

ELISpot: a new tool for the detection of nickel sensitization

M. Lindemann*, J. Böhmer†, M. Zabel† and H. Grosse-Wilde*

*Institute of Immunology, University Hospital Essen, †Department of Dermatology, Knappschafts Krankenhaus Recklinghausen, Germany

Summary

Background The standard assay for the clinical detection of contact allergy against nickel is the patch test. For research purposes, the *in vitro* lymphocyte transformation test (LTT) is mostly applied. However, the *in vivo* application of allergens includes the potential of sensitization, whereas the LTT demands 6 days of cell culture and radioactive labelling procedures.

Objective The purpose of this study was to validate an enzyme-linked immunospot (ELISpot) assay to specifically detect cellular response against nickel sulphate in correlation with patch test, anamnestic data, and LTT results.

Methods In probands with positive ($n = 60$) and negative patch test results ($n = 19$), cytokine ELISpot and LTT were performed in parallel using various concentrations of nickel sulphate, various numbers of responding peripheral blood mononuclear cells (PBMC), and various durations of cell cultures.

Results Concentrations of 50 and 75 μM nickel sulphate were found to be optimal to stimulate in the ELISpot 4×10^5 and in the LTT 2×10^5 PBMC, respectively. An increase in ELISpot sensitivity was reached by pre-incubation with nickel sulphate for 24 h prior to transfer to ELISpot plates. In nickel-sensitive probands, an average precursor cell frequency of 19×10^5 , 1.7×10^5 , and 0.7×10^5 could be defined for IFN- γ , IL-2, and IL-4 producing PBMC, respectively. In non-sensitive probands IFN- γ producing cells were detectable, but with significantly lower frequency (2×10^5 ; $P = 0.004$). The prior performance of patch tests had no significant effect on cytokine production or lymphocyte proliferation. Overall, the parameters patch test, anamnestic nickel allergy, ELISpot, and LTT results were positively correlated ($P < 0.05$).

Conclusions Based on these experiments, the quantitative *in vitro* detection of cell-mediated reactions towards nickel as well as other heavy metal ions should be possible utilizing the above-described ELISpot assay.

Keywords ELISpot, *in vitro* cytokine production, *in vitro* lymphocyte proliferation, nickel allergy, patch test

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Introduction

Nickel sulphate is the most frequent cause of allergic contact dermatitis, determined by patch test at a frequency of 13% in the German population [1, 2], and expected in the general adult population in 10% [1]. Women are over-represented in patch test-positive individuals with a frequency of 20% as compared to 6% in men [1]. Exposure to fashion jewelry is hypothesized as a major cause for this sex-related difference, and an increased susceptibility to contact sensitization in women is discussed controversially [3, 4]. Patch tests are frequently applied for clinical diagnostics of metal sensitization; for research purposes, the LTT is used to detect nickel-specific lymphocyte proliferation [5–15]. Comparing this cellular *in vitro* test with the patch test, an increase in sensitivity of up to 30% was described [16].

Another *in vitro* method for the identification of cellular immune reactions is the ELISpot, allowing the measurement of cytokine production at the single cell level [17]. Independently from our study, Jakobson et al. [15] very recently described an ELISpot assay using nickel chloride to stimulate cytokine production in a cohort of 20 probands. Owing to the low number of individuals tested, a correlation with established methods to detect nickel sensitization could hardly be determined.

The diagnosis of metal sensitization could not only be relevant for dermatologists, but also for cardiologists, angiologists, or surgeons because allergic reactions against metals are proposed to be a risk factor for in-stent restenosis and reactions to metal skin clips could result in delayed wound healing [18, 19]. In selected cases, for example, in patients suffering from additional dermatological diseases (e.g. eczema) or patients with extreme hairiness, in which skin test results are difficult to interpret, a nickel-specific ELISpot – performed by specialized laboratories – could offer an additional diagnostic tool. Compared with the patch test, the

Correspondence: H. Grosse-Wilde, Institute of Immunology, University Hospital Essen, 45122 Essen, Germany.
E-mail: immunologie@uni-essen.de

ELISpot is more convenient for longitudinal analysis and does not sensitize the proband against test substances. Therefore, it should be a suitable test, especially for monitoring in clinical studies, e.g. on the desensitization/tolerance induction by oral nickel application.

