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Reactivity of in vitro activated human T lymphocytes to p-phenylenediamine and related substances

CLAUDIA SKAZIK1,2,*, SILKE GRANNEMANN2,†, LIANE WILBERS1, HANS F. MERK1, PIETER-JAN COENRAADS3, SEBASTIAN BREUER2 AND BRUNHILDE BLOÊMEKE2

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Background: Patch tests to p-phenylenediamine (PPD) and related substances often show concurrent reactions that can be attributed to separate sensitization or cross-reactivity.

Objectives: In order to understand the health risks associated with cross-reactivity, we studied cross-reactivity of eight chemicals in vitro by measurement of T-cell proliferation of peripheral blood mononuclear cells (PBMC), T-cell lines (TCL), and T-cell clones (TCC) of subjects with a positive patch test result to PPD.

Patients/Methods: We studied PBMC from 13 patients and were able to generate TCL from seven and TCC from four patients. Their proliferative responses to the chemicals were estimated.

Results: Concurrent reactions to these compounds on the polyclonal and monoclonal level were found. A restricted T-cell receptor (TCR) Vβ16-usage was observed (5/8 clones). A detailed analysis of 34 TCL showed broad cross-reactivity (64.7%) between PPD, p-toluenediamine, Bandrowski’s Base, and p-aminoazobenzene. More restricted patterns were found in 8.8%, which responded only to compounds with two or three benzene rings, whereas 26.5% of the clones reacted specifically only to one compound.

Conclusion: More than 60% of the clones showed a broad cross-reactivity pattern. Hence, clinically observed cross-reactivity between different para-amino compounds can be based on a TCR recognizing similar epitopes of these compounds with low specificity.

Key words: cross-reaction; delayed-type hypersensitivity; para-amino compounds; p-phenylenediamine; T-cell clones; Vβ analysis.

Accepted for publication 13 May 2008
themselves, bind to proteins or peptides and modify them. The modified proteins are processed and presented as hapten-modified peptides in association with human leucocyte antigen molecules to T cells and are able to activate them (8, 9). Haptens are often derived from chemicals considered as prohaptenes, which are not chemically reactive per se and have to be metabolized into an electrophilic hapten endowed with antigenic properties first (10, 11). Moreover, it has been recently reported that chemically inert substances can bind directly to the major histocompatibility complex (MHC–peptide complex in a processing-independent way and thus can interact with the T-cell receptor (TCR) and stimulate T cells (12). We found data supporting this concept while studying the contact allergen PPD (13) showing that PPD can activate memory T cells using glutaraldehyde-fixed antigen-presenting cells (APC) that are incapable of processing. Based on this context, it is supposed that the cross-reactivity of structurally related compounds depends on the affinity, concentration, and TCR density expressed on the cell surface (14). Different mechanisms have been assumed to account for frequently observed cross-reactivity to hapten: generation of a common reactive metabolite that acts as a hapten, the T cell would then recognize identical neoantigens, T-cell recognition of cryptic self-peptides, or true cross-reactions, whereas the TCR of a T cell is incapable to distinguish between the different hapten (15). Furthermore, some evidence indicates that the concomitant reactions to PPD and textile dyes (16) may also be due to metabolic conversion of dyes to PPD at the surface by skin bacteria (17).

In patients with positive patch tests to PPD, high frequencies of simultaneous sensitivity (16, 18, 19) of memory T cells to p-toluenediamine (PTD), para-aminoazobenzene (AAB), and also azo dyes used in textiles, like Disperse Orange 3 (DO3), Disperse Yellow 3 (DY3), and Disperse Blue (DB 106/124), or Bandrowski’s Base (BB) (20), an autoxidation product of PPD (21), have been diagnosed.

