

Delayed-type hypersensitivity reaction to paraphenylenediamine is mediated by 2 different pathways of antigen recognition by specific $\alpha\beta^+$ human T-cell clones

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Background: Allergic contact dermatitis to paraphenylenediamine (PPD) is a frequent cause of morbidity and occupational disability.

Objective: The aim of the study was to characterize T-cell responses to PPD and Bandrowski's base (BB), an autooxidation product of PPD, by using polyclonal and monoclonal T-lymphocyte cultures.

Methods: PPD- and BB-driven proliferation of PBMCs and T-cell clones (TCCs) was assessed by means of tritiated thymidine incorporation. Surface markers were studied by means of flow cytometry, and cytokine generation was assessed with an ELISA. **Results:** TCCs, with one exception, were CD4⁺/CD45RO⁺, and T-cell receptors were $\alpha\beta^+$. Three of 6 TCCs expressed V β 16. TCC stimulation was HLA-DP restricted, and TCCs secreted IL-4, IL-5, and marginal levels of IFN- γ . TCCs reacted to both PPD and BB. Presentation of BB to TCCs was dependent on viable antigen-presenting cells (APCs) pulsed for 4 hours, and fixed APCs failed to stimulate TCCs. Moreover, polyclonal responses to BB were enhanced by metabolically active enzymes, such as cytochrome P450 enzymes. BB has to be metabolized and processed. In contrast, fixation of APCs did not impair their ability to present PPD to TCC, whereas pulsing of APCs with PPD failed to stimulate TCCs. Thus PPD had to be present during the process, and polyclonal stimulation was not enhanced by cytochromes.

Conclusion: These results suggest that PPD itself can be recognized by T cells through a processing-independent pathway, whereas its autooxidation product, BB, required processing and possibly metabolism to stimulate the same TCC. Our data demonstrate that 2 distinct pathways of antigen presentation to activate specific TCCs are involved in the immune response to PPD. (*J Allergy Clin Immunol* 2002;109:1005-11.)

Key words: Paraphenylenediamine, Bandrowski's base, delayed-type hypersensitivity, antigen processing, T lymphocytes, T-cell clone, PBMC, cytokine, T-cell receptor

Paraphenylenediamine (PPD) is one of the most frequently encountered contact allergens.¹ Positive skin test responses have been found in 10% of white subjects and up to 50% to 60% of subjects in certain professions. Cross-sensitization between PPD and para-aminophenol, metaphenylenediamine, or drugs such as sulfonamides and benzocaine have been reported.² Knowledge about the cellular immune response initiated by PPD is limited. PPD induces oxidative stress and intercellular adhesion molecule 1 expression^{3,4} and is acetylated in human skin and keratinocytes.⁵ Others⁶ reported that Bandrowski's base (BB), an autooxidation product of PPD, is involved in allergic contact dermatitis to PPD.

The nature of the immunogenic antigens and the mechanism behind the recognition of such small molecular compounds has not been fully elucidated. Per se reactive compounds like trinitrophenol and β -lactam antibiotics⁷ bind extracellularly to proteins, which are subsequently processed and might be presented in association with HLA class I or II molecules to specific T cells,⁸ or the HLA molecule itself can be the target of the haptenization process.⁹ In contrast, per se nonreactive compounds might need activation, and the resulting metabolites bind to intracellular proteins, which are degraded and presented as drug-modified self-peptides by antigen-presenting cells (APCs).¹⁰ An involvement of cytochrome P450 enzymes, which are expressed in APCs, has been reported.^{11,12} In addition, Schneyder et al¹³ and Zanni et al¹⁴ recently proposed an additional model. They found that sulfamethoxazole and lidocaine can bind directly to the HLA-peptide complex stimulating T lymphocytes through an HLA-restricted processing- and metabolism-independent pathway.

We analyzed the recognition and processing requirements of PPD and its autooxidation product, BB, by using PBMCs of human subjects allergic to PPD and PPD- and BB-reactive T cells from these sensitized individuals.

