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Detection of nickel and palladium contact hypersensitivity by a flow cytometric lymphocyte proliferation test

Flow cytometric detection of metal contact hypersensitivity

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Abstract

We established a flow cytometric lymphocyte proliferation test (LPT) for the detection of nickel (Ni) and palladium (Pd) sensitization. Eighty-one consecutive patients with an indication for patch test (PT) were tested by LPT with Ni (NiSO_4) and Pd (Na_2PdCl_4 and PdCl_2) salts. The imprecision of the LPT was low (coefficient of variation 7.2%). Using PT as a diagnostic reference, the sensitivity and specificity of LPT were 74.4% and 80% for NiSO_4 , 74.4% and 78.3% for Na_2PdCl_4 , and 57.2% and 85.4% for PdCl_2 , respectively. For both Ni and Pd, the likelihood ratio for a positive PT markedly increased with increasing LPT value. With medical history as a reference, the sensitivity and specificity were 40.6% and 82.1% for LPT, and 59.4% and 89.7% for PT, respectively. Combination of LPT with PT resulted in a higher specificity of 95%, albeit lower sensitivity of 34.4%. In conclusion, flow cytometric LPT represents a reliable and useful method for the detection of Ni and Pd sensitization. LPT values correlate with PT results and, when used in combination with PT, increase test specificity.

Introduction

Allergic contact dermatitis is a frequent manifestation of delayed-type hypersensitivity representing a significant health problem with 15-20% of the general population sensitized to one or more contact allergens, particularly nickel (Ni) and palladium (Pd) (1, 2). Diagnosis of delayed-type hypersensitivity is challenging due to the lack of standardized tests to unequivocally differentiate allergic from non-allergic reactions. Although generally regarded as the gold standard for the diagnosis of allergic contact sensitization, the *in-vivo* skin patch test (PT) can be false-negative, or false-positive by inducing irritant contact reactions (3). The *in-vitro* lymphocyte proliferation test (LPT) circumvents the possibility of strong local reactions and avoids the risk of patient sensitization (4-8). LPT assays are however labor-intensive and need special laboratory equipment. In addition, the classic LPT requires handling of radioactive material and only detects proliferation of a heterogeneous leukocyte population without specific identification of reacting cells, thus complicating assay standardization. Flow cytometry represents a non-radioactive method, capable of simultaneously measuring cell characteristics and proliferation triggered in LPT (9, 10). It may be better accessible and more amenable to standardization, as has already been illustrated by numerous other clinical applications of flow cytometry, such

as the basophil activation test (11). Although flow cytometric LPT has been used in numerous studies on lymphocyte function (10), reports on its clinical application in differentiating between metal-sensitized and non-sensitized individuals are scarce and limited to only beryllium (12, 13). Of note, the latter studies did not include PT as a reference. Hence, the goal of this study was to establish a flow cytometric LPT for the detection of Ni and Pd sensitization, and to determine its diagnostic performance within a dermatologic patient collective using PT and history as diagnostic references.

Methods

Patient's collective

Eighty-one consecutive adult patients (79% females, mean age 56) visiting the dermatologic clinic of the University Hospital Basel and having any indication for PT were included. In 28 (35%) individuals (25 with orthopedic implants and 3 with dental implants) a metal hypersensitivity was primarily suspected. Patients were tested with PT and flow cytometric LPT for Ni and Pd sensitization by using NiSO_4 , Na_2PdCl_4 and PdCl_2 salts (6). In 64 (79%) individuals all three metal antigens were tested, whereas 17 (21%) patients were tested for one or two antigens. Patients with a negative PT served as controls when assessing specificity of flow cytometric LPT with PT as a standard. For 78 patients a detailed history of metal allergy (e.g., contact dermatitis caused by metals, eczema after contact with costume jewelry, jeans buttons or other metal items) was taken. Individuals with a negative medical history for Ni hypersensitivity were used as controls when evaluating specificity of tests against history. All patients gave informed consent to participate and the study was approved by the local ethical committee.

