

Quantitative, Absolute Count–Based T-Cell Analysis of CD69 Upregulation as a New Methodology for In Vitro Diagnosis of Delayed-Type Hypersensitivity Reaction to Nickel

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■ Abstract

Background: T cells play a major role in delayed-type hypersensitivity reactions. Their reactivity can be assessed by measuring the upregulation of the activation marker CD69, followed by assessment of proliferation and cytokine production. The aim of our study was to develop a novel, whole blood–based, quantitative, absolute count activation index (AI) for analysis of CD69 upregulation in various subsets of T cells in nickel-hypersensitive patients and compare it with previously reported approaches.

Methods: The study population comprised 10 patients with nickel allergy and 9 healthy controls. CD69 expression of CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells in heparinized blood was determined with flow cytometry after incubation with nickel sulfate for 48 hours. The absolute count of CD69⁺ cells was determined using microbeads. Production of the cytokines IL-2, IL-5, IL-13, and IFN- γ was determined after stimulation of peripheral blood mononuclear cells with nickel sulfate for 48 hours.

Results: We showed absolute AI to be the most sensitive approach. The index was calculated as the ratio of the absolute count of nickel-stimulated CD69-positive T cells to the absolute count of CD69-positive T cells in nonstimulated blood. This novel quantitative approach was more discriminative than previously reported approaches in which the T-cell CD69 percentage AI and cytokine production are measured.

Conclusions: Our results demonstrated that measuring the absolute CD69 AI is a novel and accurate approach for quantification of antigen-specific T cells in the blood of patients with hypersensitivity reactions to nickel. This approach may be useful for better in vitro assessment of patients with delayed-type hypersensitivity reactions.

Key words: Delayed-type hypersensitivity. Nickel. Whole blood. CD69.

■ Resumen

Antecedentes: Los linfocitos T juegan un papel importante en las reacciones de hipersensibilidad de tipo retardado. Su actividad puede evaluarse midiendo la expresión del marcador de activación CD69, seguido de la proliferación y la producción de citocinas. El objetivo de nuestro estudio ha sido el desarrollar un novedoso análisis cuantitativo del índice de activación absoluto (AI) en sangre completa de la expresión de CD69, en diferentes subconjuntos de linfocitos T, en pacientes con hipersensibilidad al níquel, y compararlo con los métodos existentes.

Métodos: Se estudiaron diez pacientes con alergia al níquel y nueve controles sanos. La expresión de CD69 de los linfocitos T CD3⁺, CD3⁺CD4⁺ y CD3⁺CD8⁺ en sangre heparinizada se determinó con citometría de flujo, después de una incubación con sulfato de níquel durante 48 h. El recuento absoluto de células CD69⁺ se determinó con microesferas. La producción de las citocinas IL-2, IL-5, IL-13 e IFN- γ se cuantificó después de la estimulación de células mononucleares periféricas, durante 48 h, con sulfato de níquel.

Resultados: Se demuestra que la determinación del índice AI absoluto es la metodología más sensible. Se calculó como la relación entre el recuento absoluto de linfocitos T CD69-positivos estimulados con níquel y el recuento absoluto de linfocitos T CD69-positivos en sangre no estimulada. Este nuevo enfoque cuantitativo fue más discriminativo que los enfoques publicados previamente en los que se midió el porcentaje de CD69 de linfocitos T y la producción de citocinas.

Conclusiones: Nuestros resultados demostraron que la medición del AI absoluto de CD69 es un enfoque nuevo y preciso para cuantificar los linfocitos T específicos de antígeno en la sangre de pacientes con reacciones de hipersensibilidad al níquel. Este enfoque puede ser útil para una mejor evaluación *in vitro* de los pacientes con reacciones de hipersensibilidad de tipo retardado.

Palabras clave: Hipersensibilidad de tipo retardado. Níquel. Sangre completa. CD69.

Introduction

Nickel is one of the most common causes of allergic contact dermatitis and delayed-type hypersensitivity reactions [1,2]. A recent study showed that approximately 15% of the population are allergic to nickel sulfate [3]. Allergic contact dermatitis to nickel is caused by release of nickel-specific T cells followed by proliferation and production of cytokines [4,5]. More specifically, in the sensitization phase, nickel ions penetrate the skin, thus activating epithelial cells, which in turn produce various cytokines and chemokines. This phase is followed by complex immune responses resulting in activation of antigen-presenting cell (APCs), such as Langerhans cells and dendritic cells. Activated APCs migrate to lymph nodes, where they present the antigens to naïve T cells. Subsequent re-exposure to the same allergen during the elicitation phase leads to activation of antigen-specific T cells, which enter the bloodstream, where they activate, proliferate, and produce various inflammatory cytokines and chemokines at the site of exposure. These agents promote the allergic reaction and lead to development of characteristic skin lesions [6].

