Chapter 5

Reactivity to sodium tetrachloropalladate (Na$_2$PdCl$_4$) compared to PdCl$_2$ and NiCl$_2$ in lymphocyte proliferation tests

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5.2 Abstract

Objectives: For patch testing, replacement of the commonly used palladium dichloride (PdCl\textsubscript{2}) by sodium tetrachloropalladate (Na\textsubscript{2}PdCl\textsubscript{4}) was recently demonstrated to improve test accuracy and show a significant correlation with nickel (Ni), supporting the concept of cross-reactivity between Pd and Ni. A promising alternative to metal allergy patch testing is the \textit{in vitro} lymphocyte proliferation test (LTT). The aim of this study was to test whether Na\textsubscript{2}PdCl\textsubscript{4} is also more sensitive for diagnosing Pd allergy with a standardized LTT.

Patients/methods: After determining optimal nontoxic and non-mitogenic concentrations for Na\textsubscript{2}PdCl\textsubscript{4}, blood samples from 105 patients with clinical suspicion of metal allergy were tested with an LTT called memory lymphocyte immuno-stimulation assay for Na\textsubscript{2}PdCl\textsubscript{4}, PdCl\textsubscript{2} and NiCl\textsubscript{2}. Reaction profiles were analysed for concordant positive reactions.

Results: Using the conventional cut-off of stimulation index $\geq 3$, 74.3\% showed a positive reaction to NiCl\textsubscript{2}, 15.2\% to PdCl\textsubscript{2} and 28.6\% to Na\textsubscript{2}PdCl\textsubscript{4}. All positive results to PdCl\textsubscript{2} were covered by Na\textsubscript{2}PdCl\textsubscript{4}. From the 30 positive reactions to Na\textsubscript{2}PdCl\textsubscript{4}, 26 (87\%) were concordant for NiCl\textsubscript{2} reactivity.

Conclusion: In LTT, the use of Na\textsubscript{2}PdCl\textsubscript{4} results in more positive reactions in Pd allergy testing which are in concordance with positive reactions to PdCl\textsubscript{2} and NiCl\textsubscript{2}.
5.2 Introduction

Metal allergy has been gaining interest in the last years. In dentistry, a great variety of metals resulting in ca. 7000 different dental alloys are used to restore decayed teeth. Because of galvanic and crevice corrosion as well as the aggressive oral environment, all metals will ionize to some extent. The clinical impact from this metal exposure is mainly described in case reports. Palladium (Pd) allergy emerged in the literature after the introduction of Pd-containing dental alloys in 1973 (1). Palladium is mainly used in crown and bridge work and introduced due to rising gold prices. Furthermore, Pd and its alloys are used in jewellery and as catalysts in the chemical and automotive industries (2).

In literature, cross-reactivity between nickel (Ni) and Pd is described, although positive reactions exclusively to Pd have been reported (3). Concordant positive reactions to Ni and Pd may be explained by (i) sensitization to both metals (ii) contamination of the Pd patch test material with spurious amounts of Ni (although several studies have disputed this theory) (4) and (iii) the fact that Ni and Pd have similar chemistry and electron arrangements, which might be responsible for true cross-reactivity at the T-cell level (5-7). Hindsén et al. (8) provided evidence for cross-reactivity of Ni and Pd in vivo by systemic administration. They yielded flare-up reactions on sites previously tested with Ni and Pd after oral Ni provocation. In their study, contamination was excluded by chemical analysis. Pistoor et al. and Moulon et al. (5, 6) provided evidence for cross-reactivity on the T-cell level between Ni and Pd, as well as copper (Cu), by in vitro tests. In both studies, Ni-reactive T-cell clones proliferated upon selective Pd and Cu stimulation. In most studies concerning cross-reactivity with patch testing, nearly all Pd reactors react to Ni as well (90–100%) (9), although a lower rate has been communicated lately by Fowler and Hayden (70%) (10). Because of the higher prevalence of Ni allergy compared to Pd allergy, true cross-reactivity seems not to be the case (1).

