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

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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) V β 16-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; V β analysis. © Blackwell Munksgaard, 2008.

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Allergic contact dermatitis, a delayed hypersensitivity reaction to small molecular weight compounds (haptens), is a common skin reaction causing personal and occupational problems (1, 2). Chemicals such as *p*-phenylenediamine (PPD) and related para-amino compounds, frequently found in dyes, are raising increasing concerns due to their strong allergenicity. Recent surveys in Europe,

USA and Japan indicate that hair dyeing and skin paintings have become much more prevalent during the past 10 years; it is performed at a younger age and the proportion of men is increasing (3). Thus, contact allergy to PPD and related compounds has increased significantly in eczema patients (3, 4) and also in the general population (5) over the past decades.

So far, the mechanism of skin sensitization and elicitation to the pro- or prehapten PPD (6) has not been elucidated, but some hypotheses on the sensitization to small chemicals exist. The hapten concept (7) suggests that small chemically reactive compounds, which are not immunogenic by

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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 prohaptens, 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 haptens: 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 haptens (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 mononu-

clear 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 (hairstylist) 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 $\lambda_{\max}$ 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

Fig. 1. Chemical structures of the investigated compounds.

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 (2×10^5 cells), TCL, or TCC (1×10^5 cells) plus autologous gamma-irradiated PBMC (2.5×10^5 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 incu-

bated 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. $\pm$ 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 4×10^5 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 μ l 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-V β expression of TCC was analysed by staining using a panel of 24 monoclonal antibodies against various V β gene products (DPC Biermann, Bad Nauheim, Germany): Anti-TCR- $\alpha\beta$ (BMA031, mIgG2b), anti-TCR- $\gamma\delta$ (Immu510, mIgG1), anti-V β 1 (BL37.2, rIgG1), anti-V β 2 (mIgG1), anti-V β 3 (CH92, mIgM), anti-V β 5.1 (3D11, mIgG1), anti-V β 5.2 (36213, mIgG1), anti-V β 5.3 (3D11, mIgG1), anti-V β 7 (ZOE, mIgG2a), anti-V β 8.1/8.2 (56C5, mIgG2a), anti-V β 9 (FIN9, mIgG2a), anti-V β 11 (C21, mIgG2a), anti-V β 12 (Ver2.32.1, mIgG2a), anti-V β 13.1 (Immu222, mIgG2b), anti-V β 13.6 (JU-74, mIgG1), anti-V β 14 (CAS1.1.3, mIgG1), anti-V β 16 (TAMAYA1.2, mIgG1), anti-V β 17 (E17.5F3, mIgG1), anti-V β 18 (BA62, mIgG1), anti-V β 20 (ELL1.4, mIgG1), anti-V β 21.3 (IG125, mIgG2a), anti-V β 22 (Immu546, mIgG1), and anti-V β 23 (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,

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

Cases	Patch test reaction to PPD ^a	<i>In vitro</i> reaction to PPD SI ^b
1	+++	2.7
2	++	2.2
3	+++	13.3
4	++	2.1
5	++	4.5
6	++	<2.0
7	++	<2.0
8	++	3.3
9	+++	4.0
10	++	9.4
11	+++	6.3
12	++	2.1
13	+++	49.3

PPD, *p*-phenylenediamine; SI, stimulation indices.

^aPatch test readings were taken at D2, D3, and D7 after exposure.

^bPeripheral blood mononuclear cells were cultured with concentrations between 1 μ M and 50 μ M 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 are presented.

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

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 μ M) 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 subtraction of control without antigen (PBMC: 575–175375 c.p.m. and TCL: 520–52760 c.p.m.). Only results with SI > 2 are depicted (PBMC: 2.1–49.3 SI; TCL: 2.1–24.6 SI). TT-specific responses served as positive control for T-cell functionality, finding lower responses in TCL compared with PBMC because antigen-specific T cells accumulate during restimulation. For this reason, positive TT data in this figure derive from PBMC only.

AAB, *p*-aminoazobenzene; BB, Bandrowski's Base; BBY, Bismarck Brown Y; c.p.m., counts per minute; DDM, diaminodiphenylmethane; DO3, Disperse Orange; DY3, Disperse Yellow; MBT, mercaptobenzothiazole; PBMC, peripheral blood mononuclear cells; PPD, *p*-phenylenediamine; PTD, *p*-toluenediamine; SI, stimulation indices; TT, tetanus toxoid.

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 benzoquinone 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- $\alpha\beta$ (Fig. 3) except one TCC that was CD8 positive and expressed TCR- $\gamma\delta$. Unfortunately, we were not able to culture this clone long enough for a detailed functional analysis. Further V β chain analysis of eight TCC showed a strong expression (>90%) of one V β chain and a restricted TCR-V β usage, indicating the oligoclonality of the dye-specific immune response (Table 2). Interestingly, five of eight TCC were positive for V β 16 (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- $\alpha\beta$, and (d) CD45RO accordingly representing memory T cells.

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

T-cell clones	T-cell receptor V β chain	V β %
1	V β 16	95.34
2	V β 16	92.92
3	V β 16	97.36
4	V β 16	91.57
5	V β 16	93.78
6	V β 2	94.03
7	V β 9	88.96
8	V β 22	77.15

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 $SI \geq 3$ 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.

Fig. 4. Proliferative responses (c.p.m.) of arylamine-specific TCC from PPD-sensitized persons to various para-amino compounds/arylamines (1–50 μ 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.

AAB, para-aminoazobenzene; 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.

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

Fig. 5. Distinct proliferation patterns of representative TCC ($n = 21$) from PPD-sensitized persons divided into three groups: group I specific reaction to only one antigen ($n = 6$); group II reactions to compounds with one and more benzene rings ($n = 12$) and group III reactions to antigens with two and more benzene rings ($n = 3$). Cells were cultured *in vitro* with the different para-amino compounds for 2 days. Afterwards, T-cell responses were measured by [3 H]thymidine incorporation assay. The line in the graph indicates stimulation indices = 2, threshold for positive responses. TT served as control for cloning success.

AAB, *p*-aminoazobenzenes; BB, Bandrowski's Base; BBY, Bismarck Brown Y; DO3, Disperse Orange; PPD, *p*-phenylenediamine; PTD, *p*-toluenediamine; 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 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-amino compounds to each other. Interestingly, we found a restricted usage of V β chains in the arylamine-specific CD4⁺ TCC, predominantly V β 16, exactly as found earlier (13). This finding suggests that the V β 16 chain plays an important role in the recognition of para-amino 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 V β 17 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.

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