Here, we present our data on nickel-specific cellular *in vitro* responses in probands with positive and negative patch test results. We established and utilized IFN- γ , IL-2, and IL-4 ELISpots for the detection of nickel sensitization, and optimized the assay conditions for the LTT. In addition, we show that the results of the patch test, the anamnestic data, and cellular *in vitro* tests are significantly correlated.

Materials and Methods

Study subjects

In total, 79 patients and medical staff members (65 female, 14 male, mean age 41 years, range 14–78) from the Department of Dermatology, Knappschafts Krankenhaus Recklinghausen, were asked for clinical signs of nickel allergy and examined by patch and cellular *in vitro* tests. Heparinized blood samples were procured after informed consent. From patch test positive and negative probands, different numbers of PBMC and different concentrations of nickel sulphate were studied to define the optimal assay conditions for ELISpot and LTT. These *in vitro* assays were performed before and 1 week after skin testings to study the influence on cellular immunity. Furthermore, kinetic experiments of ELISpot and LTT were performed in parallel to optimize the discrimination between nickel-allergic and non-allergic probands.

Patch testing

Nickel sulphate (5% in vaseline) was applied by Finn chambers (Epitest Ltd Oy, Tuusula, Finland) to healthy, non-inflamed backs of probands [20]. After 48, 72 and, in dubious cases, also after 96 h the reaction was classified according to the guidelines of the German Dermatological Society as negative (0), slightly positive (+), medium positive (++), or strongly positive (+++) [21]. Positive reactions were defined as the appearance of erythema, papule, and/or vesicle. A crescendo character in patch test results was considered as typical for allergic, and a decrescendo character for toxic-irritable reactions.

Nickel-specific ELISpot assay

PBMC from heparinized blood were separated by Ficoll-Hypaque™ density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) centrifugation. The ELISpot utilized 3×10^5 and 4×10^5 PBMC, respectively, with 200 μ L culture volume in MultiScreen-HA plates (MAHAS4510, Millipore, Bedford, MA, USA) for 48 h. Seven different concentrations of nickel sulphate (6, 12, 25, 50, 75, 100, and 200 μ M, i.e. 1.6–52.5 μ g/mL of nickel(II) sulphate hexahydrate 99%, Aldrich, Steinheim, Germany) were applied. Subsequent cytokine production (IFN- γ , IL-2, and IL-4) was detected by a solid phase ELISA, using a membrane-anchored first antibody and a biotinylated second antibody at concentrations of 10 and 2 μ g/mL, respectively (purified monoclonal anti-IFN- γ , clone

1-D1K, anti-human IL-2, clone 5344.111, and anti-IL-4, clone MP4-25D2, and biotinylated anti-IFN- γ , clone 7-B6-1, anti-human IL-2, clone B33-2, and anti-IL-4, clone 12.1, both IFN- γ antibodies from Mabtech, Nacka, Sweden, both IL-2 antibodies from Pharmingen, San Diego, CA, USA, the first antibody against IL-4 from Pharmingen, the second from Mabtech). Using a modified protocol of Herr et al. [22], MultiScreen plates were coated with 60 μ L of the first antibody for 2 h at 37 °C in 5% CO₂, followed by three washings with PBS. Each well was blocked with 150 μ L of RPMI 1640 (Invitrogen, Karlsruhe, Germany), supplemented with 10% of inactivated human serum pool (HSP). After 1 h at 37 °C, the medium was discarded and PBMC and nickel sulphate were added in RPMI 1640+10% HSP. After a 48 h incubation at 37 °C, the ELISpot plates were washed six times with PBS/0.05% Tween20 (PBS/Tween). Captured cytokines were detected by incubation for 2 h with 60 μ L of the second antibody diluted in PBS/0.5% bovine serum albumin (BSA, fraction V, Calbiochem, La Jolla, CA, USA) followed by six washings with PBS/Tween. Then, 100 μ L of avidin-biotin peroxidase complex (ABC Vectastain-Elite kit, Vector Laboratories, Burlingame, CA, USA) was added at a dilution of 1:100. ELISpot plates were incubated at room temperature for 1 h and washed three times with PBS/Tween and three times with PBS. After adding the substrate 3-amino-9-ethyl-carbazole (Sigma, St Louis, MO, USA), red spots appeared within 4 min. The colour reaction was stopped by washing the plates under running water. The numbers of spots were analysed by the Bioreader 2000 (Biosys, Karben, Germany).