While metal allergies are in principle diagnosed with an epicutaneous or patch test, alternatives have been sought caused by certain drawbacks of patch testing, including its low sensitivity to certain metals such as Ni (11-13) and its potentially sensitizing character (14). Several in vitro tests have been described in the literature, of which the lymphocyte proliferation or transformation test (LTT) seems to be the most promising alternative, especially for non-dermal-related allergies (11, 12, 15-18).

Recently, sodium tetrachloropalladate (Na₂PdCl₄) and palladium dichloride (PdCl₂) were used as test salts for Pd allergy patch testing (19). It was found that Na₂PdCl₄ is more sensitive than PdCl₂, most probably due to its better solubility in water.
because of its mononuclear structure, while the molecular structure of PdCl₂ is oligo-
or polymeric and is therefore nearly insoluble in water (20, 21). In contrast to PdCl₂, 
NiCl₂ is well soluble in water and is therefore a standard test material for Ni allergy 
testing in LTT and in patch testing. As the solubility and the molecular structure of test allergens in in vitro tests is an 
important factor in antigen presentation, it was hypothesized for this study that 
Na₂PdCl₄ is also a more reactive test material than PdCl₂ for diagnosing Pd allergy by 
means of LTT. The reactivity to Na₂PdCl₄ was compared to the reactivity to, and the 
concordance with, PdCl₂ and NiCl₂, respectively, in patients with clinical suspicion of 
Pd and Ni allergy.

5.3 Materials and methods

Patients
Blood from 105 samples submitted to the Department of Immunology, Laboratory 
Center Bremen, Germany accredited according to DIN EN ISO 15189 and DIN EN 
ISO/IEC 17025, for routine LTT-MELISA® (MEmory Lymphocyte Immuno Stimulation 
Assay) diagnosis of allergy to various metals including PdCl₂ (Sigma-Aldrich Chemie 
GmbH, Steinheim, Germany) and NiCl₂ (NiCl₂.6H₂O; Sigma-Aldrich Chemie GmbH) 
was used. The LTT- MELISA® is a patented modification of the LLT using a 
standardized and validated protocol (22, 23). All patients had clinical suspicion of Ni 
and/or palladium allergy, rendering this a selected group of patients. Whenever 
sufficient lymphocytes were available, an extra LTT test was performed in parallel 
with Na₂PdCl₄ (Na₂PdCl₄.3H₂O; Sigma-Aldrich Chemie BV, Zwijndrecht, The 
Netherlands; purity: 99.998%) as test allergen. All blood samples were submitted in 
CPDA monovettes (Sarstedt AG & Co., Nümbrecht, Germany) or ACD Solution A 
vacutainer tubes (Becton Dickinson GmbH, Heidelberg, Germany) and transported by 
normal post or by private courier to arrive in the laboratory usually within 24 h, at 
most 48 h, after the blood was drawn. Peripheral blood mononuclear cells (PBMCs) 
were isolated on Ficoll Histopaque (Sigma-Aldrich Chemie GmbH, Taufkirchen, 
Germany) immediately upon arrival in the laboratory and either used immediately or 
stored in 20% medium (RPMI-1640 containing HEPES; Life Technologies, Karlsruhe, 
Germany), 8 mg.l⁻¹ gentamicin (Sigma-Aldrich Chemie GmbH), 2 mmol.l⁻¹ L-
glutamine (Biochrom seromed, Berlin, Germany) and 20% pooled, heat-inactivated 
human AB serum (Cambrex Bio Science Verviers S.p.r.l., Verviers, Belgium) overnight 
at 4 °C prior to set up.
**LTT-MELISA®**