Patch test

PTs were performed using the European Standard Series (14), including NiSO_4 (5%), and a series of metal allergens, including PdCl_2 (1%) and Na_2PdCl_4 (3%) in petrolatum (15) (Hermal-Almirall, Wallisellen, Switzerland). Application time was two days and skin reactions were scored directly after removal of the PT and at day 3 or 4, according to recommendations (16). Patients were instructed not to expose test areas to ultraviolet light for four weeks before and during PT and to stop any corticosteroid treatment ten days prior to PT.

Flow cytometric LPT

The flow cytometric LPT was adapted from the method originally described by Lyons and Parish (9). Briefly, peripheral blood mononuclear cells were isolated, labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Basel, Switzerland), and distributed at a concentration of 1×10^6 cells in 1 ml RPMI medium with 10% human AB-serum and 5mM L-glutamine in 24-well plates. Antigen concentrations were based on (6) and

adapted in preliminary experiments: 29.6, 19.7, 13.1, and 8.7 $\mu\text{g/ml}$ for NiSO_4 (Merck Millipore, Darmstadt, Germany; purity $\geq 99.0\%$); 62.5, 41.7, and 27.8 $\mu\text{g/ml}$ for Na_2PdCl_4 (Sigma-Aldrich, Buchs, Switzerland; purity $\geq 99.9\%$); and 41.7, 27.8, and 18.5 $\mu\text{g/ml}$ for PdCl_2 (Sigma-Aldrich; purity $\geq 99.9\%$). *Candida albicans* antigens (Greer, Lenoir, NC) and tetanus toxoid from *Clostridium tetani* (Statens Serum Institut, Copenhagen, Denmark) were added as positive controls at a final concentration of 4 $\mu\text{g/ml}$ and 14.7 Lf units/ml, respectively. Following incubation at 37°C and $5\% \text{CO}_2$ for 7 days, cells were harvested, stained with CD4-PE, CD8-PerCP and CD3-APC (BD Biosciences, Heidelberg, Germany), and analyzed by flow cytometry using a FACSCanto II (BD Biosciences). Per sample 100'000 CD3^+ cells were acquired and analyzed (Fig. S1). The stimulation index (SI) was calculated as the ratio of the percentage of proliferating cells (i.e., cells with decreased CFSE) upon antigen stimulation to the percentage of proliferated cells without antigen. Since the proliferative responses of $\text{CD3}^+\text{CD4}^+$ cells (Fig. 1) were consistently higher than those of $\text{CD3}^+\text{CD8}^+$ cells (Fig. S2), SIs of $\text{CD3}^+\text{CD4}^+$ cells were used throughout this study.

Results and Discussion

The LPT with control antigen *Candida albicans* resulted in an SI ranging from 20 to 6'945. The response range to *Clostridium tetani* was similar (i.e., 1-6'029) (Fig. 1A). Duplicate experiments with *Candida albicans* in 74 patients indicated a high precision of the flow cytometric LPT with a mean coefficient of variation of 7.2% ($\text{SD}=9.3$).

When evaluating metal sensitization, a positive PT (score >0) for NiSO_4 was observed in 39 (49.4%) individuals (Fig. 1B). Positive PTs with Na_2PdCl_4 and PdCl_2 were observed in 43 (65.2%) and 26 (38.8%) individuals, respectively (Fig. 1C-D). Interestingly, both Ni- and Pd-specific flow cytometric LPT showed a significant correlation with Ni- and Pd-induced PT, respectively (Fig. 1 B-D). Using PT as a reference, the diagnostic sensitivity and specificity of LPT was 74.4% and 80.0%, respectively, for NiSO_4 , 74.4% and 78.3%, respectively, for Na_2PdCl_4 , and 57.2% and 85.4%, respectively, for PdCl_2 (Fig. 1B-D). With specificity set at 95% the LPT showed sensitivities of 43.6%, 39.5%, and 42.3% for NiSO_4 , Na_2PdCl_4 , and PdCl_2 , respectively (Fig. 1B-D). Interestingly, likelihood ratios correlated with SI values (Fig. 1B-D), indicating that the higher the absolute LPT SI value, the more likely the patient demonstrates a positive PT reaction. In fact, our data show that a high LPT SI value is almost exclusively associated with a positive PT. In addition, for all investigated antigens a trend for higher SIs was observed in individuals with stronger PT reactions (Fig. S3).