For kinetic experiments, PBMC (4×10^5 /200 μ L culture volume) were pre-incubated 0, 24, 48, and 168 h (7 days) with 50 and 75 μ M nickel sulphate in round-bottom tubes (TC 163 160, Greiner Bio-One, Frickenhausen, Germany), then 4×10^5 PBMC per well were transferred to ELISpot plates and incubated for a further 48 h. To exclude the possibility that pre-incubation induced proliferation of PBMC before ELISpot, the formation of cell clusters and blast transformation were evaluated microscopically.

Nickel-specific lymphocyte transformation test

The lymphocyte transformation test was performed in microtitre plates with 1×10^5 and 2×10^5 PBMC per 200 μ L culture at the same nickel concentrations as for ELISpot experiments. Cell culture conditions and measurement of H3-TdR uptake followed a protocol described previously [23]. For subsequent LTT experiments in parallel to ELISpot kinetics, 2×10^5 PBMC and 50 and 75 μ M nickel sulphate were chosen as conditions yielding optimal results.

Statistical analysis

To analyse ELISpot and LTT data at different concentrations of nickel sulphate, the median of seven autologous (unstimulated) values was subtracted from each of seven nickel-specific values (cpm and spots increment, respectively). For all the other comparisons that were made, increment values were calculated by subtracting autologous from nickel-stimulated values, considering the second highest of seven values each. This value was chosen for calculations because

the mean or median value would mask a proportion of positive reactions; at the highest and lowest concentrations of nickel sulphate lymphocyte proliferation is usually on a level with autologous values – the dose–response curves display a typical bell-shaped form. H3-TdR uptake values were inserted into the formula $\text{Ln}(\text{second highest nickel-specific cpm value} - \text{background})$. The background was defined as the sum of mean and $3.29 \times \text{SD}$ of autologous values. This includes the variation of autologous proliferation (not just, e.g., three-fold of an autologous value) to evaluate low level nickel-specific values with greater sensitivity. The results were then converted into scores from 0 to 6: $\leq 0 \rightarrow 0$, $\leq 7 \rightarrow 1$, $\leq 8 \rightarrow 2$, $\leq 9 \rightarrow 3$, $\leq 10 \rightarrow 4$, $\leq 11 \rightarrow 5$, and $> 11 \rightarrow 6$. Cytokine production or lymphocyte transformation results before and after skin testing were compared by the Wilcoxon-matched pairs test, in probands with positive and negative patch test reactions by the Mann–Whitney test.

For kinetic experiments, two concentrations of nickel sulphate (50 and 75 μM) were chosen. The maximum of two increment values (median of triplicates) was considered for further calculation. At each time point, *in vitro* reactions of probands with positive and negative patch test results were compared by the Mann–Whitney test. Results of patch test were converted into a numeric scale (0, +, ++, +++ $\rightarrow$ 0, 1, 2, 3), and medical history was analysed dichotomously, i.e. negative (0) vs. positive (1). For mutual correlation analysis, Spearman's test was applied. To compare sensitivity and specificity of patch test and medical history with ELISpot or LTT, McNemar's test was used. Here, spots increment > 2 for IFN- γ or > 2 for IL-2 ELISpot, and LTT scores > 0 were defined as positive. Additionally, calculations were performed using a threshold value of > 20 instead of > 2 for IFN- γ spots increment. Differences were regarded as statistically significant at $P < 0.05$.