Hence, to be able to estimate and decrease the health risks associated with exposure to these substances, it is important to understand the mechanism of cross-reactivity to different para-amino compounds. In order to analyse T-cell cross-reactivity to para-amino compounds such as PTD, BB, AAB, DO3, 4,4’-diaminodiphenylmethane (DDM), Bismarck Brown Y (BBY), and DY3, and the unrelated contact allergen mercaptobenzothiazole (MBT) (Fig. 1) in humans, we investigated in vitro T-lymphocyte responses from PPD-positive cases in polyclonal and monoclonal T-cell subsets such as peripheral blood mononuclear cells (PBMC), T-cell lines (TCL), and T-cell clones (TCC).

Materials and Methods

Subjects

We investigated 13 subjects (10 females and 3 males; 11 tested with the German patch test baseline series and 2 with the Dutch standard series) showing at least ++ positive patch test reactions to PPD according to the International Contact Dermatitis Research Group’s recommendations (application period: 2 days; readings at 2, 3, and 7 days) and a strong history of allergic contact dermatitis due to personal (hair dyeing and tattoo) or occupational exposure (hairdresser) to PPD. The study was approved by the local ethics committee, and all subjects gave informed consent. Patch test materials were purchased from Hermal (Reinbek, Germany) (1% in petrolatum) and applied in Finn chambers (Epitest Ltd Oy, Tuusula, Finland).

Antigens

PPD (freebase), AAB, DO3, DY3, DDM, BBY, and 2-MBT were obtained from Sigma (Steinheim, Germany). BB was purchased from INC Biomedicals (Aurora, OH, USA) and PTD from Merck (Hohenbrunn, Germany). Stock solutions of each antigen were prepared freshly in culture medium by using light-protected tubes to avoid unspecific effects. The non-toxic antigen concentration was determined as concentration that did not affect phytohaemagglutinin (PHA)-induced proliferation of PBMC as described earlier (22). The purity of PPD and PTD was 98%, AAB was 98.9% and DDM and MBT was 99% or more based on the information given by the companies. The manufacturers of BB, DO3, DY3, and BBY did not provide the purity but provided information on the melting point and λmax or thin layer chromatography comparisons of the product with standards. We used the same substances as are commonly used in patch tests, although we are aware of the existing differences in purity (23).

Culture medium

The cell culture medium used in this study was RPMI-1640 supplemented with L-glutamine, 10% foetal calf serum (FCS) (PAA, Pasching, Austria), 1% 100 mM sodium pyruvate, 1% 10× non-essential amino acids, and 1% antibiotic-antimycotic solution (Gibco BRL, Paisley, Scotland).

For growing TCL and TCC, the complete culture medium was prepared without any
antibiotics, but with the addition of 2% heat-inactivated pooled human AB serum (Sigma).

**Proliferation assays**

PBMC were isolated by density centrifugation over Ficoll-Paque™ (Uppsala, Sweden). Antigen-specific proliferation was investigated by culturing (37°C, 5% CO₂) PBMC alone (2 × 10⁵ cells), TCL, or TCC (1 × 10⁵ cells) plus autologous gamma-irradiated PBMC (2.5 × 10⁵ cells) in the presence and absence of antigen (1–50 μM in order to avoid toxicity for PBMC, data not shown), 4.25 Lf/ml tetanus toxoid (TT) as control for specificity, and PHA as a mitogenic control in a total volume of 200 μl (96-well plates with flat bottom: Falcon BD, Cedex, France). After two (TCL and TCC) or 6 days (PBMC), cultures were pulsed with [³H]thymidine (0.5 μCi) and incubated for additional 16 hr. Cells were harvested on a glass fibre filter, and the [³H]thymidine incorporation was measured as counts per minute (c.p.m.) using a Wallac MicroBeta Counter (Perkin Elmer, Zaventem, Belgium). Stimulation indexes (SI) were calculated as c.p.m. in the presence of a compound (antigen) divided by c.p.m. in the absence of compound (mean c.p.m. ± SD of triplicate cultures). SI of greater than 2.0 were considered as significant responses.