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Abbreviations used

APC: Antigen-presenting cell
 BB: Bandrowski's base
 PPD: Paraphenylenediamine
 SI: Stimulation index
 TCC: T-cell clone
 TCR: T-cell receptor
 TT: Tetanus toxoid

METHODS**Reagents**

PPD, paraformaldehyde, dimethyl sulfoxide, Percoll (Sigma Chemical Co), and BB (ICN Biomedicals Inc) were purchased. Stock solutions of antigens were prepared shortly before the experiment by using light-insensitive tubes to avoid unspecific effects. Nontoxic antigen concentrations were determined as concentrations that did not affect PHA-induced proliferation of PBMCs.

Culture medium

The culture medium was RPMI-1640 complete (50 IU/mL benzylpenicillin, 50 µg/mL streptomycin, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1× mixture of nonessential amino acids [Gibco BRL], 10% FCS, and 2% heat-inactivated pooled human AB serum [Sigma Chemical]).

Proliferation assays

Proliferation assays were performed as previously described.⁷ PBMCs (1.5×10^6 cells/mL) from patients with positive patch test reactions to PPD ($n = 11$) and healthy volunteers ($n = 8$) were incubated with and without antigens (37°C, 5% CO₂). Final concentrations ranged from 0.01 to 20 µg/mL, except for PPD, which could be tested only at 0.01 to 5 µg/mL. After 6 days, each culture was pulsed with tritiated (³H) thymidine (0.5 µCi), incubated for an additional 16 hours, harvested (Dunn Labortechnik), and counted for 1 minute (β counter, Pharmacia-Upjohn). T-cell proliferation was expressed as the stimulation index (SI), the ratio of ³H thymidine incorporation in cultures with and without antigen. SIs of greater than 2.0 were considered positive responses.

Clonal T cells (5×10^4) were incubated with 3×10^4 x-irradiated (5000 rad) PBMCs in the presence and absence of the antigens or tetanus toxoid (TT) at indicated concentrations (final volume of 200 µL in 96-well round-bottom plates) to determine the specificity of T-cell clones (TCCs) to PPD and BB. After 48 hours, ³H thymidine (0.5 µCi) was added, and the cultures were harvested 16 hours later.

The influences of metabolic enzymes (P450s) on T-cell proliferation was determined as follows. The reaction mixture contained pooled human liver microsomes (20 µg of protein per milliliter, Gentest, Natutec), with the antigen and cells in a buffer containing 100 mmol/L potassium phosphate buffer (pH 7.4) with a reduced nicotinamide adenine dinucleotide phosphate-generating system consisting of 0.5 mmol/L NADP⁺, 5 mmol/L glucose 6-phosphate, and 0.5 units of glucose-6-phosphate dehydrogenase per milliliter. Incubations were carried out at 37°C for 24 hours and terminated by means of centrifugation of the cells (1 minute at 150g) and removal of the supernatants. The recovered cells were resuspended in fresh medium, and proliferation was determined as described.

Generation of TCCs

After primary culture of PBMCs, 20 IU/mL rhIL-2 (Biotest, Dreieich, Germany) was added to cells, and stimulation was conducted for 5 days. Cells were cloned by limiting dilution. T-cell blasts were seeded at 0.6 cells/well in round-bottom microtiter

plates (Falcon) in the presence of 1 µg/mL PHA (Gibco BRL), 20 IU/mL rhIL-2, and 2.5×10^5 autologous, x-irradiated (5000 rad) PBMCs in complete culture medium (10% FCS and 2% pooled human AB serum). TCCs were maintained by means of restimulation with autologous x-irradiated PBMCs, PHA, and IL-2.

Flow cytometry

TCCs were stained with FITC-labeled mAbs at 4°C for 30 minutes. Cells were then washed and analyzed by means of flow cytometry on a FACScan (Becton Dickinson). Monoclonal antibodies (anti-CD4, CD8, CD45RO, CD45RA, T-cell receptor [TCR] αβ, and TCRγδ) and isotype controls were purchased from Becton Dickinson.

PCR-based Vβ analysis

Total RNA was isolated from 6 TCCs (High Pure RNA-isolation kit, Roche Diagnostics). Reverse transcription of RNA (0.5 µg/10 µL) was performed (RT-PCR kit, Perkin Elmer). Generally, 4 µL of diluted cDNA (dilution factors, 1:400–4000; final volume of 25 µL) was amplified in the presence of specific primers (20 pmol)¹⁵ in buffer (10 mmol/L Tris-HCL [pH 8.3], 50 mmol/L KCL, and 1.5 mmol/L MgCL₂), 2'-deoxynucleoside 3'-triphosphates (0.2 mmol/L), and AmpliTaq Gold (1.25 U, Perkin Elmer). The PCR reaction began at 95°C for 9 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 90 seconds, and, finally, 72°C for 5 minutes. DNA fragments were separated on an ethidium bromide-stained 2.2% agarose gel.