Nickel allergy is mainly diagnosed using patch testing or the in vitro lymphocyte transformation test, which exploits the proliferative potential of antigen-specific T cells [7-11]. However, given that the lymphocyte transformation test imposes limitations in terms of using radioactive isotopes, there is a need for other methods that are easier to implement. Therefore, CD69 has been identified as the earliest marker of T-lymphocyte activation [12]. An alternative approach, the lymphocyte activation test (LAT), comprises flow cytometry analysis of in vitro CD69 upregulation in T lymphocytes [12-14]. LAT studies have applied analysis of the percentage activation index (AI), which is the ratio of the percentage

of CD69-positive cells stimulated with nickel sulfate to the percentage of CD69-positive cells stimulated with culture medium only. An AI >2 is considered the threshold for positivity [13,15]. However, the main limitation of this approach is that the difference between the percentage of CD69-positive cells stimulated with a test substance and the percentage of CD69-positive cells in nonstimulated blood is often small (<2-fold). This percentage-based AI also depends on the percentage ratio of other lymphocyte populations and frequently results in an unclear interpretation (AI slightly greater than 2), with no clear distinction between positive and negative results. These limitations could be overcome with a more precise and specific measurement of the absolute number (cells per μL) of antigen-specific CD69-positive T cells following in vitro antigen stimulation in whole blood.

The aim of our study was to develop a novel, whole blood-based, quantitative, absolute count AI for analysis of CD69 upregulation in different subsets of T cells in nickel-hypersensitive patients and compare it with the previously reported method in which the CD69 percentage AI and cytokine production are measured [16-20].

Methods

Study Population and Blood Collection

The study population comprised 10 patients with a clinical history of hypersensitivity to nickel. Eight of the 10 patients had a positive patch test result to nickel sulfate (7 +++ and 1 ++) [21]. Nine healthy controls with no previous evidence of nickel allergy were also included. Before recruiting patients/healthy controls, 9 controls were tested for quantitative, absolute count-based analysis of CD69 upregulation after