Results

The use of different concentrations of nickel sulphate in probands with positive patch test results ($n = 47$) yielded a dose-dependent IL-2 production and lymphocyte transformation response (Figs 1 and 2) with maximum responses at 50 and 75 μM . While IL-2 production mirrored lymphocyte transformation, IFN- γ showed no clear dose-dependence. It is obvious from the panels of Fig. 2 that the number of responding cells was positively correlated with the specific results.

In probands with negative patch test ($n = 14$), IFN- γ and IL-2 production, and lymphocyte transformation were significantly lower ($P < 0.01$) than those in positive probands (Fig. 3).

Experiments before and 1 week after patch tests ($n = 23$) showed that the *in vivo* application of nickel sulphate had no significant influence on ELISpot or LTT results. Furthermore, there was no influence of the interval after patch testing (mean 2.2 years, range 1 day – 19 years) on the cellular *in vitro* parameters (data not shown).

ELISpot experiments revealed that IFN- γ , IL-2, and IL-4 were secreted with different kinetics and that pre-incubation of 24 h enhanced the discrimination between patch test

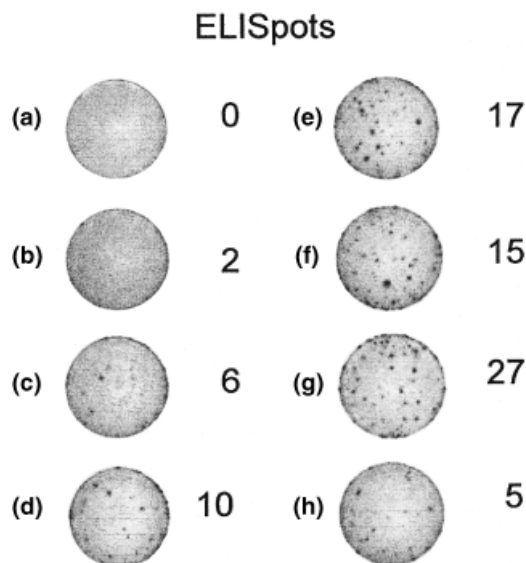


Fig. 1. IL-2 ELISpot results (3×10^5 PBMC/well) in a patch test positive proband using seven different concentrations of nickel sulphate (A: autologous control; B: 6 μM ; C: 12 μM ; D: 25 μM ; E: 50 μM ; F: 75 μM ; G: 100 μM ; H: 200 μM nickel sulphate).

positive and negative probands (Fig. 4). Based on the maximum responses, IFN- γ -producing T cells appeared with a mean frequency of 19×10^5 in nickel-reactive probands; the average numbers of IL-2 and IL-4-secreting cells were 1.7×10^5 and 0.7×10^5 , respectively. In skin test anergic probands, IFN- γ -producing cells were only detectable after pre-incubation of 48 h, and the precursor frequency was approximately eight times lower (2×10^5 ; $P = 0.004$) than that in reactive probands. Interestingly, nickel-specific IL-4 production could be observed after pre-incubation for 24 and 48 h, which was almost undetectable without pre-incubation (Fig. 4c). Microscopic analysis revealed that nickel-specific cell clusters and blast transformation started to appear after a pre-incubation period of 96 h, indicating that the frequency of cytokine producing lymphocytes following pre-incubation for 24 and 48 h was not elevated.