Cytokine secretion

Supernatants from TCCs (72 hours after stimulation with antigen [5 µg/mL] and 2.5×10^5 autologous, x-irradiated PBMCs as APCs) were analyzed for IL-4, IL-5, and IFN-γ concentrations (ELISA, Coulter Immunotech). The levels of detection were 5 pg/mL for IL-4 and 50 to 100 pg/mL for IL-5 and IFN-γ.

HLA restriction (antibody-blocking experiments)

We used the anti-HLA class II mAbs (1 µg/mL, PharMingen) L243 (anti-DR), B7.21 (anti-DP), or SK10 (anti-DQ) to study HLA class II restriction. TCCs were stimulated with mAbs and antigens (5 µg/mL for 3 days). The mAbs were tested at the same concentration on unrelated clones without showing any toxicity on TCCs.

Glutaraldehyde fixation and pulsing of APCs

APCs were fixed as previously described.¹⁶ APCs were resuspended (0.5 mL of serum-free medium) and fixed with 0.05% glutaraldehyde (30 seconds at 22°C). After addition of 0.2 mol/L L-glycine (1 mL for 45 seconds), APCs were washed 4 times before use. In addition, we preincubated our APCs (4 hours) with antigens, removed soluble antigens by using extensive washing steps, and cocultured them with TCCs.

RESULTS**In vitro stimulation of PBMCs from patients allergic to PPD and control subjects**

PBMCs from 11 patients (8 female and 3 male patients) and 8 healthy donors were cultured in vitro with PPD and BB (0.01–20 µg/mL). All patients had a strong positive history of allergic contact dermatitis to PPD after occupational exposures or as a result of cosmetic products, such as hair dyes. Surprisingly, PBMCs of all patients revealed positive reactions to PPD, BB, or both.

Two patients reacted only to PPD, and one person reacted only to BB. SIs varied between 2.1 and 40.2 for PPD and between 2.6 and 34.2 for BB. Optimal proliferation was observed for PPD in the range of 0.1 to 5.0 $\mu\text{g/mL}$. BB was tested in the range of 0.1 to 20.0 $\mu\text{g/mL}$. To exclude unspecific stimulations, we studied PBMCs from 2 types of control subjects: presumably unexposed healthy volunteers ($n = 5$, 3 female and 2 male subjects) and exposed female subjects ($n = 3$, exposed to hair dyes) without clinical symptoms. Seven of 8 control subjects did not show a proliferation to any compound, and 1 reacted only to BB (data not shown). All patients showed a proliferative response to TT (data not shown).

TCCs generated against PPD and BB

PBMCs from these patients were stimulated with PPD and BB, and 25 PPD-derived, as well as 28 BB-derived, specific TCCs could be generated by limiting dilution. TCCs were not stimulated by TT. SIs for all 53 TCCs varied between 2.4 and 151.1. PPD-derived TCCs recognized BB, and BB-derived specific TCCs were stimulated by PPD. Representative examples are depicted in Fig 1. The vast majority of TCCs (41/53) showed similar proliferation rates to PPD (SI, 5.8-151.3) and BB (SI, 6.6-116.4). A second group (6/53; 5 BB and 1 PPD TCCs) responded more strongly to BB than to PPD (SI, 14.19-104.3 vs 2.5-7.6), and 6 TCCs (6/53; 3 BB and 3 PPD TCCs) showed higher proliferative responses to PPD than to BB (SI, 15.8-66.3 vs SI 3.1-3.5). Phenotypic analysis (FACS analysis) demonstrated that all TCCs were $\text{CD4}^+/\text{CD45RO}^+$, except for one CD8^+ clone. Unfortunately, we could not propagate this CD8^+ clone in culture. All clones expressed a TCR of the $\alpha\beta$ repertoire (data not shown).