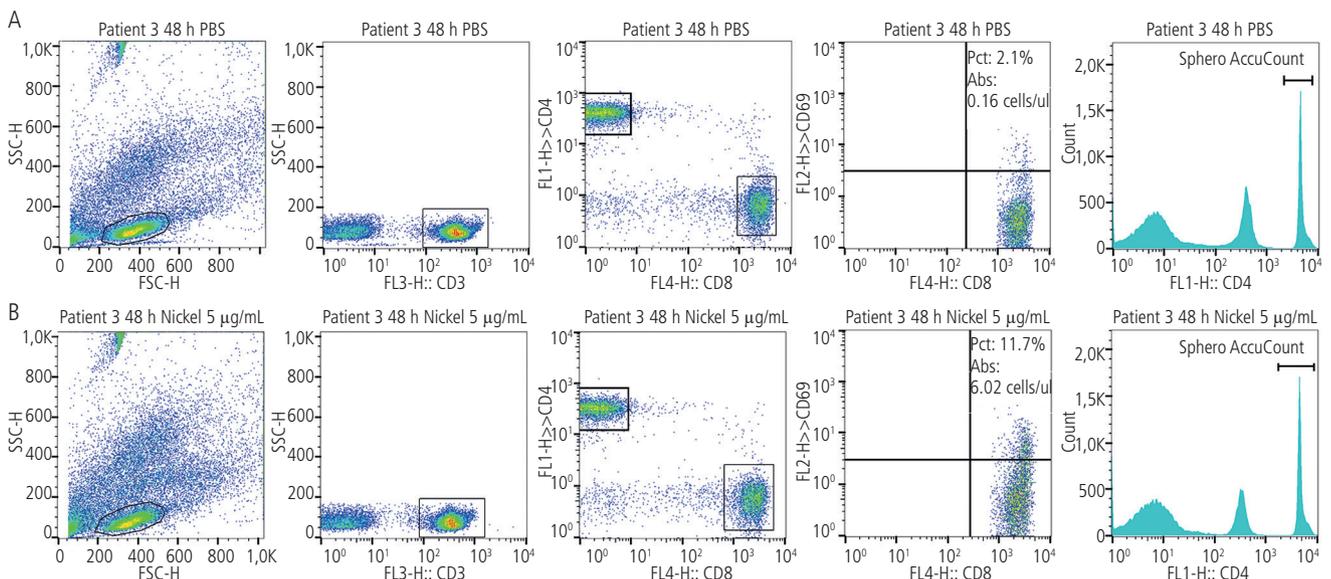


Figure 1. Representative flow cytometry analysis in absolute count-based and percentage-based T-cell analysis of CD69 upregulation in CD3⁺CD8⁺ cells for 1 patient after 48 hours of stimulation of whole blood with (A) PBS or (B) 5 $\mu\text{g/mL}$ nickel sulfate. The absolute cell count was determined with SPHERO AccuCount Fluorescent Particles, which were added to antibody-labeled blood samples prior to flow cytometry analysis.

stimulation with phytohemagglutinin (PHA), which served as a positive and methodological control of the novel test approach. Whole blood samples were collected in heparinized blood tubes (BD Vacutainer) and were processed within 1 hour.

Measurement of CD69 Activation

The next step involved incubation of 225 μ L of heparinized whole blood with 25 μ L of nickel sulfate hexahydrate (Sigma Aldrich) at a final concentration of 0.5 or 5 μ g/mL or 25 μ L in phosphate-buffered saline (PBS) as a negative stimulation control. For the positive control, the same amount of heparinized blood was stimulated with PHA (Sigma Aldrich) at a final concentration of 20 μ g/mL. All samples were incubated for 48 hours at 37°C in 5% CO₂. Next, CD69 expression of CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells was determined using flow cytometry from 50 μ L of stimulated/nonstimulated blood and antihuman CD4-FITC, CD3-PerCP, CD8-APC, and CD69-PE (all BD Biosciences). The absolute cell count was determined with SPHERO AccuCount Fluorescent Particles (Spherotech). Whole blood probes were lysed, washed, fixed, and analyzed within 2 hours on a FACSCalibur flow cytometer (BD Biosciences). Moreover, 15 000 events were acquired in forward and side scatter lymphocyte gates in each tube analyzed. Figure 1 shows representative flow data and gating strategy from the absolute cell count assay. The percentage AI (Pct AI) was calculated as the ratio of the percentage of CD69-positive CD3⁺, CD3⁺CD4⁺, or CD3⁺CD8⁺ T cells stimulated with nickel sulfate to the percentage of the corresponding CD69 cells in nonstimulated blood (PBS alone). The absolute AI (Abs AI) was calculated as the ratio of the absolute number of CD69-positive CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells stimulated with nickel sulfate to the absolute number of the corresponding CD69 cells from nonstimulated blood.

Measurement of Cytokine Secretion

Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Paque density gradient, which was washed twice with PBS and resuspended in cell culture medium consisting of RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM of L-glutamine, and 1% penicillin/streptomycin (all Sigma Aldrich). PBMCs were seeded at a concentration of 2×10^5 cells per well and

incubated with nickel sulfate hexahydrate (Sigma Aldrich) at a final concentration of 0.5 or 5 μ g/mL or cell culture medium as negative stimulation control for 48 hours at 37°C in 5% CO₂. Four cytokines, namely, IL-2, IL-5, IL-13, and IFN- γ , were quantified in the supernatants using the cytometric bead array system (BD Biosciences) according to the manufacturer's protocol. The results were normalized to nonstimulated (cell culture medium) cytokine secretion. Samples were analyzed within 2 hours on a FACSCalibur flow cytometer (BD Biosciences).

Statistical Analysis

The Mann-Whitney test was used to compare AIs and cytokine responses between patients and controls. Receiver operating characteristic (ROC) curve analysis was used for the flow cytometric assay of CD69 upregulation or cytokine production to predict clinical history. We used the nickel sulfate concentration of 5 μ g/mL for statistical analysis. A *P* value below .05 was considered significant. All statistical analyses were carried out using GraphPad Prism software (version 5). All reported *P* values are 2-tailed.