The LTT-MELISA® was performed in the Laboratory Centre Bremen essentially as described by Stejskal et al. (22) and modified for routine clinical testing by Valentine-Thon (23). Briefly, 1x10⁶ PBMCs (monocyte reduced by plastic adherence) in 1 ml of 10% medium were pipetted into the wells of a 24-well cell culture plate pre-coated with two to three serial (1 : 2) dilutions of metal salt solutions in the following initial concentrations: PdCl₂, 125 µg.ml⁻¹ (0.71 mM); NiCl₂.6H₂O, 100 µg.ml⁻¹ (0.42 mM); Na₂PdCl₄.3H₂O, 125 µg.ml⁻¹ (0.36 mM). Activated monocytes produce prostaglandins (24), which negatively affect lymphocyte activation (25). During preparation procedures the number of monocytes increases. In order to normalize this number and to exclude possible negative effects of produced prostaglandins, partial monocyte depletion is carried out. The concentrations used were determined according to standard procedures applied in LTT to reveal nontoxic and non-mitogenic reactions. All salts used were of ‘pro analysi’ grade, and the diluent was sterile double-distilled water. To dissolve PdCl₂ in 10 ml of water, 250 µl of 30% HCl was needed. No HCl was needed to dissolve Na₂PdCl₄ in water. Solubility was macroscopically checked. The plates were incubated for 5 days at 37 °C with 5% CO₂. Three negative controls (lymphocytes in 10% medium without antigen) and one positive control (lymphocytes in 10% medium plus 2 µg.ml⁻¹ pokeweed mitogen (PWM); Sigma-Aldrich Chemie GmbH) were incubated on each plate. After 5 days, 600 µl of cell suspension from each well was transferred to a new 24-well plate, and the cells pulsed for 5 h with 3 µC methyl-³H-thymidine (Amersham Buchler, Brunswick, Germany; specific activity 185 GBq/mmol). The radioactivity [counts per minute (cpm)] was measured in a liquid scintillation counter (1450 Microbeta Trilux, Wallac Distribution GmbH, Freiburg, Germany). A reaction was considered positive when one or more of the test concentrations gave a stimulation index (SI) ≥ 3, calculated as follows:

\[
SI = \frac{\text{cpm in test well}}{\text{the average cpm in negative control wells}}
\]

A SI < 2 was considered negative, SI ≥ 2 but <3 was interpreted as a ‘possible sensibilization’, and a SI ≥ 3 was interpreted as positive, i.e. evidence of sensibilization.

Cells from the 5-day cultures were additionally analysed morphologically after staining cytospin preparations with rapid differential haematology staining solutions (Dade Behring AG, Marburg, Germany). Only tests in which the radioactively positive results showed the presence of lymphoblasts and radioactively negative results showed only viable, small lymphocytes (non-cytotoxicity and non-mitogenic stimulation) were accepted as valid. Statistical analysis For statistical analysis, the obtained SI values were converted to binary values. To evaluate concordance
(Na₂PdCl₄ with PdCl₂ and NiCl₂), the Spearman’s rank order correlation (Sigmasstat 3.0; SPSS Inc., Chicago, IL, USA) was used. p<0.05 was considered significant.

5.4 Results

The mean SI of 105 patients classified in four different groups are summarized in Table 5.1. As cut-off values of LTT results to discriminate between a positive and a negative test reaction are still debated, the results are presented separately using two different cut-off values: SI ≥ 3 and SI ≥ 5. The average cpm of the negative controls was 792, while the positive pokeweed controls showed in nearly all cases a SI > 30.

Table 5.1 The mean Simulation Index (SI) and the standard deviations in parentheses of 105 patients classified in four different groups.