HLA restriction of PPD- and BB-specific TCCs

HLA restriction of the TCCs was studied by means of mAb blocking experiments. In 2 independent experiments we found that the proliferative response of TCCs ($n = 10$) was strongly inhibited by anti-HLA-DP (80%-95%, $P = .001$) and, to a lesser extent, also by anti-HLA-DR (25%-35%, $P = .41$) but not by HLA-DQ ($P = .98$). This finding was independent of the TCC type and antigen used (data not shown).

Cytokine secretion from TCCs

Table I shows the influence of PPD and BB (5 $\mu\text{g/mL}$ for 72 hours) on cytokine protein secretion from TCCs. Cytokine release was not induced by TT treatment of TCCs. The majority of the clones secreted IL-4 (range, 6.8-520 pg/mL) and high levels of IL-5 (range, 0.1-35 ng/mL), and 10 of 15 clones also secreted low levels of IFN- γ (range, 0.03-0.560 ng/mL). Overall, the cytokine levels were positively correlated with the extent of proliferative T-cell responses. There was only a statistically significant difference between the 2 antigens for IL-4. IL-4 secretion was enhanced after TCC stimulation with PPD ($P = .024$). We investigated the clonality of 6 representative CD4^+ clones (2 of each group in Fig 1) by

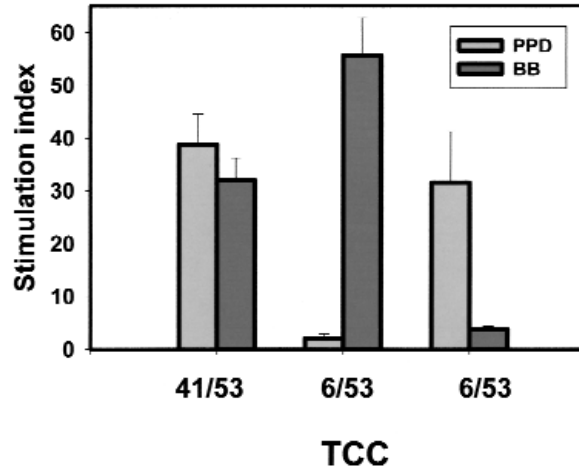


FIG 1. Distinct proliferation rates of TCCs to presented PPD and BB (5 $\mu\text{g/mL}$). TCCs are grouped according to their responses to PPD and BB. Proliferative responses (^3H thymidine uptake) are given as SIs. Mean values $\pm$ SE of all clones in each group are shown.

studying the expression of 24 TCR $\text{V}\beta$ chains (RT-PCR). All clones gave a strong positive signal only to one $\text{V}\beta$ chain (data not shown). Interestingly, expression of $\text{V}\beta$ 16 was found in 3 of 6 TCCs (50%), and the remaining 3 clones expressed $\text{V}\beta$ 6, 9, and 13.

Presentation of PPD and BB

Irradiated autologous fixed APCs or pulsed APCs were tested for their ability to activate TCCs to investigate whether processing is required for presentation of the antigen. Glutaraldehyde-fixed APCs were washed extensively and used to stimulate TCCs with and without addition of soluble PPD and BB (Fig 2, A). Fixed APCs were able to present PPD to TCC, whereas stimulation of TCCs by BB was significantly reduced in the presence of fixed APCs. Additionally, we performed the experiments with APCs preincubated (pulsing) with haptens. This is based on the knowledge that preincubation of proteins or peptides for about 4 hours leads to uptake, processing, and presentation of the antigen at the surface of APCs. Pulsing of APCs with PPD failed to stimulate all clones studied, whereas BB-pulsed APCs stimulated TCCs (Fig 2, B). This was found independently from their origin (PPD- or BB-derived TCC). Apparently, PPD had to be present throughout the assay period. These results suggest that BB, but not PPD, required at least uptake and possibly metabolism and processing to stimulate T cells.

