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CONTACT DERMATITIS

Allergic contact dermatitis to nickel: modified *in vitro* test protocols for better detection of allergen-specific response

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To date, no *in vitro* test is suitable for routine diagnosis of contact allergy. The aim of our study was to establish improved *in vitro* test protocol for the detection of antigen-specific responses of lymphocytes from patients with allergic contact dermatitis to nickel (Ni-ACD). Blood leucocytes from 14 Ni-ACD patients and 14 controls were cultured in the presence of 'cytokine cocktails' skewing lymphocytes towards 'type 1' [interferon-γ (IFN-γ)-secreting] or 'type 2' [interleukin (IL)-5 and IL-13-secreting] phenotypes. The cocktails consisted of IL-7 and, respectively, either IL-12 or IL-4. Cell responses to nickel were measured with enzyme-linked immunospot assay (ELISpot), enzyme-linked immunosorbent assay (ELISA), and lymphocyte proliferation test (LPT). Significant differences between patients with Ni-ACD and controls were found for the 'type 2' cytokines IL-13 and IL-5, with further increase of allergen-specific responses occurring when cultures were supplemented with IL-7 and IL-4. No significant differences were found for IFN-γ. The best correlate to clinical diagnosis was LPT with 'type 2' skewing ($r = 0.739$, $P < 0.001$), followed by IL-13 ELISpot with 'type 2' skewing ($r = 0.654$, $P < 0.001$). The non-radioactive method that correlated best with LPT was IL-2 ELISpot ($r = 0.809$, $P < 0.001$). Overall, we conclude that combining ELISpot assay with proposed modifications of culture conditions improves detection of specific lymphocyte responses in contact allergy to nickel.

Key words: allergic contact dermatitis; cytokine cocktails; enzyme-linked immunosorbent assay (ELISA); enzyme-linked immunospot assay (ELISpot); lymphocytes; lymphocyte proliferation test (LPT); phenotype skewing. © Blackwell Munksgaard 2007.

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For more than a century, patch tests have been the method of choice for the diagnosis of allergic contact dermatitis (ACD). Though indispensable, they have, however, certain limitations, such as interobserver variability (1), site-to-site variability (2), and test-to-test variability (3). Patch test results may be influenced by the time of reading (4, 5), quality of allergens used (6), ultraviolet irradiation (7), topical and oral steroids (8, 9). In some cases, excessive irritation of the skin makes the interpretation of patch tests difficult or impossible, a situation referred to as 'angry back' or 'excited skin syndrome' (10, 11). Taking the above limitations into account, a reliable *in vitro* test for contact allergy would be greatly appreciated.

In the past decades, various *in vitro* tests have been used for the detection of contact allergy, starting with macrophage migration inhibition test (12) and lymphocyte blastic transformation test (13). Lymphocyte proliferation test (LPT) was introduced in the 1970s (14) and has been used until today, however, mainly for experimental purposes. Later, the above-mentioned methods were followed by analyses of cytokine and chemokine secretion, surface cell markers and gene expression. Unfortunately, none of these methods have proven sufficient for diagnostic use, mainly due to poor sensitivity and/or specificity. Combinations of 2 or 3 different *in vitro* methods or parameters have been proposed to overcome this problem (15, 16), however, also this approach has not found its way into routine clinical applications.

In a previous study (17), we have shown that skewing lymphocytes towards 'type 1' [interferon-γ

(IFN-γ)-secreting cells] and 'type 2' [interleukin (IL)-5 and IL-13 secreting cells] could improve detection of nickel-specific T-cell response in contact allergy. Observations were also published suggesting that enzyme-linked immunospot assay (ELISpot) could offer advantages in detection of allergen-specific responses than other *in vitro* assays (18–20). In the present study, we combined both the above-mentioned approaches to see whether this could offer any progress to the present state of the art. The aim was to determine whether combining modified culture conditions with ELISpot assay could improve the detection of nickel-specific response *in vitro*, as compared to the previously described methods.

Patients and Methods

Combinations of particular culture conditions and *in vitro* assays are referred to as 'test protocols' in this study. The design of the study and data analysis were aimed at answering the 3 following questions: Q1 'In which test protocol the differences between patients with allergic contact dermatitis to nickel (Ni-ACD) and controls are most pronounced and significant?', and Q2 'In which test protocol the results correlate best with clinical diagnosis?'. As LPT has probably been the most popular *in vitro* assay in contact allergy for past decades, the question Q3 was 'Which non-radioactive test protocol produces results that correlate best with LPT?'.