In a total of 61 probands after patch tests, Spearman's correlation analysis between ELISpot without pre-incubation and LTT results revealed r_s values of 0.56 for IFN- γ ($P < 0.0001$) and of 0.54 for IL-2 ($P < 0.0001$) (Table 1). Kinetic experiments indicated that pre-incubation of 48 h was optimal for IFN- γ ($r_s = 0.71$, $P < 0.0001$, Fig. 5a), and of 24 h for IL-2 production ($r_s = 0.67$, $P = 0.0005$, Fig. 5b). The nickel-specific IL-4 production and T cell proliferation after 24 h of pre-incubation were only weakly correlated ($r_s = 0.44$, $P = 0.04$). Furthermore, the most prominent r_s value of 0.74 ($P < 0.0001$) was observed for patch test and LTT (Fig. 5c). Taken together, patch test, medical history, ELISpot (especially after pre-incubation), and LTT were significantly correlated.

Using McNemar's test to compare a standard method (patch test or medical anamnesis) with ELISpot and LTT results, ELISpot without pre-incubation was significantly less sensitive ($P = 0.02$ in each case) and equally specific as compared to LTT. Considering ELISpot data after pre-incubation (48 h for IFN- γ and 24 h for IL-2), both *in vitro*

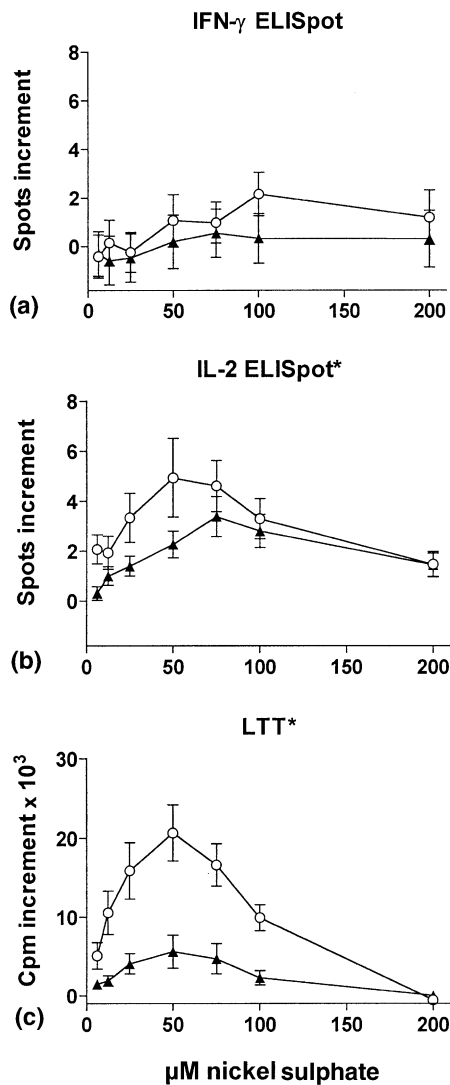


Fig. 2. Dose-response curves of ELISpot and LTT results in 47 patch test positive probands using different concentrations of nickel sulphate. In panels a and b, open circles represent 4×10^5 , filled triangles 3×10^5 PBMC/well, and in panel c 2×10^5 and 1×10^5 PBMC/well, respectively. The values are given as mean $\pm$ standard error of the mean (SEM). *Two probands not tested.

methods yielded comparable results, either using >2 or >20 spots increment as cut-off value for IFN- γ .

In four probands with a positive patch test but no known history of nickel allergy, the LTT results were significantly lower than in those with nickel allergy (0.1 ± 0.5 vs. $21.2 \pm 3.5 \times 10^3$ cpm increment, $P = 0.003$). The same trend holds for IFN- γ and IL-2 ELISpot (0.7 ± 0.7 vs. 2.8 ± 0.6 and 0.0 ± 1.8 vs. 4.6 ± 1.0 spots increment, respectively); vice versa, one patch test negative proband reported a history of nickel allergy, but nickel-specific *in vitro* tests revealed no positive reaction. Furthermore, ELISpot after pre-incubation periods as indicated for McNemar's test was positive in 87% (13/15) of the probands with positive, and in 10% (1/10) with negative patch test and anamnesis (>20 spots increment for IFN- γ were defined as positive). When setting a threshold value of >2 IFN- γ spots increment instead, the sensitivity increases to 100% but the specificity decreases to 40%. In addition, LTT was positive in 91% (41/45) of the probands