<table>
<thead>
<tr>
<th></th>
<th>NiCl₂</th>
<th>PdCl₂</th>
<th>Na₂PdCl₄</th>
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<tbody>
<tr>
<td>SI &lt; 3</td>
<td>1.6 (0.6) [n = 27]</td>
<td>1.3 (0.6) [n = 89]</td>
<td>1.6 (0.7) [n = 74]</td>
</tr>
<tr>
<td>3 ≤ SI &lt; 5</td>
<td>3.8 (0.6) [n = 17]</td>
<td>3.8 (0.8) [n = 3]</td>
<td>3.6 (0.5) [n = 13]</td>
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<tr>
<td>SI ≥ 3</td>
<td>23.6 (35.9) [n = 78]</td>
<td>12.4 (14.0) [n = 16]</td>
<td>11.5 (20.1) [n = 30]</td>
</tr>
<tr>
<td>SI ≥ 5</td>
<td>29.1 (38.8) [n = 61]</td>
<td>14.4 (14.9) [n = 13]</td>
<td>17.6 (25.4) [n = 16]</td>
</tr>
</tbody>
</table>

*Stimulation index ≥ 3*

Twenty-three samples (21.9%) did not show a positive proliferation reaction to any of the investigated metal salts, while 78 cases (74.3%) showed a positive proliferation reaction to NiCl₂, 16 (15.2%) to PdCl₂, and 30 (28.6%) to Na₂PdCl₄ (Fig. 1). All positive results to PdCl₂ were covered by Na₂PdCl₄. Twenty-six of the 30 samples positive to Na₂PdCl₄ (87%) showed concordant reactions to NiCl₂. Furthermore, four exclusively Pd-positive (Ni-negative) reactions were observed towards Na₂PdCl₄, of which one was also positive to PdCl₂. Spearman’s rank order correlation between Na₂PdCl₄ and PdCl₂ was 0.640. Although the correlation is not very strong, it is significant (p<0.05). No statistically significant correlation was found between Na₂PdCl₄ and NiCl₂.

*Stimulation index ≥ 5*

Forty-two samples (40.0%) did not show a positive reaction to any of the investigated metal salts, while 61 cases (58.1%) showed a positive proliferation reaction to NiCl₂, 13 (12.4%) to PdCl₂ and 16 (15.2%) to Na₂PdCl₄ (Fig. 2). Interestingly, not all the positive reactions to PdCl₂ are covered by Na₂PdCl₄ when
Na₂PdCl₄ in lymphocyte proliferation test

using this cut-off value. Three samples (1.9%) showed positive proliferation towards PdCl₂ and NiCl₂, but not to Na₂PdCl₄. Furthermore, one sample reacted positively only to Na₂PdCl₄. Finally, one sample was negative to NiCl₂, but positive to PdCl₂ and Na₂PdCl₄. Spearman’s rank order correlation between Na₂PdCl₄ and PdCl₂ at cut-off value ≥ 5 is not strong (0.645) but still significant (p<0.05). Again, no statistically significant correlation was found between Na₂PdCl₄ and NiCl₂.

Figure 5.1  Positive reactions to the materials investigated from 105 patients when cut-off value is SI ≥ 3. Areas of vertical overlap represent concordant reactions.

Figure 5.2  Positive reactions to the materials investigated from 105 patients when cut-off value is SI ≥ 5. Areas of vertical overlap represent concordant reactions (* represents one sample only).
5.5 Discussion

The aim of this study was to evaluate the reactivity of Na$_2$PdCl$_4$ as a test material when LTT-MELISA$^\circledR$ is used on blood samples derived from Pd and Ni allergy susceptible patients, as the patients were submitted for testing based on clinical suspicion of a Ni and Pd allergy. No attempt was made to investigate the clinical relevance of positive LTT-MELISA$^\circledR$ results, as detailed anamnestic data concerning exposure and patch test results were not available on all of these patients. However, there are several studies confirming the clinical relevance of LTT-MELISA$^\circledR$ published in the past (17, 26, 27). These publications showed a decrease in SI after removal of the insulting antigen, mostly dental restorative materials. Lymphocyte transformation test in general seems to be promising, especially for the detection of non-dermally induced metal sensitization, as described for bronchial or oral mucous membrane contact with beryllium, titanium and other dental metals (28), as well as for orthopaedic implant-related sensitization (16, 18).