Study group

14 female patients with confirmed Ni-ACD and 14 female controls were studied. The inclusion criteria for the Ni-ACD group were clear history of metal dermatitis and an at least ++ patch test reaction to 5% $NiSO_4$ petrolatum (pet.) after 2, 3 and/or 6 days ('golden standard' for this study). The control group consisted of 7 patients with eczema and 7 healthy volunteers, with no signs of metal intolerance and negative patch test to nickel. Three patients among Ni-ACD and 4 among controls had positive history of atopic diseases. The age range was 20–59 years (median 36.5) in the Ni-ACD group and 25–62 years (median 36.5) among controls. The participants gave informed consent, and the study was accepted by the local ethics committee.

Cell cultures

Peripheral blood mononuclear cells (PBMC) were separated from the blood samples of participants in Ficoll–Paque Plus (Amersham, Uppsala, Sweden) and cryopreserved in liquid nitrogen until testing. The cells were cultured in Iscove's modified Dulbecco's medium with penicillin, streptomycin, and 1,4-dithiothreitol at 37 celsius, 95% RH, 5% CO_2. The final cell density was 10^4 cells/well for IFN-γ ELISpot and 2×10^5 cells/well for all remaining ELISpot, enzyme-linked immunosorbent assay (ELISA), and LPT assays. The cells were cultured in triplicate both with and without the presence of $NiSO_4$ (50 μM), and both with and without the addition of IL-7 and IL-4 or IL-12, referred to as 'cytokine cocktails'. The selection of nickel concentration was based on study results of Lindemann et al. (19), who compared 7 different concentrations of nickel sulfate (6–200 μM) and found 50 μM most suitable for cell cultures, in terms of specific lymphocyte stimulation versus cytotoxicity. Two 'cytokine cocktails' were used in the study: '7/4 cocktail' – a 'type 2'-skewing combination of IL-7 and IL-4, and '7/12 cocktail' – a 'type 1'-skewing combination of IL-7 and IL-12. All cytokines used were human recombinant proteins (Strathmann Biotec, Hannover, Germany) with specific activities of 5×10^7 U/mg for IL-7, 2×10^7 U/mg for IL-4, and 1×10^7 U/mg for IL-12. Final concentrations of the cytokines in cell cultures were 240 U/ml IL-4, 0.5 U/ml IL-7, and 10 U/ml IL-12. These concentrations were established in a series of introductory tests and proved effective in preceding studies carried out in our group (17, 21). A similar approach was also used by Jennes et al. for improving detection of virus-specific lymphocytes through culturing PBMC with IL-7 and IL-15 (22).

Enzyme-linked immunospot assay

The cells were cultured initially for 20 hr in round-bottom 96-well culture clusters in order to enable good antigen presentation. Subsequently, the cells were moved to 96-well polyvinylidene fluoride (PVDF) microfilter plates (Millipore, Molsheim, France), each coated with respective antibodies. After additional 40 hr of culture for IFN-γ, and 5 days for IL-2, IL-5, and IL-13, the ELISpot assay was done following the manufacturers' guidelines. Antibodies for IFN-γ, IL-5, IL-13 ELISpot, and streptavidin-alkaline phosphatase conjugate were from Mabtech (Näcka, Sweden), antibodies for IL-2 ELISpot were from R&D (Minneapolis, MN, USA), the BCIP/NBT colour AP substrate was from Bio-Rad (Hercules, CA, USA). Spots were counted automatically using the AID ELISpot Reader (AID, Strassberg, Germany).

Enzyme-linked immunosorbent assay and LPT

Cultures for ELISA and LPT were grown in round-bottom 96-well culture clusters for 6 days. IFN-γ in supernatant was measured with antibody pairs from Sanquin (Amsterdam, the Netherlands) and IL-5 with antibodies from BD (San Diego, CA, USA) according to the manufacturers' recommendations. Colour reaction was developed with horseradish peroxidase (s-HRP) polymer (Sanquin) with OPD/H_2O_2 substrate, and measured with Microplate Autoreader EL311 (Bio-Tek, Winooski, VT, USA). ^{3}H-thymidine (Amersham, Little Chalfont, UK) was added to cell cultures for the last 5 hr, after which the amount of incorporated radioactivity was measured using TopCount NXT from Packard Biosciences (Downers Grove, IL, USA).