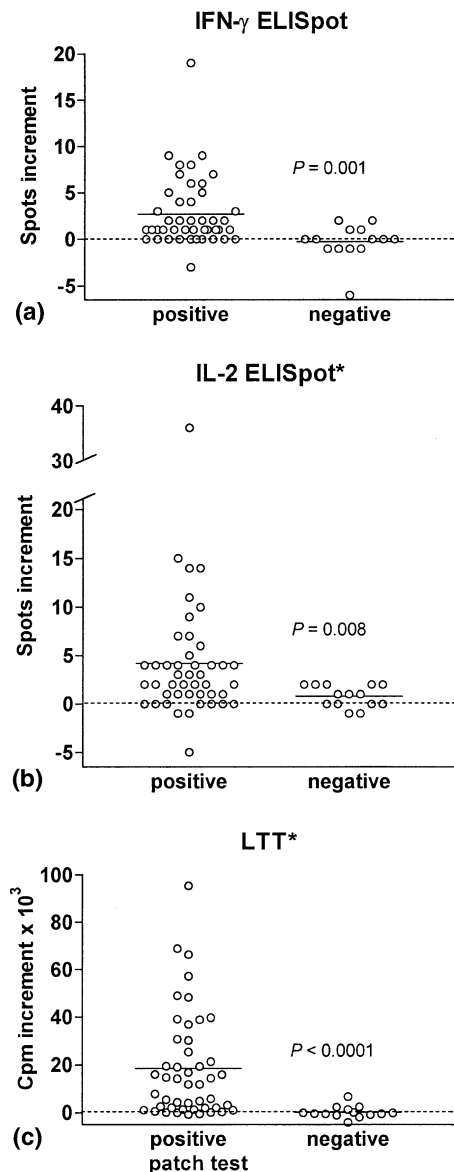


Fig. 3. ELISpot and LTT results in probands with positive ($n = 47$) and negative ($n = 14$) patch test results and their statistically significant (Mann-Whitney test) differences. For ELISpot 4×10^5 , and for LTT 2×10^5 PBMC/well were used. Horizontal lines indicate mean values for both proband groups. *Two probands not tested.

with positive and in 7% (1/14) with negative patch test results. Based on anamnesis, the LTT was positive in 95% (40/42) of allergic and in 12% (2/17) of non-allergic probands. Taken together, under optimized conditions, the sensitivity of ELISpot and LTT is 87% and 91%/95%, and the specificity 90% and 93%/88% (based on patch test/anamnesis), corresponding to positive predictive values of 93% and 98%/95% and negative predictive values of 82% and 76%/88%, respectively.

Discussion

In this report, we focused on the establishment and comparative validation of an ELISpot assay to analyse