Results

Lymphocyte Activation Test

The CD69 Pct AI (percentage-derived) in response to 5 μ g/mL nickel sulfate was significantly higher in patients than in healthy controls for CD3⁺ cells (median 2.62 vs 0.96; *P*=.002), CD3⁺CD4⁺ cells (median 4.17 vs 1.00; *P*=.006), and CD3⁺CD8⁺ cells (median 1.73 vs 0.95; *P*=.0002). The median increase in Pct AI was 2.73-fold for CD3⁺ cells, 4.17-fold for CD3⁺CD4⁺ cells, and 1.82-fold for CD3⁺CD8⁺ cells.

The CD69 Abs AI (absolute count-derived) in response to 5 μ g/mL nickel sulfate was significantly higher in patients than in healthy controls for CD3⁺ cells (median 6.93 vs 0.92; *P*=.0004), CD3⁺CD4⁺ cells (median 14.59 vs 0.93; *P*<.0001), and CD3⁺CD8⁺ cells (median 3.14 vs 1.03; *P*<.0001). The median increase in Abs AI was 7.53-fold for CD3⁺ cells, 13.66-fold for CD3⁺CD4⁺ cells, and 3.04-fold for CD3⁺CD8⁺ cells. Flow cytometry Pct and Abs data are presented in Table 1.

Table 1. Flow Cytometry Percentage and Absolute Data for CD69 Measurements After Stimulation (0.5, 5 μ g/mL) or No Stimulation (PBS) of Whole Blood With Nickel Sulfate for 48 Hours

	PBS, Median (IQR)	0.5 μ g/mL Nickel Sulfate, Median (IQR)	5 μ g/mL Nickel Sulfate, Median (IQR)
CD69-Positive Cells, %			
CD3 ⁺	0.975 (0.600-1.418)	1.020 (0.698-1.590)	2.695 (1.193-5.640)
CD3 ⁺ CD4 ⁺	0.350 (0.258-0.448)	0.435 (0.205-1.043)	1.850 (0.673-5.135)
CD3 ⁺ CD8 ⁺	1.645 (1.298-2.218)	1.805 (1.370-2.203)	2.855 (2.433-5.948)
Absolute Count of CD69-Positive Cells/ μ L			
CD3 ⁺	0.141 (0.044-0.252)	0.112 (0.079-0.308)	1.322 (0.050-5.013)
CD3 ⁺ CD4 ⁺	0.018 (0.008-0.076)	0.044 (0.009-0.131)	0.488 (0.171-3.080)
CD3 ⁺ CD8 ⁺	0.158 (0.044-0.317)	0.141 (0.072-0.273)	1.200 (0.232-4.663)

Abbreviation: PBS, phosphate-buffered saline.

