

Analysis of effector and regulatory immune reactivity to nickel

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Summary

Background Diagnostic patch testing in allergic contact dermatitis faces the risk of boosting existing hypersensitivities or active sensitization. Risk-free and reliable *in vitro* assays using peripheral blood are, therefore, wanted.

Objectives Here, we studied new approaches for *in vitro* monitoring of nickel-specific effector and regulatory cell functions in allergic patients and potentially tolerized individuals.

Methods Lymphocyte proliferation assays were carried out with the allergen and additional IL-12/IL-7 or IL-4/IL-7 cytokine supplements. Release of IFN- γ and IL-5 were assessed as measures for type-1 and type-2 effector T cell function, respectively, and IL-10 and TGF- β_1 to monitor possible regulatory T cell function reflecting immunological tolerance. After optimization of *in vitro* cut-off values, potency of these parameters was evaluated as compared with conventional nickel patch testing.

Results One hundred and fifty six outpatients were included in this study, 74 of whom presenting with a positive history of nickel allergy. Nickel-sulphate patch test results showed positive reactions in 43 patients, of whom 40 had a positive history (test sensitivity 54%; specificity 96%; overall accuracy 76%). Proliferation tests without cytokine supplementation showed an accuracy of 68%, which was further improved by supplementing IL-4/IL-7 (82%). IFN- γ and IL-5 cytokine production, as revealed in IL-12/IL-7 and IL-4/IL-7 supplemented cultures, respectively, showed accuracies of 70% and 83%. As to the production of putatively immunoregulatory cytokines, IL-10 was most informative, with highest production rates in nickel-skin test negative individuals with long-lasting mucosal metal contact preceding skin piercing.

Conclusions These results indicate that measuring both T cell proliferation and cytokine secretion profiles, in particular IL-5 release using IL-4/IL-7 supplemented medium, offers a promising improvement of the *in vitro* diagnostic options in monitoring nickel contact sensitization. Since oral nickel contact has been shown earlier to induce active tolerization, nickel-induced *in vitro* IL-10 production may help identify nickel-tolerized individuals.

Keywords contact allergy, *in vitro* detection, lymphocyte proliferation test, nickel, patch test, tolerance

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Introduction

Allergic contact dermatitis (ACD) is a common skin disease, because of an acquired T cell immune response to highly reactive small molecules, termed haptens [1, 2]. The most frequent cause of contact allergy in western countries is sensitization to nickel [3]. About 20% of the female and 6% of the male population are sensitized to nickel [4]. Epidemiological studies have revealed that sensitization to nickel is, in general, the result of a prolonged direct epidermal exposure, especially after skin piercing [3, 5]. Contact allergy to nickel is currently diagnosed by epicutaneous hapten

exposure, in particular by patch testing as developed by Jadassohn [6]. Patch testing, however, holds distinct disadvantages. In particular, both false-negative and false-positive reactions can frequently be encountered. False-negative reactions may result from slow allergen penetration, low inflammatory capacity of the skin test site (e.g. post-UV radiation), or generally reduced immune reactivity (e.g. by use of immunosuppressive medication). On the other hand, false-positive reactions can be because of 'angry back'- and 'excited skin'-like phenomena, and result from locally increased skin irritability. Another concern with skin testing is the risk of interference with the patients' immune status. Existing, but clinically silent hypersensitivities, maintained by long-living central memory T cells with a high activation threshold [7], can be boosted or, even worse, patients can be sensitized *de novo* to the applied haptens [3, 8].

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Hence, the necessity of more reliable and safe diagnostic approaches has been felt for some time [3, 9–11]. Preferably, such tests aim at the detection of allergen-specific T cells under harmless *in vitro* conditions. Using patients' blood samples, such cells can be detected by analysing proliferation and mediator release in response to allergen exposure [3, 9]. Classically, contact allergy mediating T lymphocytes have been regarded to be type-1 T cells [12, 13]. However, in the last few years studies with T cell lines and fresh, patient-derived T cells (Moed et al., unpublished data) have also shown significant numbers of type-0 and type-2 T cells in nickel-allergic patients [14–17]. As yet this knowledge has not been translated into novel diagnostic *in vitro* procedures. Moreover, culture conditions have not yet been optimized to facilitate detection of circulating, allergen-specific memory T cells. Thus, improvement of antigen-specific T cell detection, might be achieved by supplementing cell cultures with cytokines now known to enhance (1) antigen-presentation; (2) expression of crucial costimulatory and adhesion molecules; (3) proliferation, and (4) mediator release of specifically activated T cells.