Compared to patch test results in general (29), a great number of Ni and Pd-positive reactions are found in this study. This is mainly explained by the inclusion of patients with a specific suspicion of a Ni and Pd allergy. In the patch testing study (19) as mentioned before, positive reactions to Pd increased from 1.8% to 14%, because of the use of Na$_2$PdCl$_4$ compare with PdCl$_2$. This drastic increase is most likely explained by two advantages of Na$_2$PdCl$_4$ compared with PdCl$_2$. First, Na$_2$PdCl$_4$ is better soluble in water because of its mononuclear structure, as PdCl$_2$ forms oligo- or polymeric structures in water(20, 21). Secondly, because of the smaller molecular volume of Na$_2$PdCl$_4$, skin penetration will be easier compared to PdCl$_2$. With respect to Ni, a comparative study of Ni chloride, bromide, iodide and dioctanoate showed that not the polarity but the molecular volume was the decisive factor for membrane diffusion (30).

The use of Na$_2$PdCl$_4$ compared with PdCl$_2$ in LTT nearly doubled the positive reactions to Pd, from 15.2% to 28.6% (cut-off value $\geq$3). As skin penetration is not required in LTT, this may explain the less pronounced increase in positive reactions. All positive reactions to PdCl$_2$ are covered by Na$_2$PdCl$_4$. Except from four positive reactions to Na$_2$PdCl$_4$, all were concordant to NiCl$_2$ (87%). These findings are in line with the former reports on concordant reactions between Ni and Pd. Although from these results, it seems that Na$_2$PdCl$_4$ is more sensitive than PdCl$_2$ for Pd allergy testing; more research is needed to elucidate the clinical relevance of these positive reactions.

The lack of statistically significant correlation between Ni and Pd-positive reactions can be explained by the remarkably high number of positive reactions to Ni. Almost
all Pd-positive subjects reacted positively to Ni as well (87%; SI ≥ 3), whereas more than half of Ni reactors did not react to Pd. A similar trend was observed in patch testing, although less pronounced (19). A likely explanation is that to obtain a positive reaction in LTT, the subject has to be exposed to the relevant allergen to have sufficient specific T-cells in the blood circulation. When a current exposure is lacking or is insufficient, not enough T-cells might be circulating to give rise to a positive test result. As exposure to Pd is much more limited than to Ni in daily life, fewer positive reactions to Pd would be expected. On the other hand, as in patch testing nearly all positive reactions to Pd are concordant to Ni, but not the other way around as a result of the much higher prevalence of Ni allergy itself. Indeed, these results do not confirm cross-reactivity between Ni and Pd as stated by Aberer et al. earlier (1) Thus, Pd allergy might be caused by concomitant sensitization, and clinical relevance might be overlooked by the lack of knowledge on chronic Pd exposure as for example because of Pd-containing dental restorations.

When the cut-off value is ≥ 5, 3 PdCl₂-positive reactions, concordant to NiCl₂ but not to Na₂PdCl₄, were found. It looks like Na₂PdCl₄ might be more sensitive especially in the area of weak to moderate positive reactions in LTT-MELISA® and probably in all LTTs. The most appropriate cut-off will be clearly that which is most clinically relevant.

In summary, the results show that Na₂PdCl₄ clearly induced more positive reactions than the commonly used PdCl₂, not only in patch testing but also in LTT as well. This is most probably caused by the better solubility in water of Na₂PdCl₄. The larger difference in patch testing is probably as a result of the additional advantage of the better skin penetration kinetics of Na₂PdCl₄ caused by the smaller molecular volume. All positive reactions to PdCl₂ were covered by Na₂PdCl₄ (cut-off ≥ 3). Furthermore, nearly all samples with positive reactions to Na₂PdCl₄ show concordant positive reactions to NiCl₂ (87%). Although the results do not support the theory of cross-reactivity between Ni and Pd, they are in line with other studies *in vitro* and *in vivo*. Further research on Na₂PdCl₄ as a test allergen in LTT is recommended in order to obtain information on the clinical relevance of these positive Pd reactions and the true sensitivity of Na₂PdCl₄ in Pd allergy testing.
5.6 References