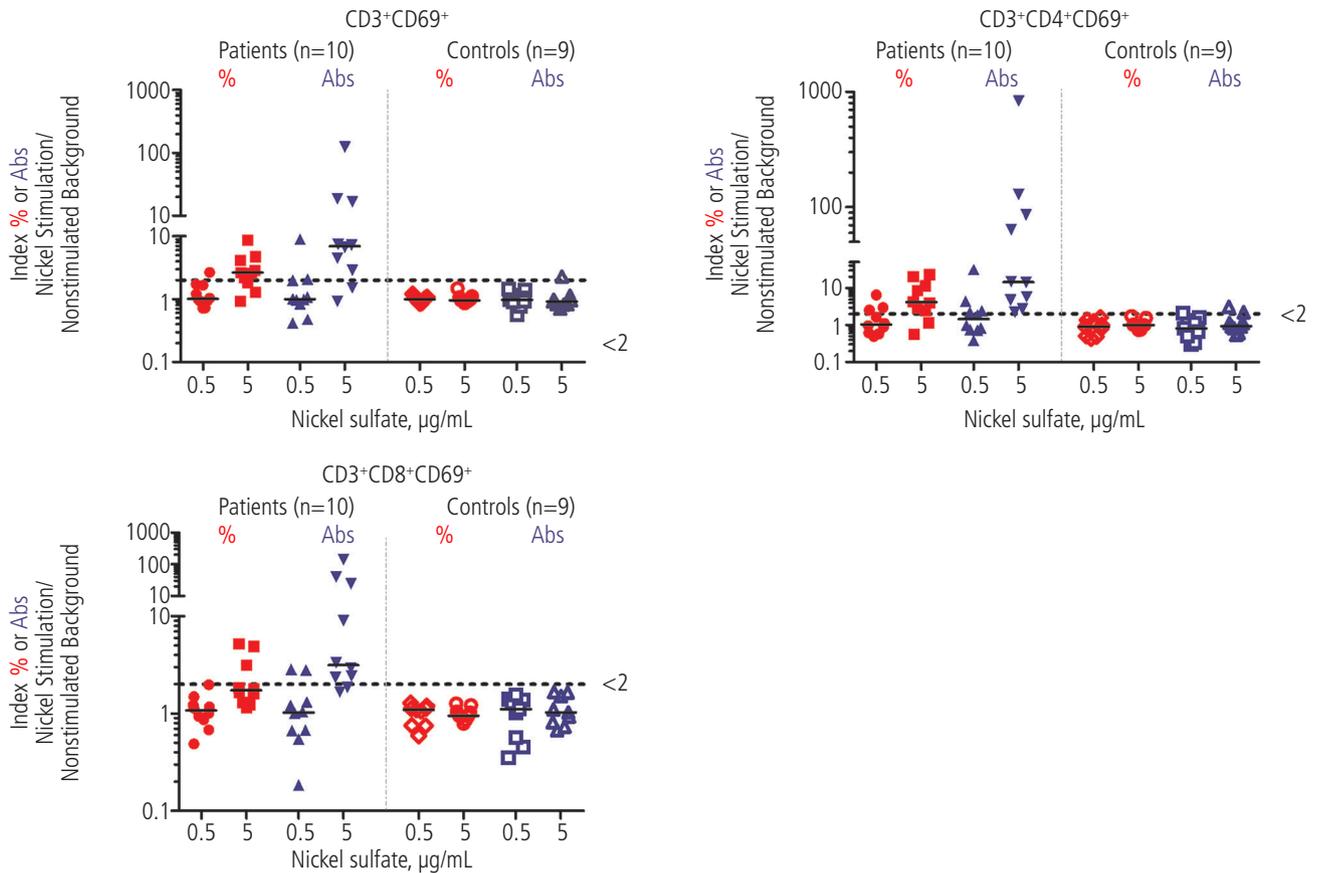


Figure 2. CD69 activation in CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells after stimulation of whole blood with nickel sulfate (0.5 and 5 µg/mL) for 48 hours. The activation index is expressed as either a percentage (red) or absolute count (blue). AI indicates activation index; Pct, percentage; Abs, absolute.

Given a previously accepted threshold of AI > 2 for positivity [15], 7/10 (70%) patients were CD3⁺CD69⁺, 8/10 (80%) were CD4⁺CD69⁺, and 3/10 (30%) were CD8⁺CD69⁺ when Pct AI was applied. In comparison, 8/10 (80%) patients were CD3⁺CD69⁺, 10/10 (100%) were CD4⁺CD69⁺, and 8/10 (80%) were CD8⁺CD69⁺ when Abs AI was applied (Figure 2).

ROC analysis demonstrated an AUC of 0.91 for CD3⁺CD69 Pct AI and an AUC of 0.94 for CD3⁺CD69 Abs AI. We found an AUC of 0.87 for CD3⁺CD4⁺CD69 Pct AI, an AUC of 0.98 for CD3⁺CD4⁺CD69 Abs AI, an AUC of 0.97 for CD3⁺CD8⁺CD69 Pct AI, and an AUC of 0.9 for CD3⁺CD8⁺CD69 Abs AI (Figure 3).

The best diagnostic cut-off values for CD3⁺CD4⁺CD69⁺ Abs AI were obtained at 2.24 with 100% sensitivity and 89% specificity and for CD3⁺CD8⁺CD69⁺ Abs AI at 1.76 with 90% sensitivity and 100% specificity (Table 2).

In addition, strong CD69 positivity in all T-cell subsets was demonstrated for all 9 controls after stimulation of whole blood with PHA for 48 hours. Median CD69 Abs AI in response to 20 µg/mL PHA was 2650.0 (range, 236.3-9441.0) in CD3⁺ cells, 10031.0 (204.6-31629.0) in CD3⁺CD4⁺ cells, and 1126.0 (47.3-2037.0) in CD3⁺CD8⁺ cells. Median CD69 Pct AI was 52.3 (17.4-95.7) in CD3⁺ cells, 108.4 (14.7-182.7) in CD3⁺CD4⁺ cells, and 33.3 (7.7-46.8) in CD3⁺CD8⁺ cells, respectively (Supplementary Figure 1).