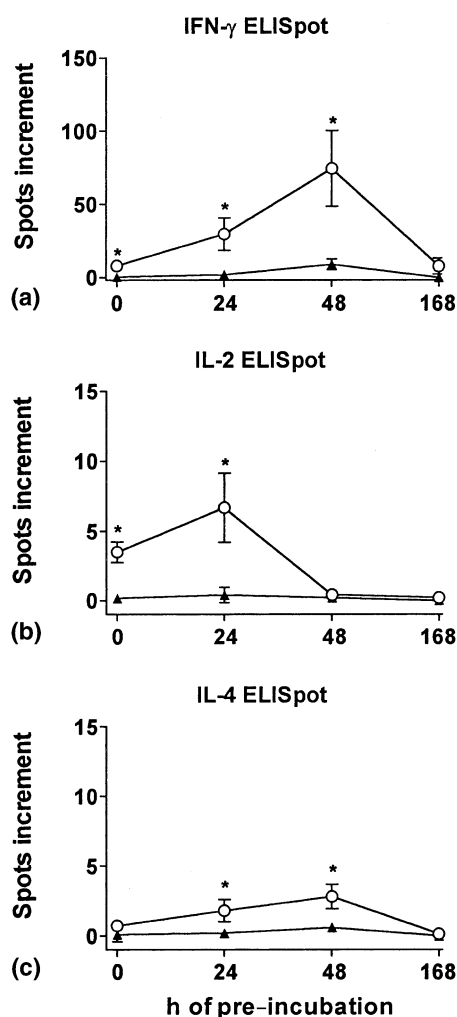


Fig. 4. Results of time kinetics for ELISpots (4×10^5 PBMC/well) in probands with positive ($n = 11$; open circles) and negative ($n = 10$; filled triangles) patch test. ELISpot results are given as mean $\pm$ SEM after the respective pre-incubation periods. * $P < 0.05$ (Mann-Whitney test).

nickel-specific cytokine production at the single cell level. As the IFN- γ , IL-2, and IL-4 ELISpot results revealed highly significant correlations with patch test results and medical history, these ELISpots are additional tools for clinical trials on nickel sensitization.

In allergic probands, the precursor frequency of nickel-specific cells detected by ELISpot is in the same range as that determined by the limiting dilution technique [24]. Using ELISpot we observed a mean precursor frequency of 19×10^5 IFN- γ producing PBMC, and Cavani et al. [24] reported a frequency of 28×10^5 for nickel-specific CD4 $^+$ cells and 5×10^5 for CD8 $^+$ T cells.

Different patterns of cytokine production, following stimulation of PBMC or T cell clones with nickel sulphate, are described. Using ELISpot, a specific increase in IFN- γ and IL-4 production was most recently reported by Jakobson et al. [15] in nickel-allergic probands. However, this group did not analyse the production of IL-2 – in our hands a very suitable cytokine. In sensitized probands, nickel-specific IL-2 secretion, but only modest or non-existent IFN- γ production of PBMC were found using ELISA [25]. Furthermore,

Table 1. Spearman correlation analysis for patch test, medical history, and cellular *in vitro* parameters.

Parameter 1	Parameter 2	Without pre-incubation		With pre-incubation*	
		r_s	P-value	r_s	P-value
Patch test	Medical history	0.73	<0.0001	0.89	<0.0001
Patch test	IFN- γ ELISpot	0.36	0.005	0.67	0.0002
Patch test	IL-2 ELISpot	0.44	0.0005**	0.65	0.001**
Patch test	IL-4 ELISpot	Not tested		0.54	0.008**
Patch test	LTT	0.74	<0.0001**	0.64	0.0006
Medical history	IFN- γ ELISpot	0.29	0.03	0.66	0.0004
Medical history	IL-2 ELISpot	0.42	0.001**	0.73	<0.0001**
Medical history	IL-4 ELISpot	Not tested		0.42	0.047**
Medical history	LTT	0.65	0.001**	0.70	<0.0001
LTT	IFN- γ ELISpot	0.56	<0.0001**	0.71	<0.0001
LTT	IL-2 ELISpot	0.54	<0.0001**	0.67	0.0005**
LTT	IL-4 ELISpot	Not tested		0.44	0.04**

*In 61 probands, ELISpot assays were performed without, and in 25 with pre-incubation (48 h for IFN- γ , 24 h for IL-2 and IL-4). For ELISpots, 4×10^5 , for parallel LTT, 2×10^5 PBMC/well were applied.

**Two probands not tested.

significant differences in IL-4 production of T cell clones or PBMC from metal-allergic vs. non-allergic probands were observed by other groups [7, 8, 26]. On the contrary, Kapsenberg et al. [27] found substantial amounts of IFN- γ and IL-2 and low levels of IL-4 production in nickel-specific CD4 $^+$ T cell clones of nickel-allergic and also non-allergic probands. This is in line with the study of Cavani et al. [24], also demonstrating similar nickel-specific responses of CD4 $^+$ but not CD8 $^+$ T cell clones. Our own data indicate that PBMC of nickel-allergic probands produce significantly higher levels of IFN- γ , IL-2, and IL-4 following specific stimulation than non-allergic ones. But IFN- γ production was also detectable in non-allergic probands, which supports the data of Kapsenberg et al. [27]. Most probably, the above differences in cytokine patterns are attributable to variable assay conditions (e.g. number of responding cells, PBMC vs. T cell clones, duration of cell culture). Our ELISpot kinetics indicate that the duration of cell culture has great impact on the quantitative pattern of the cytokines produced.