**Generation of TCC**

TCC were generated using the limiting dilution technique as described (13). Briefly, chemical-specific TCL (PTD, BB or PPD, and only a few with AAB or BBY) were diluted to a cell density of 0.5 cell/well and cultured in 96-well round-bottom culture plates with 4 × 10⁴ autologous-irradiated
PBMC in the presence of antigen and interleukin (IL)-2 (50 U/ml; Endogen, Woburn, MA, USA) in complete culture medium (10% FCS and 2% pooled human AB serum). Growing TCC were maintained in complete culture medium by periodic restimulation in the presence of irradiated PBMC, antigen, and IL-2. The specificity was tested as described above.

Flow cytometric analysis
Aliquots that contained 10^5 cells were stained with fluorochrome conjugated antibodies in 50 μl of buffer (phosphate-buffered saline with 1% FCS and 0.02% NaN₃) for 30 min at 4°C and analysed on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany). Anti-CD3 (HIT3a, mIgG2a), anti-CD8 (HIT8a, mIgG1), anti-CD4 (RPA-T4, mIgG1), anti-CD45RA (HI100, mIgG2b), and anti-CD45RO (UCHL1, mIgG2a) were purchased from BD Biosciences. TCR-Vβ gene products (DPC Biermann, Bad Nauheim, Germany): Anti-TCR-αβ (BMA031, mIgG2b), anti-TCR-γδ (Immu510, mIgG1), anti-Vβ1 (BL37.2, rlgG1), anti-Vβ2 (mIgG1), anti-Vβ3 (CH92, mIgM), anti-Vβ5.1 (3D11, mIgG1), anti-Vβ5.2 (36213, mIgG1), anti-Vβ5.3 (3D11, mIgG1), anti-Vβ7 (ZOE, mIgG2a), anti-Vβ8.1/8.2 (56C5, mIgG2a), anti-Vβ9 (FIN9, mIgG2a), anti-Vβ11 (C21, mIgG2a), anti-Vβ12 (Ver2.32.1, mIgG2a), anti-Vβ13.1 (Immu222, mIgG2b), anti-Vβ13.6 (JU-74, mIgG1), anti-Vβ14 (CAS1.1.3, mIgG1), anti-Vβ16 (TAMAYA1.2, mIgG1), anti-Vβ17 (E17.5F3, mIgG1), anti-Vβ18 (BA62, mIgG1), anti-Vβ20 (ELL1.4, mIgG1), anti-Vβ21.3 (IG125, mIgG2a), anti-Vβ22 (Immu546, mIgG1), and anti-Vβ23 (AF-23, mIgG1).

Results
T-cell responses to para- amino compounds including some azo dyes were characterized using PBMC of 10 females and 3 males (mean age = 50 years, range: 26–93) based on at least ++ positive patch test reactions to PPD and a history of allergic contact dermatitis due to personal or occupational exposures. Specific proliferative responses of PBMC to PPD were observed in 11 cases (SI = 2.2–49.3; Table 1). No associations with sex and age were observed. Thus, we found a good correlation (83%) between our in vitro results and the in vivo test.

Further proliferation studies on the polyclonal level found similar SI (2.1–49.3) for BB, PTD, PPD, p-phenylenediamine; SI, stimulation indices.

<table>
<thead>
<tr>
<th>Cases</th>
<th>Patch test reaction to PPD a</th>
<th>In vitro reaction to PPD SI b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
<td>2.2</td>
</tr>
<tr>
<td>3</td>
<td>+++</td>
<td>13.3</td>
</tr>
<tr>
<td>4</td>
<td>++</td>
<td>2.1</td>
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<td>++</td>
<td>4.5</td>
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<td>++</td>
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<td>7</td>
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<td>12</td>
<td>++</td>
<td>2.1</td>
</tr>
<tr>
<td>13</td>
<td>+++</td>
<td>49.3</td>
</tr>
</tbody>
</table>

AAB, DO3, DDM, and BBY as classical para-amino compounds (Figs 1 and 2) and azo dye DY3. There was also a similar SI for MBT as a contact allergen that does not belong to any of the highest indices are presented.