Data analysis

Altogether, results of 15 *in vitro* test protocols were compared: IL-2 ELISpot in cultures without and with the '7/12 cocktail', IL-5 ELISpot and ELISA without and with the '7/4 cocktail', IL-13 ELISpot without and with the '7/4 cocktail', IFN-γ ELISpot and ELISA without and with the '7/12 cocktail' and finally LPT without cocktail, with the '7/4 cocktail', and with the '7/12 cocktail'. To answer question Q1 (influence of *in vitro* test protocols on measurable differences between patients with Ni-ACD and controls), test protocols, which produced significant differences ($P \leq 0.05$, Mann–Whitney '*U*' test, 2-tailed), were ranked according to magnitude of differences between median results in Ni-ACD and Controls, expressed as per cent of the median result in Ni-ACD group. To answer question Q2 (concordance of *in vitro* results with clinical diagnosis), *in vitro* test protocols were ranked according to the coefficient of correlation (Pearson's correlation, significance test 2-tailed) with the golden standard (positive history of nickel intolerance combined with at least a ++ patch test to Ni). To answer question Q3 (concordance between non-radioactive test protocols and the LPT), non-radioactive test protocols were ranked from the highest to the lowest coefficient of correlation with LPT (Pearson, 2-tailed). Statistical package SPSS+ for Windows (SPSS Inc, Chicago, IL, USA) was used for the above analyses.

Results

Influence of protocol on differences between patients with Ni-ACD and controls (Q1)

In 6 of 15 *in vitro* protocols tested, differences between median results for patients with Ni-ACD and controls were larger than 90% and significant at $P \leq 0.05$ (Table 1). The differences of more than 100% in the case of IL-13 ELISpot with '7/4 cocktail' are due to the fact that numbers of cells secreting IL-13 in response to nickel were decreased in PBMC cultures from non-allergic subjects after addition of '7/4 cocktail' (possibly a cytotoxic effect; compare Fig. 1). Individual results for IL-5, IL-2, and LPT test protocols are shown in Figs 2–4. Regarding secretion of the 'type 1' cytokine IFN-γ, no relevant differences were observed between Ni-ACD and controls in any of the test protocols studied. IFN-γ ELISA without cytokine cocktails was closest to the selected significance level with $P = 0.062$.

Concordance between in vitro *results and clinical diagnosis (Q2)*

The strongest correlation with 'golden standard' was observed in the case of LPT with the '7/4 cocktail',

Table 1. In vitro test protocols with significant differences between patients with Ni-ACD and controls. ELISpot results are shown as numbers of secreting cells per 10^6 PBMC, ELISA results are in pg/10^6 PBMC. LPT results are values of the SI. Only results are shown, for which the difference between Ni-ACD and controls was statistically significant ($P \leq 0.05$)

In vitro test protocol	Median result		Difference between Ni-ACD and controls (%)[a]	*P*
	Controls	Ni-ACD		
IL-13 ELISpot with '7/4 cocktail'	−7.5	135	105	<0.001
IL-13 ELISpot without cocktail	0	17.5	100	0.001
IL-5 ELISA without cocktail	0	14	100	0.001
IL-5 ELISpot without cocktail	0	10	100	0.007
IL-5 ELISpot with '7/4 cocktail'	10	140	93	<0.001
IL-2 ELISpot with '7/12 cocktail'	5	70	93	0.001
IL-2 ELISpot no cocktail	7.5	67.5	89	<0.001
IL-5 ELISA with '7/4 cocktail'	33	266	88	0.014
LPT without cocktail	1.4	5.5	74	<0.001
LPT with '7/12 cocktail'	1.2	2.3	48	0.001

ELISpot, enzyme-linked immunospot assay; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPT, lymphocyte proliferation test; Ni-ACD, allergic contact dermatitis to nickel; SI, stimulation index; PBMC, peripheral blood mononuclear cells.
[a]The way of calculating the difference is described in Patients and Methods.