The parallel use of two Pd salts in LPT as well as in PT allowed direct comparison of both salts as test reagents. Almost all (5 of 6) individuals with a positive LPT for PdCl_2 , but a negative PT for this salt demonstrated a positive LPT reaction to Na_2PdCl_4 (Fig 1 C-D, yellow). Of note, all these patients showed a positive PT for

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Na₂PdCl₄. In addition, almost all (8 of 10) individuals with a negative LPT, but a positive PT for PdCl₂ demonstrated a positive reaction to Na₂PdCl₄ in both LPT and PT (Fig 1 C-D, red). These results are in line with previous studies reporting a relatively poor reactivity to PdCl₂ in PT and LPT as compared to Na₂PdCl₄ (6, 15). It should however be mentioned that the increased sensitivity of Na₂PdCl₄ might be at the cost of specificity since all (5 of 5) individuals with a positive LPT, but a negative PT for Na₂PdCl₄ did not react to PdCl₂ in both LPT and PT (Fig 1 C-D, blue), and almost all (9 of 10) individuals with a positive PT for Na₂PdCl₄, but a negative LPT for this salt did not react to PdCl₂ in both LPT and PT (Fig 1 C-D, green). Our data are in line with recent findings indicating Na₂PdCl₄ to be a more sensitive test allergen than PdCl₂ (15), and suggest that besides PT also LPT should be performed with both Pd salts.

We also examined the efficiency of LPT and PT in detecting metal sensitization by using patient history as a reference (3). This was done for individuals where patient history was conclusive for a diagnosis of metal allergy. Hence, 32 patients with Ni allergy and 39 patients without any indication for Ni sensitization were evaluated. PT showed a higher sensitivity and specificity (59.4% and 89.7%, respectively) compared to LPT (40.6% and 82.1%, respectively) (Fig. 2). Importantly, however, when a positive LPT was combined in a patient with a positive PT the specificity for Ni allergy increased from 89.7% for PT alone to 95.0%, albeit that sensitivity of this combined testing was relatively low (34.4%).

In conclusion, our results show that flow cytometric LPT may be of help for the detection of Ni and Pd sensitization. Patients with high LPT values have a high probability for having a positive PT and a positive clinical history. Therefore, a high LPT value may represent a promising alternative for PT, for example in such cases where PT is contraindicated or not possible. In case flow cytometric LPT and PT are used concurrently, combined positivity of both tests increases specificity. Although the sensitivity of combined positivity is relatively low, the finding of a positive LPT in a patient with a positive PT could thus be helpful in the differential diagnosis of metal contact hypersensitivity.

Author contributions

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Conflicts of interest

The authors have no relevant conflicts of interest to disclose.