Here, distinct recombinant cytokines, in particular IL-4, IL-7 and IL-12, were utilized to improve *in vitro* detection methods. Of note, IL-4 promotes general T cell proliferation and release of type-2 cytokines such as IL-5 [18–20]. Moreover, IL-4 can improve antigen-specific T cell activation by enhancing MHC-dependent antigen-presentation and up-regulation of essential costimulatory molecules of the early T cell activation phase, such as CD29/CD49d [21–23]. IL-7 is known to augment proliferation [24–27] and cytokine release of activated T cells [28–31]. IL-12 induces, next to proliferation of type-1 T cells, differentiation and release of type-1 cytokines, notably IFN- γ [28, 32–34]. Actually, IL-7 also is known to increase T cell proliferation by up-regulation of costimulatory molecules, such as CD80 and CD86 [35, 36].

In preliminary experiments testing wide concentration ranges of IL-4, IL-7, IL-12 and their combinations, in particular IL-7 with either IL-12 or IL-4, to supplement culture media, we observed considerable improvement of nickel-allergy detection. Apart from superior nickel-specific proliferation, nickel-specific production of IFN- γ in IL-12/IL-7, and IL-5 in IL-4/IL-7 supplemented cell cultures were found. Hence, detection of nickel allergy in cytokine-supplemented *in vitro* assays might, next to improved accuracy, facilitate characterization of the predominantly reacting type-1 or type-2 T cell subpopulations. Besides revealing effector T cell functions in allergic patients, *in vitro* tests might help to identify tolerized individuals, which has, as yet, not been possible. Earlier epidemiological studies from our group revealed that oral mucosal nickel contacts preceding skin piercing can reduce the frequency of nickel allergy by inducing immunological tolerance [37]. We hypothesized that *in vitro* tests might also help to detect tolerance mediating T cells that are characterized by the release of immunomodulatory cytokines such as IL-10 and TGF- β_1 [2]. The results of the present study support the diagnostic applicability of cytokine-supplemented *in vitro* lymphocyte activation tests for detecting nickel allergy in patients routinely visiting the patch test clinic, as well as for the identification of tolerized individuals.

Materials and methods

Patients

Patients ($n = 156$) visiting the Free University medical centre patch test clinic participated in this study after informed consent. From all patients a detailed medical history was taken, including history of nickel allergy, i.e. former positive nickel patch tests and/or nickel-induced eczematous skin reactions. Furthermore, history of nickel exposure, in particular skin piercing, and wearing of metal dental braces was recorded. None of the patients included in this study had recently used immunosuppressive medication, underwent UV radiation or suffered from recent or actual inflammatory skin diseases. To avoid the possible impact of positive patch test reactions, blood samples were taken directly before epicutaneous testing.

Patch testing

All patients were skin patch tested with the European standard series of contact allergens, including 5% nickel sulphate (NiSO_4) in petrolatum (Hermal, Hamburg, Germany). In addition, in all but one patient 5% nickel chloride (NiCl_2) in aqua (Hermal) was tested. According to the recommendations of the International Contact Dermatitis Research Group (ICDRG), skin tests were evaluated after 48 and 72 h, and, in addition, after 168 h (7 days) [3, 38]. Patch tests were regarded to be positive if at least at one reading a positive reaction to one nickel salt occurred.

Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated from 40 mL-heparinized blood by density centrifugation with Lymphoprep (Nycomed AS, Oslo, Norway) [39] and directly used or cryopreserved until further testing. In each experiment, a vial of cells from a large batch of frozen nickel-sensitive PBMC was included to provide an inter-assay standard (data not shown). PBMC were seeded in round-bottom 96-well culture plates (Costar, Cambridge, MA, USA) at 2×10^5 cells/well in Iscove's modified Dulbecco's medium (BioWittaker, Verviers, Belgium) supplemented with 10% human-pooled serum (CLB, Amsterdam, the Netherlands), 50 I.E./mL sodium penicillin-G, 50 $\mu\text{g}/\text{mL}$ streptomycin sulphate and 50 μM β -mercaptoethanol (Merck, Darmstadt, Germany) at 37 °C in a 5% CO_2 -containing humidified atmosphere. Cells were stimulated in six replicates by the addition of 50 μM nickel sulphate hexahydrate (Merck) and cultured in the absence or the presence of cytokine supplements consisting of IL-4 (0.01–100 ng/mL; CLB), IL-7 (0.01–30 ng/mL; Genzyme, Cambridge, MA, USA), IL-12 (0.01–30 ng/mL; Hoffman-LaRoche, Nutley, NJ, USA), or combinations of these cytokines. Triplicate PBMC cultures supplemented with pokeweed mitogen served as positive controls. At day 7, 100 μL of the culture supernatants was removed, pooled per test condition and stored at -80°C until further use, and T cell proliferation was measured by [^3H]-thymidine incorporation analysis.

Analysis of cellular proliferation

During the last 5 h, [^3H]-thymidine was added (0.5 $\mu\text{Ci}/\text{well}$; Amersham, Aylesbury, UK), after which the cells were

harvested onto fibreglass filters and [^3H]-thymidine incorporation was determined using a flatbed liquid scintillation counter (Wallac, Türkü, Finland).