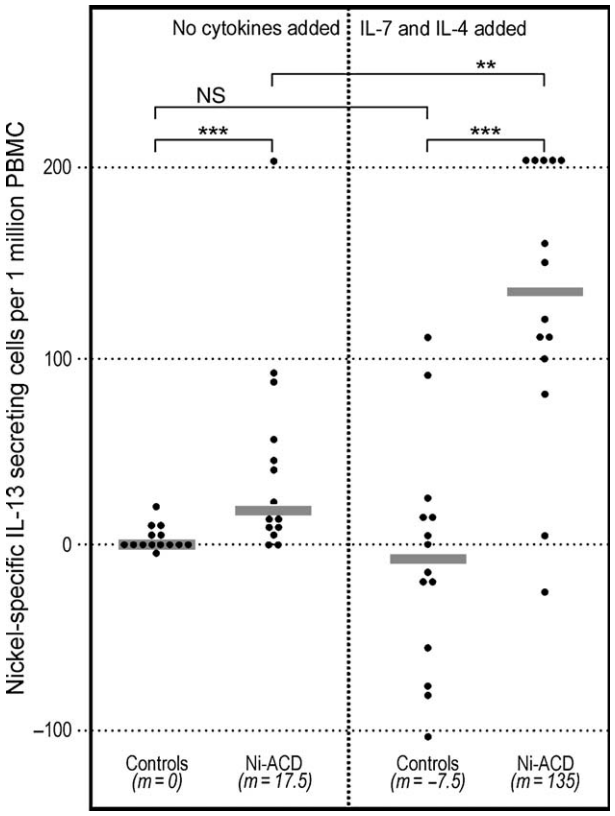

Fig. 1. Interleukin-13 secretion in response to nickel [enzyme-linked immunospot assay (ELISpot)]. The numbers of secreting cells were significantly higher among patients with allergic contact dermatitis to nickel than among controls. Addition of the '7/4 cocktail' further increased the difference. The results were calculated as difference between the number of cells secreting cytokine in the presence of nickel minus the number of cells secreting the cytokine spontaneously in the absence of nickel (background) expressed per 10^6 peripheral blood mononuclear cells. $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant; m, median. Horizontal bars represent medians.

followed by IL-13 ELISpot with '7/4 cocktail' (Table 2). For all other protocols analysed, correlation coefficients *r* were below 0.5, with the lowest correlation observed for IFN-γ ELISpot.

Concordance between non-radioactive test protocols and the LPT (Q3)

The strongest correlation was found between LPT and IL-2 ELISpot without cytokine cocktails, followed by IL-13 ELISpot with and without '7/4 cocktail' (Table 3). The correlation coefficients between LPT and IFN-γ ELISpot and ELISA assays were low ($r \leq 0.051$) and not significant.

Discussion

Nickel is the most important contact allergen with sensitization rates estimated at 17% among adults (23) and 8–10% among children (24, 25). In the present study, we compared clinical diagnosis of ACD with results of 15 *in vitro* test protocols, i.e. combinations of 3 *in vitro* assays (ELISpot, ELISA, and LPT) with 3 various culture conditions (standard culture conditions, and 2 modifications with cytokine cocktails). Both cytokine cocktails used contained IL-7, which together with IL-4 or IL-12 enhances priming of naive cells (26, 27). IL-7 also counteracts apoptosis of allergen-specific naive and effector T lymphocytes (28). The development of 'type 1' and 'type 2' lymphocyte subpopulations was supported by adding IL-12 or IL-4, respectively (17). There is ongoing discussion, which lymphocyte subsets act as effector cells in ACD: T-helper (Th) cells ($CD4^+$), T cytotoxic cells ($CD8^+$), other lymphocytes like NK or NKT cells, or maybe more than one subpopulation (29). Although this study was not specifically aimed at addressing this question, our results show that IL-5 and IL-13 production was the most prominent response to Ni, thus indicating on the prevailing 'type 2' activation [possible effector cell phenotypes Th2, Tc2, natural killer (NK)2, and/or NKT2]. The exact phenotype of these effector cells will be subject to further investigations.

Despite ACD been traditionally regarded as an IFN-γ-driven disease, we have not observed any significant differences between patients with Ni-ACD and controls regarding IFN-γ production in response to nickel. Technically, this could be explained through, for example, a high spontaneous secretion of IFN-γ (possibly by NK cells), which could 'obscure' the secretion by nickel-specific lymphocytes. However, there were previous hints on preferred 'type 2' response to nickel of peripheral blood lymphocytes (30, 31), and also of Ni-specific lymphocytes from isolated eczematous skin (32, 33). The 'type 2' cytokine pattern in response to Ni seems independent of atopic status of the sensitized person (34, 35). Moreover, this pattern seems not to be restricted to nickel only: Masjedi et al. found that allergen-specific IL-13 production discriminated best between cultures of PBMC from people with contact allergy to methylisothiazolinones and from controls (36). This encourages further studies of 'type 2' test protocols also with other contact allergens.