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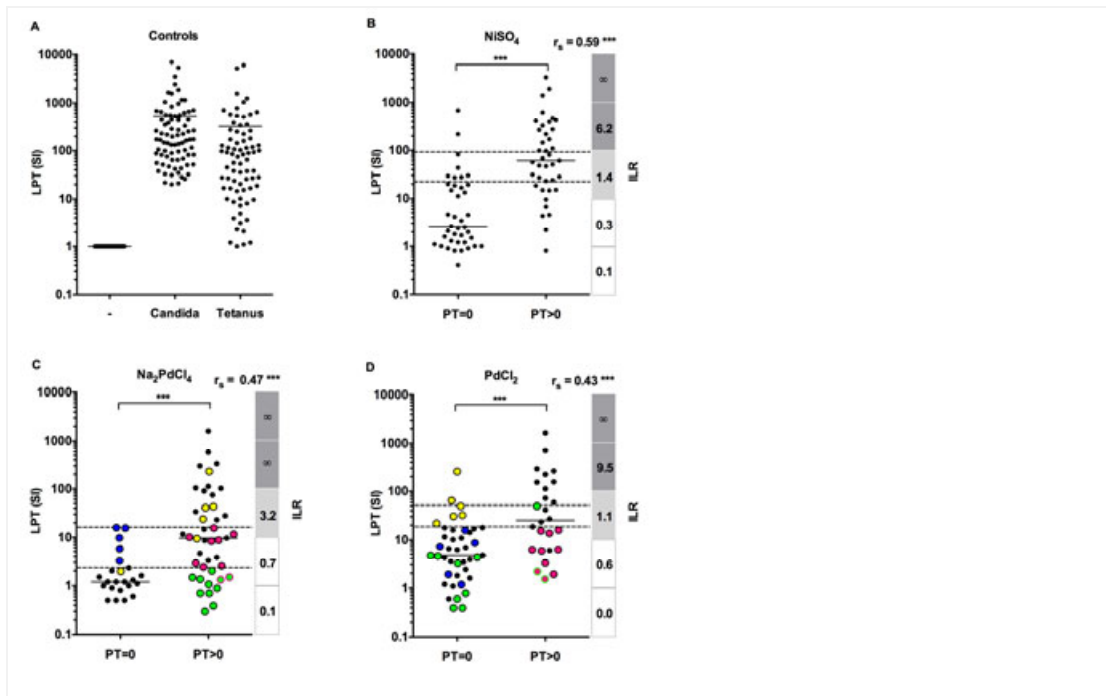


Figure 1: Lymphocyte proliferation test (LPT) results. Stimulation index (SI) of $CD3^+CD4^+$ cells without antigen (-) and stimulated with *Candida albicans* or tetanus toxoid control antigens (A). SI of $CD3^+CD4^+$ cells stimulated with $NiSO_4$ (B), Na_2PdCl_4 (C), or $PdCl_2$ (D) in individuals with negative (PT=0) and positive (PT>0) skin patch test (PT) results. For each individual, maximal SIs of LPT with different antigen concentrations are shown. Horizontal solid lines indicate median LPT values. Dotted lines represent cutoff values calculated by receiver-operating characteristic (ROC) analysis for maximal sensitivity and specificity (lower lines) or 95% specificity (upper lines). Individuals with negative PT to $PdCl_2$, but positive LPT to $PdCl_2$ are highlighted in yellow. Individuals with positive PT to $PdCl_2$, but negative LPT to $PdCl_2$ are highlighted in red. Individuals with negative PT to Na_2PdCl_4 , but positive LPT to Na_2PdCl_4 are highlighted in blue. Individuals with positive PT to Na_2PdCl_4 , but negative LPT to Na_2PdCl_4 are highlighted in green. Interval likelihood ratio (ILR) values (right, y-axis) for different LPT SI intervals (left, y-axis) are shown (B-D). Statistical significance of the differences between SIs of individuals with PT=0 and PT>0 was calculated by the Wilcoxon rank sum test. Spearman's rank correlation coefficient (r_s) between LPT and PT is given in the upper right corner of graphs. The level of significance is indicated (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