Influence of metabolic enzymes on T-cell stimulation by PPD and BB

To further study the possible involvement of metabolically active enzymes for presentation of PPD and BB, we performed experiments with antigen-modified human microsomes, which are enriched in P450s. Initially, we attempted to do these experiments with TCCs, but P450s were toxic to TCCs. Therefore we switched to PBMCs from sensitized individuals and established nontoxic

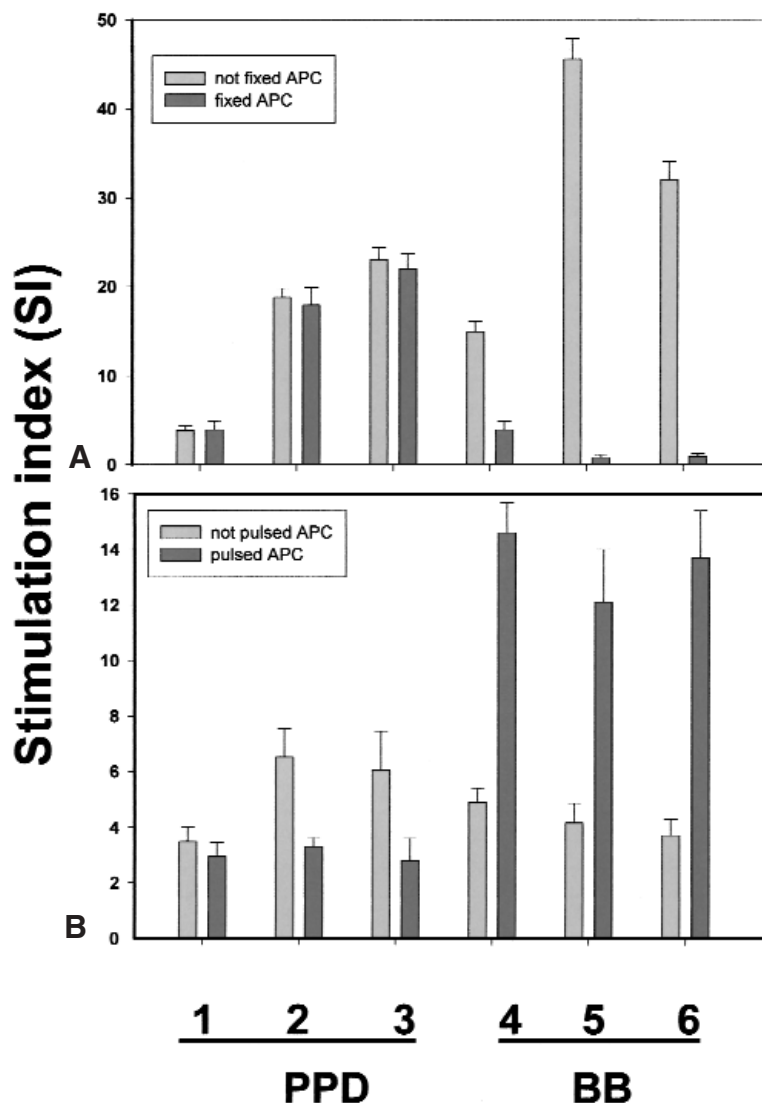


FIG 2. Modulation of proliferative responses of the same TCCs by PPD and BB in the presence of native irradiated autologous APCs compared with glutaraldehyde-fixed APCs (**A**) and in the absence and presence of pulsed APCs (**B**). Proliferative responses (SIs) are given as means $\pm$ SE.

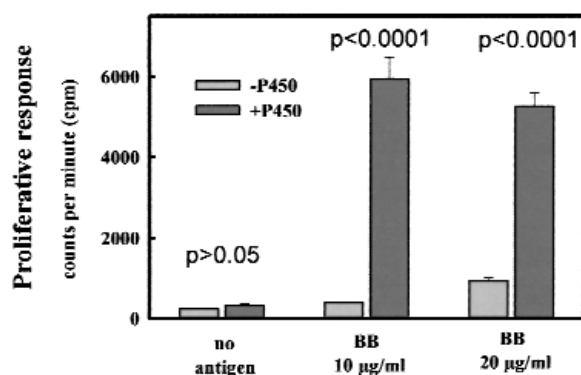


FIG 3. Influence of metabolizing enzymes (microsomes) on the proliferative response of PBMCs from sensitized individuals by means of BB ($n = 7$ individuals). Proliferative responses are given as means $\pm$ SE of all individuals tested.

assay conditions. We coincubated PPD or BB in the presence and absence of P450s and performed proliferation assays. We found that P450s enhanced the proliferative response of BB but not that of PPD ($n = 7$). The results are shown in Fig 3. The addition of an irrelevant protein, such as BSA, microsomes, or TT, or the addition of heat-inactivated P450s did not enhance T-cell stimulation to BB (data not shown). These data provide preliminary evidence that processing of BB possibly requires metabolic activation by P450s.