The *in vitro* test protocol that correlated best with clinical diagnosis ('golden standard') was LPT done with 'type 2' skewing (addition of '7/4 cocktail'). This observation was disappointing to some extent, as one of the aims of the study was to identify a non-radioactive alternative for the LPT. However, the 'classical' LPT, i.e. done without enhancement with cytokine cocktails, was outperformed by IL-13 ELISpot with '7/4 cocktail'.

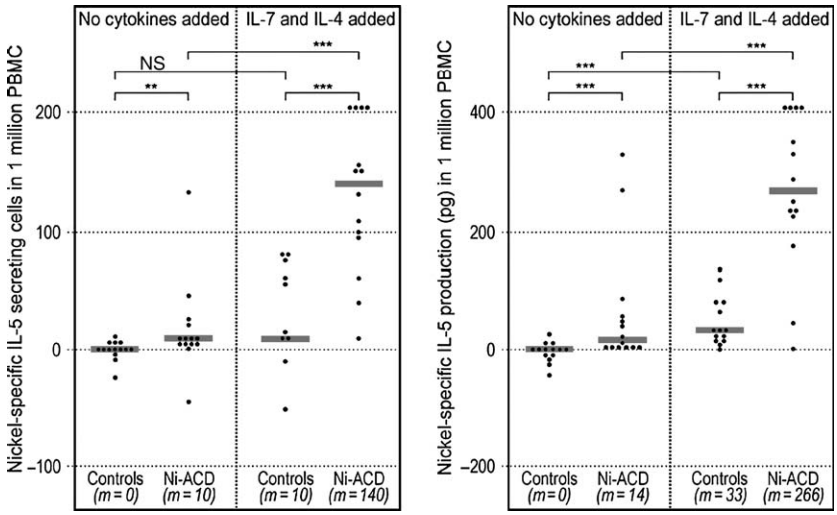

Fig. 2. Analysis of nickel-specific interleukin (IL)-5 secretion by means of enzyme-linked immunospot assay (ELISpot) (left) and enzyme-linked immunosorbent assay (ELISA) (right). The numbers of cells secreting IL-5 (ELISpot) and the overall production of IL-5 (ELISA) in response to nickel were significantly higher among patients with allergic contact dermatitis to nickel than among controls. Addition of the '7/4 cocktail' further increased the difference between groups. ELISpot results were calculated as in Fig. 1. ELISA results were presented as cytokine concentration in supernatants from cultures with nickel minus the concentration in cultures without nickel (spontaneous secretion), expressed per 10^6 peripheral blood mononuclear cells. $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant; m, median. Horizontal bars represent medians.