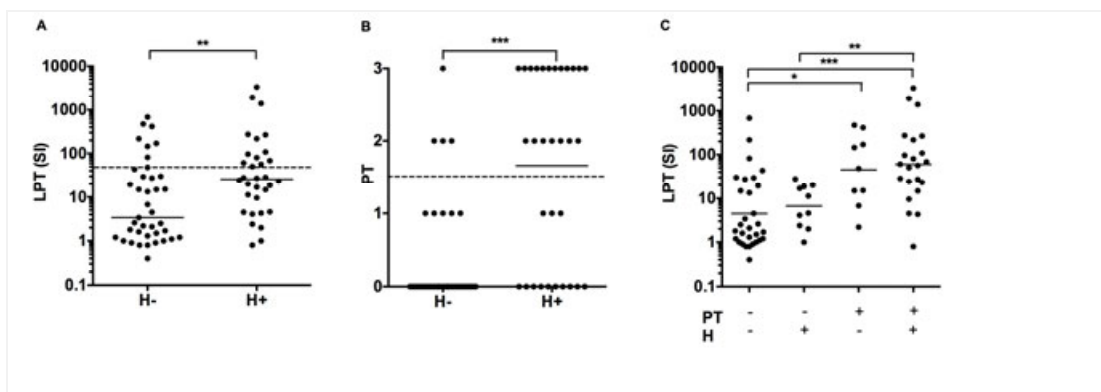
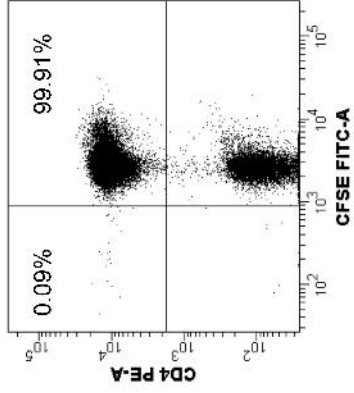
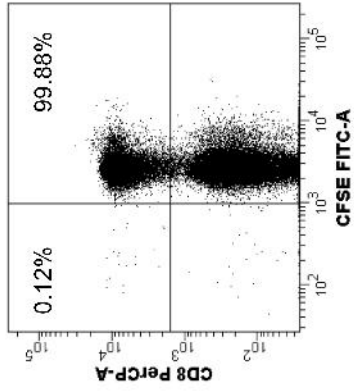
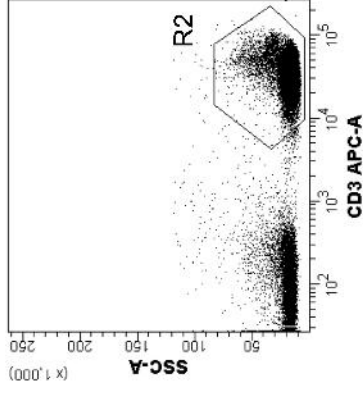
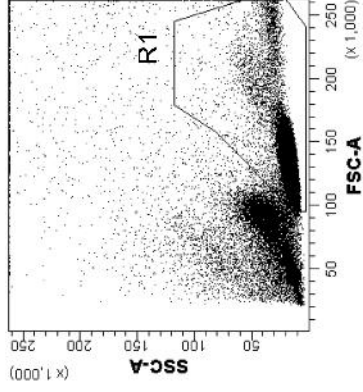


Figure 2: Lymphocyte proliferation test (LPT) results (A) and skin patch test (PT) results (B) in individuals with a conclusive medical history positive (H+) or negative (H-) for nickel (Ni) sensitization. LPT results in individuals with a negative or positive PT result and a negative or positive medical history (C). For each individual, maximal PT responses (no reaction (0), 1+ reaction (1), 2+ reaction (2) or 3+ reaction (3)) and maximal stimulation indices (SIs) of CD3⁺CD4⁺ cells in LPT with different NiSO₄ concentrations are shown. Horizontal solid lines indicate median LPT and PT values. Dotted lines represent cutoff values calculated by receiver-operating characteristic (ROC) analysis for maximal sensitivity and specificity. Statistical significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) was calculated by the Wilcoxon rank sum test (A and B) or the Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon rank sum test (C).

Control



Nickel