Only few authors have focused on a correlation analysis of nickel-specific LTT and patch test results or medical history; either the group of allergic probands studied was too small [11] or the proliferative responses were also observed frequently in probands with negative patch test results [6, 13]. Although unusual, we included the medical history in the analysis because a few patients report a typical anamnesis of nickel allergy but do not show positive patch test reactions and vice versa, indicating that also the 'gold standard', the patch test, could rarely have problems in detecting nickel allergy. Here, our data indicate that – after optimized assay conditions – ELISpot was positive in 87% and 10% of the probands with positive and negative patch test results and anamnesis, and LTT was positive in 91% and 7% of the probands with positive and negative patch test results, and in 95% and 12% of the probands with or without an anamnesis of nickel allergy, respectively.

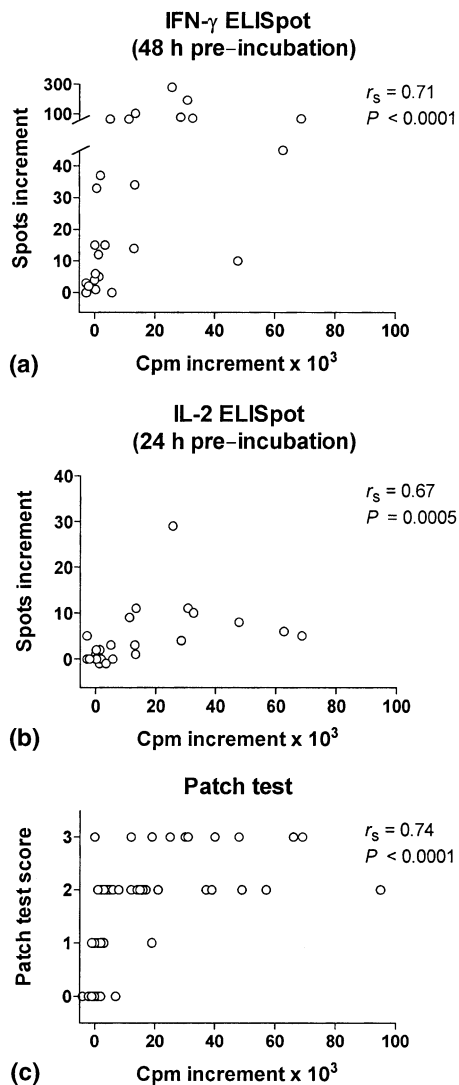


Fig. 5. Correlation analysis between ELISpot or patch test results and LTT responses. a: IFN- γ ELISpot after 48 h 'priming' and H3-TdR uptake ($n = 25$); b: IL-2 ELISpot after 24 h 'priming' and H3-TdR uptake ($n = 23$); c: Patch test scores and H3-TdR uptake ($n = 59$). Spearman's correlation analysis was applied.

The immunologic mechanisms in hypersensitivity towards metal ions have been recently reviewed [28], indicating that still many aspects of metal allergy remain unclear. Regarding the molecular interaction of metal ions with immune cells, it is suggested, that the TCR of CD4⁺ cells binds to 'metal-modified' HLA class II-peptide complexes [29, 30], or to the α - or β -chain of HLA class II heterodimers [14]. Most probably, nickel ions will bind to histidine residues of these molecules, thereby inducing a specific T cell activation.

Based on our experimental data with nickel sulphate, the detection and quantification of sensitization towards other heavy metals should also be possible utilizing the above-described ELISpot assay. This can be especially relevant for the monitoring of cellular immunity against potassium dichromate in bricklayers, where it is a common phenomenon that patch tests reveal negative results despite a typical anamnesis.

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