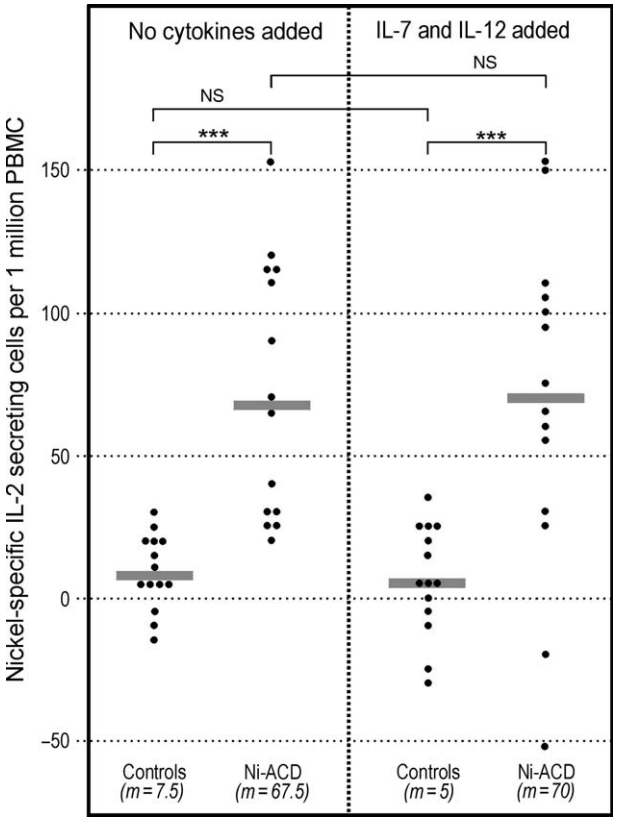

Fig. 3. IL-2 secretion in response to nickel [enzyme-linked immunospot assay (ELISpot)]. The numbers of IL-2 secreting cells [enzyme-linked immunospot assay (ELISpot)] were significantly higher among patients with allergic contact dermatitis to nickel than among controls. Addition of the '7/12 cocktail' did not change the difference. The results were calculated as in Fig. 1. $^{***}P < 0.001$; NS, not significant; m, median. Horizontal bars represent medians.

Finally, when looking for a non-radioactive alternative for the 'traditional' LPT, the highest correlation was found between this method and IL-2 ELISpot (without cocktail). This finding fits well to the knowledge that memory cells secrete IL-2 to stimulate proliferation and differentiation after encounter with specific antigen (37). Relatively high correlations were also observed between

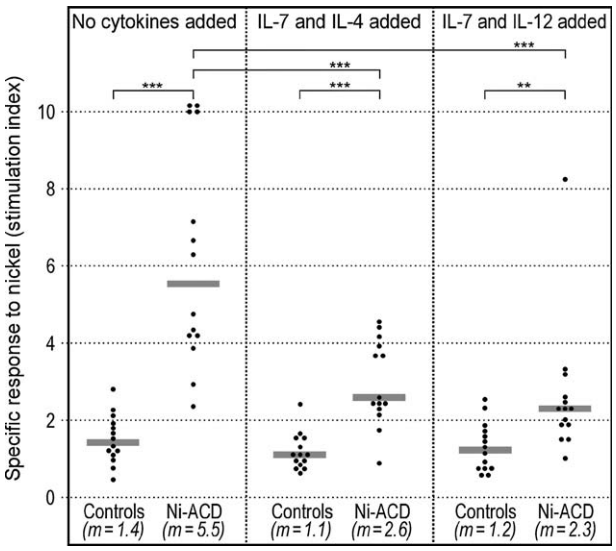

Fig. 4. Lymphocyte proliferation test in response to nickel. Results are shown as stimulation indexes, i.e. radioactivity of cultures with nickel divided by radioactivity of cultures without the presence of nickel. $^{**}P < 0.01$; $^{***}P < 0.001$; m, median. Horizontal bars represent medians.

Table 2. In vitro test protocols with highest ($r > 0.5$) correlation with clinical diagnosis (positive history and patch test)

In vitro test protocol	*r*	*P*
LPT with '7/4 cocktail'	0.739	<0.001
IL-13 ELISpot with '7/4 cocktail'	0.654	<0.001
LPT without cocktail	0.612	0.001
IL-5 ELISpot with '7/4 cocktail'	0.551	0.002
IL-2 ELISpot with '7/12 cocktail'	0.544	0.003

ELISpot, enzyme-linked immunospot assay; IL, interleukin; LPT, lymphocyte proliferation test.

Table 3. Non-radioactive test protocols with highest ($r > 0.5$) correlation with the LPT

In vitro test protocol	*r*	*P*
IL-2 ELISpot without cocktail	0.809	<0.001
IL-13 ELISpot without cocktail	0.778	<0.001
IL-13 ELISpot with '7/4 cocktail'	0.778	<0.001
IL-5 ELISA without cocktail	0.669	<0.001
IL-2 ELISpot with '7/12 cocktail'	0.587	0.001

ELISpot, enzyme-linked immunospot assay; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPT, lymphocyte proliferation test.

LPT and IL-13 ELISpot and IL-5 ELISA, but again not in case of the 'type 1' cytokine IFN-γ.

In concluding the above results, combining ELISpot assay with modified culture conditions may constitute a relevant progress in the detection of contact allergy to nickel *in vitro*. Among the test protocols analysed, IL-13 ELISpot in cultures with '7/4 cocktail' was most effective in detecting Ni-specific response and should be further evaluated regarding its possible use for *in vitro* diagnosis of contact allergy.

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