![Fig. 2. Proliferative responses (c.p.m.) of PBMC (6 days) and TCL (2–3 days) from 11 PPD-sensitized persons to different para-amino compounds/arylamines (1–50 μM) and the chemically unrelated MBT. Antigen-specific T-cell proliferation was measured by [3H]thymidine incorporation assay. In this graph, the results are shown as proliferation after subtration of control without antigen (PBMC: 2.1–49.3 pM and 50 μM of PPD for 6 days and the proliferative responses were measured by [3H]thymidine incorporation assay. Positive reactions were defined as SI > 2 only the highest indices were presented.](https://example.com/fig2.png)

Table 1. Comparison of the patch test results and in vitro proliferative responses to PPD

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**AAB**, **DO3**, **DDM**, and **BBY** as classical para-amino compounds (Figs 1 and 2) and azo dye **DY3**. There was also a similar SI for **MBT** as a contact allergen that does not belong to any of the highest indices are presented.

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<td>1</td>
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<td>2.1</td>
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<tr>
<td>13</td>
<td>+++</td>
<td>49.3</td>
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the mentioned chemical groups. Antigen concentrations between 1 μM and 50 μM were applied. TT-specific responses served as positive control for T-cell functionality, finding as expected lower responses in TCL compared with PBMC, because antigen-specific T cells accumulate during restimulation. For this reason, positive TT data in Fig. 2 derive from PBMC only.

Overall, on the polyclonal level, most concomitant reactions to PPD arose for AAB (92%) followed by DO3 (85%). Furthermore, we found co-reactions to BB (69%), DDM (62%), DY3 (54%), BBY (46%), PTD (39%), and MBT (23%). These in vitro results confirmed earlier clinical observations and patch-testing studies in PPD-sensitized subjects that display reactions to more than just one substance.

In order to validate our findings on the monoclonal level, we aimed for generation of TCC from all PPD-allergic persons by restimulation of their PBMC with different antigens, mainly PPD, PTD, DO3, AAB, and BB. Overall, 35 TCC from four subjects were successfully generated by limiting dilution. TT was used as control for cloning success, finding no responses in specific TCC. Moreover, we only included TCC that showed no reaction to MBT in further experiments (n = 34) because MBT is structurally not related to the other tested substances and no cross-reactions with para-amino compounds are expected. Nine TCC were additionally tested for reactions to benzoinone and did not react to this compound (data not shown). A subgroup of 18 TCC was analysed by flow cytometry (fluorescence-activated cell sorter). The tested clones were CD4 positive and expressed TCR-αβ (Fig. 3) except one TCC that was CD8 positive and expressed TCR-γδ. Unfortunately, we were not able to culture this clone long enough for a detailed functional analysis. Further Vβ chain analysis of eight TCC showed a strong expression (>90%) of one Vβ chain and a restricted TCR-Vβ usage, indicating the oligoclonality of the dye-specific immune response (Table 2). Interestingly, five of eight TCC were positive for Vβ16 (62.5%).

A summary of the TCC responses to the compounds (SI = 2–62, n = 190) is depicted in Fig. 4, whereas antigen concentrations between 1 μM and 50 μM were applied. Surprisingly, the pattern was comparable to our findings on the polyclonal level. Against the overall idea that one clone reacts to one specific antigen, we found that the TCC

![Fig. 3. Characterization of the generated T-cell clones through flow cytometry. (a) untagged, (b) CD4+ T lymphocytes, (c) expressed T-cell receptor-αβ, and (d) CD45RO accordingly representing memory T cells.](image-url)
reacted to several of the tested related antigens independent of the antigens they were generated with. Therefore, in the context of our research question, we defined ‘antigen specific’ as the proliferative response of a TCC with a specific reaction pattern to one or more antigens.