DISCUSSION

The prevalence of allergy to PPD, an arylamine dye, is common, and sensitization rates are high in patients with occupational exposures. In this study we found that PBMCs from patients with positive patch test responses

TABLE I. Phenotypes of TCCs

Clone	SI		Cytokines					
			IL-4 (pg/mL)		IL-5 (ng/mL)		IFN- γ (pg/mL)	
	PPD	BB	PPD	BB	PPD	BB	PPD	BB
PPD1	40.7	37.7	28.4	6.8	20.2	21.5	0.05	0.07
PPD2	20.1	10.7	118.0	84.0	19.6	7.5	0.08	<
PPD3	15.8	15.0	108.8	45.2	12.8	10.2	<	<
PPD4	20.6	27.8	287.3	125.2	26.3	16.3	0.03	<
PPD5	26.0	30.4	23.6	35.6	30.5	20.2	<	<
PPD6	12.2	20.4	71.6	52.4	13.2	11.4	<	<
PPD7	24.5	16.7	96.0	38.4	34.8	14.8	0.12	0.07
BB1	2.4	42.1	370.8	125.2	0.02	35.2	<	<
BB2	26.6	12.1	178.4	<	7.1	2.5	<	<
BB3	14.7	16.5	171.0	71.2	10.2	8.6	0.06	0.06
BB4	53.5	90.9	89.6	70.0	15.7	6.6	0.15	0.07
BB5	10.6	11.5	71.2	68.0	4.4	5.2	0.18	<
BB6	29.7	16.9	520.0	37.0	11.1	4.6	0.03	0.13
BB7	2.5	35.2	404	70.4	0.1	6.9	<	0.56
BB8	35.7	25.7	58.0	<	26.6	4.1	0.03	0.41

Culture supernatants were obtained 72 hours after stimulation with 5 μ g/mL antigen in the presence of autologous, x-irradiated PBMCs as APCs. Cytokines were determined by means of ELISA.

reacted to both PPD and BB. These results are contradictory to the results of Krasteva et al,¹⁷ who reported that in their 7 patients PPD failed to induce lymphocyte responses. In this context it should be noted that in our study only 5 of 11 patients showed reactions to PPD with SIs of greater than 3.5 patients showed SIs of greater than 2, and 1 patient did not react to PPD. Therefore the small number of patients studied thus far might be partly responsible for these differences. In addition, we made several unsuccessful attempts to explore the discrepancy. Neither varying the antigen concentration, testing antigens from different manufacturers, nor variation of the length of the time period (1, 2, or 6 days) the PBMCs were cocultured with PPD and BB could explain the different findings. Krasteva et al considered PPD to be nonimmunogenic on the basis of their findings with PBMCs and cross-challenge of one PPD-specific and one BB-specific T-cell line. We obtained similar data from 6 TCCs, but 47 TCCs reacted to PPD and BB. Taken together, it is likely that testing of many TCCs gives more insight into the mechanism of T-cell stimulation by PPD.

In the present study we found that PPD and BB stimulated the proliferation of T cells recovered from sensitized human subjects. An earlier study in guinea pigs suggested BB as the hapten rather than PPD.⁶ The authors concluded this because in guinea pigs BB acted as a stronger antigenic compound than PPD. We believe that the existence of multiple coexisting pathways leading to T-cell stimulation by arylamines is a likely explanation for this obvious discrepancy. Initially, benzoquinone was believed to be the nominal hapten for a range of 1,4-substituted benzenes. Basketter and Goodwin² and Basketter and Liden¹⁸ demonstrated, with guinea pigs and in human subjects, respectively, that this

is, in general, not the case. PPD is easily oxidized by atmospheric oxygen, as well as metabolized by human skin,⁵ which eventually might give rise to several hap- tens. Strength of immunogenic responses to substances like PPD should be taken with care because it is very difficult to ensure the chemical purity necessary to rule out any BB contamination. Finally, these authors provide considerable evidence that guinea pigs respond in a different manner than human subjects.