Cytokine Secretion Measurements

The 4 cytokines measured were IL-2 and IFN- γ , which are specific for the T_H1 immune response, and IL-5 and IL-13, which are specific for the T_H2 immune response. We found significantly increased secretion of IL-2 (median 182 vs 3 ng/mL; $P=0.0004$), IL-5 (median 9 vs 0 ng/mL; $P=0.0025$), and IL-13 (median 36 vs 0.5 ng/mL; $P=0.0004$) in response to 5 µg/mL nickel sulfate in patients compared with healthy controls. Moreover, cytokine secretion in patients was concentration-dependent. In contrast, no difference was found for IFN- γ (median 2 vs 0 ng/mL; $P=0.34$) (Figure 4).

Discussion

In this study, we present a novel, whole blood, quantitative, absolute count-based T-cell analysis of CD69 upregulation as a new methodology for in vitro diagnosis of delayed-type hypersensitivity to nickel. The test has better diagnostic value than previously reported analyses based on Pct AI. Importantly, our Abs AI analysis showed much larger differences between stimulated and nonstimulated samples than Pct AI analysis for all subsets of T cells (CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺). We also compared our results for activation of CD69 upon secretion of cytokines (IL-2, IL-5, IL-13, and IFN- γ) and found increased secretion of IL-2, IL-5, and IL-13. These

observations suggest that absolute counting of antigen-specific, CD69-positive T cells could be a novel approach in the diagnosis of delayed-type hypersensitivity reactions.

CD69 is the earliest marker of activation of T cells in vivo and in vitro and is detectable within hours of binding to the T-cell receptor [22]. Our study of CD69 measurement suggests that