In a subset of five TCC, we examined the occurrence of concomitant reactions to PPD. All nine substances were tested and most reactions besides PPD were found for AAB, DO3, PTD (three of five TCC), and BB (two of five TCC). Only one co-reaction was found for DY and DDM and no responses occurred to BBY and MBT. First, we concentrated further studies on PPD, PTD, BB, and AAB and tested 34 TCC for cross-reactions. Beside PPD and its autoxidation product BB, we included PTD because of its structural similarity to PPD, and AAB, which exhibits only one free amino group (Fig. 1). The results lead to the identification of certain reaction patterns: group I (26.5%, 9) reacting specifically only to one antigen, group II (64.7%, 22) reacted to compounds with one as well as two or three benzene rings, and group III (8.8%, 3) TCC responded only to compounds with two or three benzene rings. Hence, the following frequency distribution arose: group II > group I > group III. Secondly, we tested the validity of these groups in 21 TCC and included two textile dyes DO3 and BBY (Fig. 5). DO3 differs from AAB only concerning one amino group; BBY was chosen because it consists of three benzene rings like BB, but in addition contains two azo groups (Fig. 1). We validated our initial findings and even the extension to nine tested antigens had no effect on the grouping (data not shown). Moreover, independent evaluation by considering responses only with a stimulation index of SI > 2 instead of ≥2 did not change the results. These results confirm and extend our previous results showing that PPD-specific monoclonal T cells can clearly respond to several related para-amino compounds.

**Discussion**

Concomitant positive reactions to PPD and other arylamines, para-amino compounds, and some azo dyes are frequently reported in patch test studies. Theoretically, concomitant reactions can be explained by concomitant sensitization or by true cross-reactions (18, 24, 25). In addition, in the case of azo dyes, simultaneous reactions may result from metabolic conversion of dyes to the corresponding monomers (17, 26). Furthermore, early cross-reactivity analysis in inbred guinea-pigs indicates that the recognizing ability of hapten-specific lymphocytes varies according to the position of the functional group and its type (27). Thus, the aim of this study was to explore concomitant reactions and cross-reactivity patterns among PPD and para-amino compounds in vitro. We tested whether PPD-specific T cells also reacted to BB, PTD, AAB, DO3 (Fig. 1) on the polyclonal (PBMC and TCL) and monoclonal T cell level in order to better understand if this finding is due to cross-reactions or simply due to co-sensitization.

We found that all substances showed concomitant reactions to PPD, basically confirming in vivo patch test data. Seidenari et al. (18) showed in patch tests highest concomitant reactions to PPD for AAB (75%) and DO3 (66%). We observed

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**Table 2.** V\(\beta\) chain expression in a subset of the generated TCC \((n = 8)\) estimated by flow cytometric analysis. In total, 21 V\(\beta\) chains were analysed

<table>
<thead>
<tr>
<th>T-cell clones</th>
<th>T-cell receptor V(\beta) chain</th>
<th>V(\beta)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>V(\beta)16</td>
<td>95.34</td>
</tr>
<tr>
<td>2</td>
<td>V(\beta)16</td>
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<td>V(\beta)16</td>
<td>91.57</td>
</tr>
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<td>93.78</td>
</tr>
<tr>
<td>6</td>
<td>V(\beta)2</td>
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<td>7</td>
<td>V(\beta)9</td>
<td>88.96</td>
</tr>
<tr>
<td>8</td>
<td>V(\beta)22</td>
<td>77.15</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Proliferative responses (c.p.m.) of arylamine-specific TCC from PPD-sensitized persons to various para-amino compounds/arylamines (1–50 \(\mu\)M). Antigen-specific T-cell proliferation was measured after 2 days by \[^{3}H\]thymidine incorporation. In this graph, the results are shown as proliferation after subtraction of control without antigen (c.p.m. = 960–64050). Only results with SI > 2 are depicted (SI = 2.1–62). TT served as control for cloning success, finding no responses in specific TCC. Overall, the pattern of TCC reactivity was similar to the polyclonal level but, as expected, the clones did not react to the unrelated MBT. The one TCC that reacted to MBT was excluded from further analysis.

**References:**

AAB, para-aminodibenzene; BB, Bandrowski’s Base; BBY, Bismarck Brown Y; c.p.m., counts per minute; DDM, diaminodiphenylmethane; DO3, Disperse Orange; DY3, Disperse Yellow; MBT, mercaptobenzothiazole; PPD, \(p\)-phenylenediamine; PTD, \(p\)-toluenediamine; SI, stimulation indices; TCC, T-cell clones; TT, tetanus toxoid.
in vitro on the polyclonal level the highest correlation rates between positive responses to PPD and AAB (92%) respectively DO3 (85%), too. However, we cannot confirm other patch test studies noticing that the stronger a patient reacts to PPD, the higher is the probability for further reactions to different substances (28).