TCCs obtained from PBMCs revealed an almost homogenous T-cell involvement. All but one specific TCC were of the CD4⁺/CD45RO⁺ phenotype and HLA class II restricted. This observation is in line with the common model that elicitation of dermal hypersensitivity reactions depends primarily on CD4⁺ memory helper T cells.^{19,20} However, studies in the mouse model²¹ and human studies with uroshiol,²² nickel,^{23,24} and lidocaine²⁵ have demonstrated that CD8⁺ cells are also involved in the disease. In agreement with this, we found one cytotoxic CD8⁺ clone, which we could not propagate in culture.

Initially, T_H1-like cytokine patterns have been found in TCCs involved in contact dermatitis,²⁶ but more recently, T_H2-like cytokines were also reported for nickel²⁷ and lidocaine-specific^{25,28} TCCs. Furthermore, repeated application of TNCB, a typical contact allergen, caused a shift of the cutaneous cytokine profile from T_H1 to T_H2.^{29,30} Moreover, Padovan et al³¹ reported that the T_H1 profile shifted to a T_H2 profile depending on the allergen concentration. The high IL-4 and IL-5 secretion observed in our study has not been reported for contact allergens thus far and requires additional investigation. Fragrance-specific TCCs³² and peptide-specific TCCs³³ thus far established in our laboratory never reached such levels.

On the other hand, the observed amounts compare favorably with values found for drugs such as sulfamethoxazole or lidocaine.³⁴ TCCs did not release significant levels of IL-10 (data not shown), and therefore we can exclude that these cells represent regulatory T cells.

The majority of T lymphocytes in T-cell responses to nickel and other contact allergens or drugs were $\alpha\beta^+$ T cells.²⁶ Fifty percent (3/6) of the $\alpha\beta^+$ TCCs studied expressed only V β 16, possibly suggesting that this chain might be important for hapten recognition. Because antigen presentation is HLA II restricted, we studied the influence of the different classes. Presentation of contact allergens, such as nickel or trinitrophenol, is predominantly restricted by HLA-DR or HLA-DQ and less frequently by HLA-DP molecules.^{19,23,35,36} Our finding, associating PPD and BB T-cell stimulation with certain restriction molecules (HLA-DR, HLA-DQ, and HLA-DP), indicated a strong involvement of HLA-DP in the presentation of these 2 antigens. However, the relevance of this HLA association for a possible use as a susceptibility marker requires the identification of the sequence motif and evaluation in a population-based study.

A remarkable finding is that recognition of PPD, unlike recognition of BB, by the studied TCCs recovered from PBMCs of patients with positive patch test responses does not require processing. The latter has been considered as a prerequisite to stimulate T cells.³⁷ Although the structure of the PPD antigen epitope still has to be defined, it is likely to be extracellularly generated because we found that to stimulate specific T cells, PPD antigen has to be present in a soluble form for more than 4 hours. Indeed, fixed APCs were able to induce T-cell stimulation, whereas pulsed APCs were not. In contrast, the BB antigen is likely to be generated inside the APC because APCs pulsed for 4 hours were able to stimulate TCCs, whereas fixed APCs could not induce a T-cell response. In addition to processing, it is commonly postulated that per se nonreactive small molecular compounds gain chemical reactivity on metabolism.^{38,39} We have found that the presence of P450s enhance the T-cell stimulation of BB antigen, suggesting that metabolism of this antigen is required. Again, active metabolism by APCs was not found for PPD antigen. However, more evidence is needed to elucidate these aspects, especially because we can not exclude metabolism of PPD through other pathways (ie, either spontaneous oxidation or metabolism by T cells). On the other hand, these data are in accordance with data presented by Zanni et al⁴⁰ and Schnyder et al.⁴¹ They found that drugs such as sulfamethoxazole or lidocaine bind predominantly directly to the HLA-peptide complex.

In summary, we found that PPD antigen and BB antigen, 2 molecules in one metabolic chain, can be presented by 2 distinct pathways to specific T cells. In addition, our data provide evidence that these essentially diverse antigens are recognized by the same T cell. Our current hypothesis is that PPD is capable of behaving as a hapten and binds extracellularly to a molecule that functions as

a classical antigen-presenting molecule (ie, that binds the antigen and interacts with the TCR). Then BB is considered the prohaptent, giving rise to a hapten possibly containing a similar or even identical epitope after processing and metabolism.

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