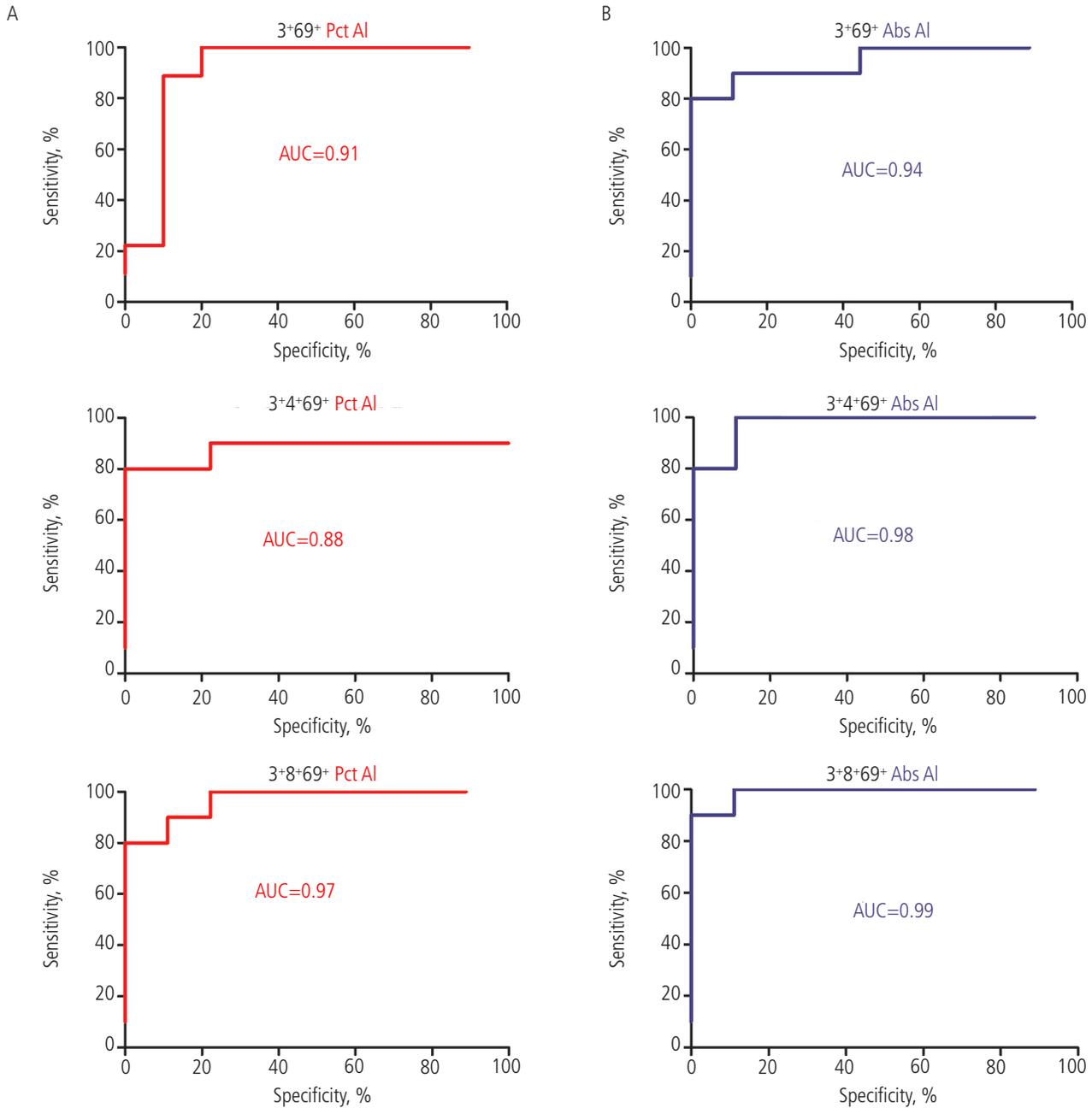


Figure 3. ROC analysis of the CD69 activation index of CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells after stimulation with 5 µg/mL nickel sulfate for 48 hours. The activation index is expressed either as A, a percentage (red) or B, an absolute count (blue). AUC indicates area under the ROC curve; AI, activation index; Pct, percentage; Abs, absolute.

Table 2. Optimal Cut-offs for the Parameters of CD69 Upregulation After Stimulation of Whole Blood With Nickel Sulfate (5 µg/mL) for 48 Hours

Parameters	AUC ROC	Cut-off	Sensitivity, %	Specificity, %
CD3 ⁺ CD69 ⁺ Abs AI	0.94	1.33	90.00 (55.50-99.75)	88.89 (51.75-99.72)
CD3 ⁺ CD4 ⁺ CD69 ⁺ Abs AI	0.98	2.24	100.00 (69.15-100.0)	88.89 (51.75-99.72)
CD3 ⁺ CD8 ⁺ CD69 ⁺ Abs AI	0.99	1.76	90.00 (55.50-99.75)	100.00 (66.37-100.0)

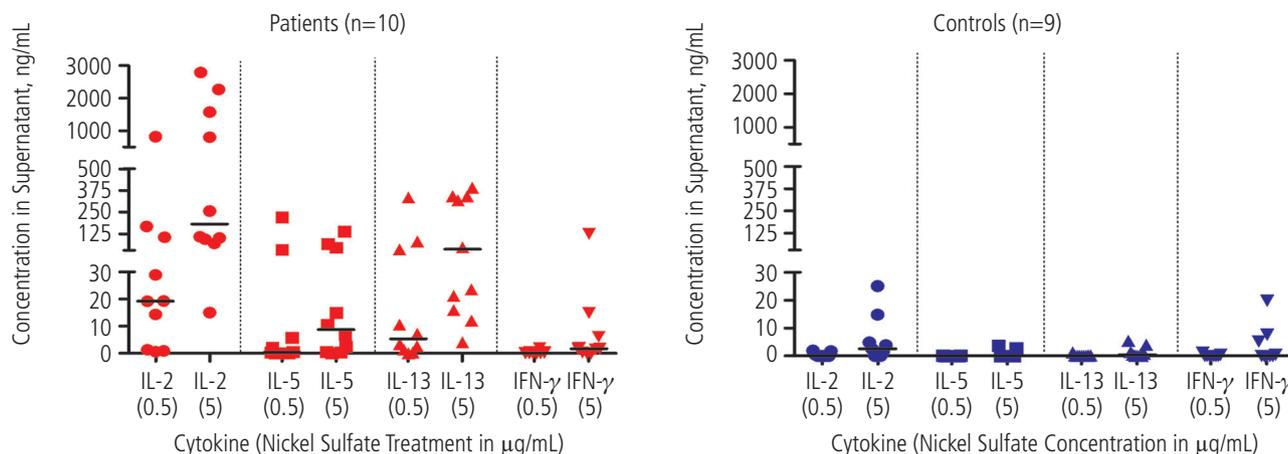


Figure 4. IL-2, IL-5, IL-13, and INF- γ cytokine expression after stimulation of PBMCs with nickel sulfate (0.5 and 5 $\mu\text{g/mL}$) for 48 hours.

