Human exposure to PPD is an almost unavoidable occurrence. Consequently, it is difficult to define the nature of the antigenic determinant that stimulates naïve T-cells using human samples. Thus, the aim of the present study was to explore the immunogenic potential of PPD and BB, the functionality of antigen-specific T-cells and mechanism of antigen presentation to T-cells using a mouse model. BALB/c strain mice were immunized with either PPD or BB via a single sub-cutaneous injection, and antigen-specific T-cell proliferation was analyzed after 7 days. This immunization protocol avoids potential PPD air (auto) oxidation processes on the skin or during cutaneous absorption, which is thought to be important for obtaining concentrations of PPD derivatives needed to induce skin sensitization (Aeby et al., 2008).

2. Materials and methods

2.1. Chemicals, radiochemicals and reagents

Dimethyl sulfoxide (DMSO), L-glutamine, glutaraldehyde, HEPES, penicillin, streptomycin, RPMI 1640 medium, [^{3}H]thymidine, foetal bovine serum, and PPD were obtained from Sigma–Aldrich (Poole, Dorset, UK). BB was obtained from ICN Biomedicals Inc. (Aurora, OH). Lymphoprep was obtained from Nycomed (Birmingham, UK). [^{14}C]BB was purchased from Selcia Ltd. (Essex, UK).

2.2. Sensitization protocol for determination of immune activation in the mouse

PPD and BB (5–25 mg/kg, 25% (v/v) DMSO in PBS; 50 μl) were administered, in the presence or absence of complete Freunds adjuvant, via a single subcutaneous injection to young adult (8–12 weeks old) female BALB/c stain mice (Charles River UK Ltd., Kent, UK) for analysis of immunogenicity. The adjuvant served to deliver the co-stimulatory signals that may not be provided by the chemical *per se*. All experiments were carried out under the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986. Concurrent controls received the appropriate vehicle with or without adjuvant. Seven days after initial chemical exposure, mice were sacrificed and the spleen removed.

2.3. Generation of bone marrow derived dendritic cells

Dendritic cells were generated from femur and tibia bone marrow cells. Marrow was flushed from bones with culture medium and cells (3×10^6) cultured in Petri dishes containing medium enriched with murine GM-CSF (20 ng/ml). Culture medium was refreshed on days 3 and 6. On day 8 cells were harvested and used as a source of antigen presenting cell in the proliferation assay. Dendritic cells routinely expressed high levels of CD11c and LPS inducible MHC class II, CD40 and CD86 (data not shown).

2.4. Determination of the in vitro proliferative response of splenocytes to p-phenylenediamine and Bandrowski's base

A suspension of single spleen cells was prepared under sterile conditions by gentle disaggregation through sterile mesh. The lymphocyte fraction was isolated by centrifugation through Lymphoprep. Cells with viability greater than 95% were suspended in RPMI-1640 supplemented with HEPES (25 mM), streptomycin (400 μg/ml), penicillin (400 μg/ml) and 10% heat inactivated foetal bovine serum and incubated (1.5×10^5) in 96-well U-bottom cell culture plates with either PPD (0.01–10 μg/ml) or BB (0.01–10 μg/ml) at 37 °C under 5% CO_2. In certain experiments, the compounds were incubated with cells in media containing glutathione (1 mM), which has recently been shown to prevent the conversion of PPD to BB (Coulter et al., 2007). After 3 days, proliferation was measured by the addition of [^{3}H]thymidine (0.5 μCi) for the final 16 h of culture. Cells were harvested, and incorporated radioactivity was measured as counts per minute on a beta counter (PerkinElmer Life Sciences, Cambridge, UK). Proliferative responses were calculated as stimulation indices (SI; cpm in treated cultures/cpm in cultures containing vehicle alone).

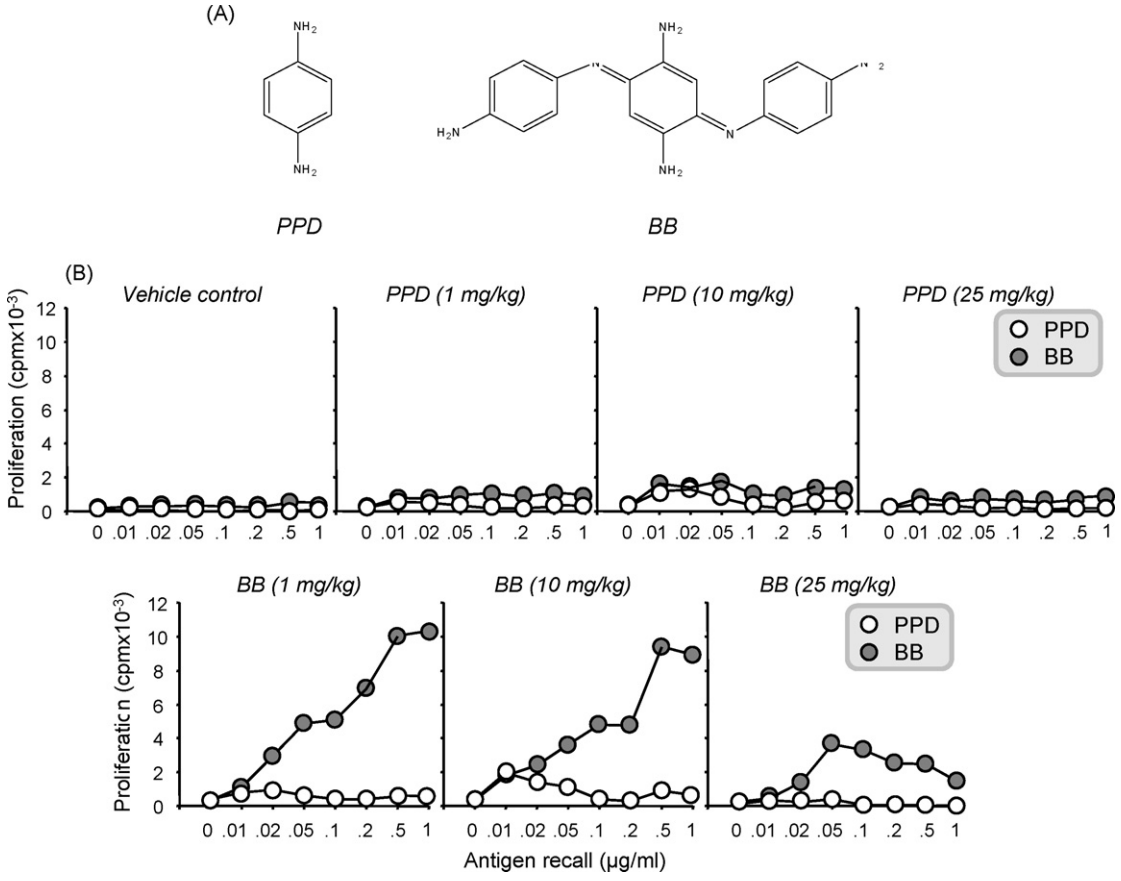

Fig. 1. Immunogenicity of PPD and BB in the mouse. (A) Structures of PPD and BB. (B) Proliferative response of spleen cells from vehicle control, PPD and BB-treated mice cultured with PPD or BB. Proliferation was determined by incorporation of [^{3}H]-thymidine over an additional 16 h. Results are presented as mean proliferative response from four mice, incubations carried out in triplicate.