In our study, BB, DDM, DY3, and BBY likewise showed various parallel reactions to PPD, whereas in patch tests, no concomitant responses to BBY were found in one study (16). Furthermore, this in vitro study found similar to others (29) concomitant responses for BB (69%), while only 16% of PPD-positive cases were positive to BB by patch testing even when PPD reactions were strong (20). This discrepancy may indicate different bioavailability.

To provide further laboratory evidence of the immune pathogenesis of allergic contact dermatitis, we cloned arylamine-specific T cells from the peripheral blood of PPD-sensitive patients and analysed their phenotype, specificity in vitro, and the requirements of TCR activation by stimulating clones with up to eight structurally related compounds.

We found that monoclonal T cells can be activated by various para-amino compounds similar to the pattern found in polyclonal cultures (compare Figs 2 and 4). Some TCC showed very specific reactions to only one substance, but most TCC displayed responses to more than one antigen. Usually, antigens are presented in the context of MHC expressed on the surface of APC, but some chemicals for instance isopentenyl pyrophosphate and fluorescein can activate T cells.
without the involvement of MHC molecules through a TCR-dependent process (30, 31). In addition, some drugs are able to elicit hypersensitivity reactions without evidence for metabolism or covalent binding to a carrier protein (32, 12). Furthermore, for these drugs, the kinetics of TCR downregulation is too fast for the involvement of antigen processing. Due to these aspects, an additional model was proposed, referred to as ‘pharmacological interaction of drugs with immune receptors’ (p-i concept) (33). The idea is that some chemicals bind reversibly to immune receptors like MHC or TCR thereby stimulating T cells. However, we found no proliferation when TCC were incubated with the substances in the absence of APC. These data indicate that our isolated TCC do not present para-amine compounds to each other. Interestingly, we found a restricted usage of Vβ chains in the arylamine-specific CD4+ TCC, predominantly Vβ16, exactly as found earlier (13) This finding suggests that the Vβ16 chain plays an important role in the recognition of para-amine compounds, although further studies are required. So far, no comparable data are available for PPD, but for nickel-specific CD4+ T cells, it has been suggested that Vβ17 is involved in antigen recognition and possibly severity of disease (34,35).

With the gathered data, we identified three types of TCC. This grouping was valid in tests with four, six, or nine substances and was also found on the polyclonal level. This proves the grouping to be no in vitro artefact and not to be restricted to in vitro-generated TCC. The compounds we used in our study consist of prohaptens, so that it is possible that these substances are presented to a TCC through different pathways including the p-i concept (reviewed in 33). It is conceivable that both pathways, with and without antigen processing, are involved in the antigen presentation of the tested arylamines, but more detailed investigations are warranted.

Overall, for each molecule, activity and potential to give rise to cross-reactions in vivo will depend on skin penetration capacities including resorption, and the balance between routes of individual skin metabolism. The latter may also account for the discrepancy between in vitro and in vivo findings for BB in humans as described above.

In summary, the para-group allergy is characterized in vitro by three types of specific CD4+ T cells, while in vivo also CD8+ T cells play an important role. The majority (>60%) of the CD4+ T cells bears a TCR with minor specificity. T cells with a low specificity TCR likely account for the in vivo observed cross-reactions between related chemicals. Our observed broad cross-reactivity pattern suggests an explanation for the in vivo observed predisposition to immune reactivity after a single exposure to related compounds.

Acknowledgements

We are grateful to the staff of the clinics (Aachen and Gröningen) for performing the patch tests and blood collection. C. S. was supported by a grant from the Interdisciplinary Centre for Clinical Research ‘BIOMAT’ within the faculty of Medicine at the RWTH Aachen University (Germany).

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