between 0.6% and 10.0% of CD3⁺ T cells reacted to stimulation with nickel, consistent with the results of Beeler et al [15], who reported between 0.5% and 3% drug-reactive CD3⁺CD4⁺ T cells. In contrast to other studies of CD69 upregulation on T cells [12,13,15], our approach utilizes whole blood and therefore does not involve isolation of PBMCs, which is time consuming and does not allow for exact cellular quantification and absolute counting of drug-activated T cells. Importantly, 48-hour incubation of nonstimulated heparinized whole blood resulted only in minimal activation of T-cell subsets (approximately 1% for CD3⁺, 0.3% for CD3⁺CD4⁺, and 1.5% for CD3⁺CD8⁺).

To normalize for background activation, the CD69 results are analyzed as the AI, which is calculated as activation in the presence of the tested substance (stimulated) divided by activation in the absence of the tested substance (nonstimulated). Previous CD69 T-cell studies of delayed-type hypersensitivity used Pct AI values [13,15]. The novelty of our study is the use of Abs AI values, which showed higher AUC and diagnostic utility than Pct AI for all analyzed T-cell subsets. The main advantage of the Abs AI approach is the much larger differences between stimulated and nonstimulated samples in hypersensitive patients, with comparable AI values observed in controls. Consequently, there is greater discrimination between patients and controls. Accordingly, we showed a median 7.5-fold increase in CD3⁺ in patients compared with controls when Abs AI was applied and only 2.9-fold when Pct AI was applied. Similar differences were also observed for the CD3⁺CD4⁺ and CD3⁺CD8⁺ subsets.

An AI of 2 was previously accepted as the cut-off value for discriminating between nonreactive and reactive T cells in CD69 LAT testing [15]. According to our ROC analysis, a very similar cut-off was also demonstrated for Abs AI, indicating that a cut-off of >2 might also be suitable for Abs analysis. It seems that CD3⁺CD8⁺CD69⁺ Abs AI and CD3⁺CD4⁺CD69⁺ Abs AI have the highest diagnostic accuracy. This observation also suggests that both helper and cytotoxic T cells contribute to the hypersensitivity reaction to nickel. A previous report failed to detect a change in CD69 upregulation in CD4⁺CLA⁺ T cells from nickel-hypersensitive patients [14]. However, the authors used a less sensitive method to detect CD69-positive, antigen-specific T cells than the approach we used.

We also demonstrated increased secretion of IL-2, IL-5, and IL-13 in nickel-hypersensitive patients. This result is comparable with the result of Minang et al [23], who also demonstrated IL-13 secretion, and suggests that nickel-induced cytokine production in PBMCs could be used as a marker of nickel hypersensitivity [23]. These observations indicate that both T_H1 and T_H2 cytokines may contribute to delayed-type hypersensitivity reactions to nickel. In contrast to our data and those of Minang et al, some previous studies suggested that nickel-induced allergic contact dermatitis is mediated mainly by T_H1-type cytokines [24,25], whereas T_H2-type cytokines and IL-10 could have downregulatory effects [4,26].

Our study is subject to a series of limitations, which may hamper interpretation of the results. The main limitation applies to the small number of persons/samples involved. Therefore, larger studies to confirm the methodological and clinical utility of this approach are needed. In addition, the specificity and sensitivity of this novel method should also be determined in patients with equivocal patch test results. Baseline data on CD69 activation may also be affected by time since last nickel exposure: this was a prospective study and all patients had a recent history (median 1 year) of nickel allergy and were tested for CD69 activation shortly after the initial clinical diagnosis and patch testing (median 6 months). Accordingly, baseline CD69 counts were comparable between patients.

In conclusion, our results point to the potential of quantitative, microbead-based T-cell analysis of CD69 upregulation to be an accurate new approach for identification of antigen-reactive T cells in the peripheral blood of patients with nickel hypersensitivity reactions. Abs AI seems to be more discriminative than Pct AI and complements analysis of cytokine secretion. These findings suggest the sufficient diagnostic utility and technical reproducibility of CD69 Abs AI analysis. Our findings should be further assessed in validation and replication studies, especially in clinically complex patients with hypersensitivity reactions to drugs.

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Conflicts of Interest

The authors declare they have no conflicts of interest.

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