Nickel-specific proliferation was assessed by the stimulation ratio (SR), calculated from the ratio of mean [^3H]-thymidine uptake in stimulated to non-stimulated cultures.

Cytokine measurements

In culture supernatants, IFN- γ , IL-5, IL-10 and TGF- β_1 were measured by commercially available ELISA kits (IFN- γ kit by Medgenix, Fleurus, Belgium; lower detection level 40 pg/mL; IL-5 kit by Pharmingen, San Diego, CA, USA; lower detection level 8 pg/mL; IL-10 kit by Medgenix; lower detection level 10 pg/mL; TGF- β_1 detection antibody pair by Pharmingen; lower detection level 10 pg/mL), according to the manufacturer's guidelines. Nickel-specific cytokine release (Δ) was assessed by subtracting the cytokine concentration of non-stimulated from stimulated culture supernatants.

Statistical analysis

For each of the *in vitro* parameters, optimal cut-off values had to be determined first. Therefore, receiver operator characteristics (ROC)-curve analyses were performed (MedCalc statistical software, Mariakerke, Belgium). In brief, the percentages of true positives (= sensitivity) and false positives (= 100 specificity) were plotted on the y - and x -axes, respectively (Fig. 2) [40]. If sensitivity and specificity are ranked equivalently important, the optimal cut-off value is the point of the curve nearest to the upper left corner (i.e. highest sensitivity and specificity). The 'truly positive' reference group was defined as the group of patients who showed positive nickel skin tests, suffered from nickel allergy and were skin pierced ($n = 31$). The 'truly negative' reference group consisted of those patients negative for these parameters ($n = 38$). The diagnostic values of the various tests are expressed by calculating sensitivity, specificity, positive and negative predictive value (i.e. the probability that a patient, who has nickel allergy is tested positive and a patient, who is not nickel allergic is tested negative, respectively) and accuracy (power of a test to find the correct diagnosis).

For statistical analyses the Fisher's exact test and, where appropriate, the likelihood is expressed by the odds ratio; $P < 0.05$ was considered significantly different.

Results

Nickel allergy can be frequently missed by patch testing

The current 'golden standard' of detecting nickel allergy is the common nickel-patch test. In order to validate this method in our hands, patients ($n = 156$; 74 with and 82 without a medical history of nickel allergy, as defined by former positive nickel patch tests and/or nickel-induced eczematous skin reactions) visiting the patch test clinic were tested epicutaneously. Out of both groups, 43 tested positive to nickel sulphate (Fig. 1). To nickel chloride tested simultaneously, 41 of these patients showed positive reactions (concordance 93%). Of note, 34 of the 74 patients with a history of nickel allergy did not show positive patch test reactions to nickel

sulphate or nickel chloride (Fig. 1), resulting in a 54% sensitivity score for patch testing. All but three patients with positive nickel patch reactions reported on a history of nickel allergy (specificity: 96%). Hence, the accuracy of patch testing was determined to be 76% (Table 2), in line with results from earlier studies describing accuracies of 70–80% for patch testing with nickel sulphate [41–43].

From the medical histories, the major risk factor for developing nickel allergy was found to be skin piercing (relative risk, i.e. the odds ratio of 10.1) and the female gender (odds ratio 21.5), whereas the presence of atopic disorders did not influence the frequency of nickel allergy (odds ratio, 1.1; Table 1), also supporting earlier reports [3, 5]. Remarkably, the relative risk for developing nickel allergy was significantly reduced in patients with long-lasting mucosal metal exposure before receiving skin piercings (odds ratio, 0.2).

Detection of nickel allergy in improved proliferation assays

In lymphocyte proliferation tests (LPTs), PBMC are cultured in the presence or the absence of nickel, and nickel-specific responsiveness has been coined with ratios of proliferation in nickel-stimulated to non-stimulated cultures (SR) exceeding 2.0 [11]. First, pilot experiments were carried out to further improve the detection of nickel-specific reactivity. *In vitro* proliferation of specific T cells was optimized by supplementing cell cultures with single or combinations of recombinant cytokines, i.e. IL-4, IL-7 and IL-12. All cytokines tested stimulated general T cell proliferation in a dose-dependent fashion. In particular, the combinations of IL-4 (3 ng/mL) and IL-12 (0.1 ng/mL) with IL-7 (0.1 ng/mL) were found to provide superior enhancement of nickel-specific cell proliferation (T. Rustemeyer, unpublished results). Secondly, optimal cut-off values were established for each test condition. Therefore, as defined in the Materials and methods, we selected groups of 'truly' nickel-allergic ($n = 31$) and non-allergic ($n = 38$) donors. Based on ROC-curve analysis, optimal LPT cut-off values to distinguish positive from negative patients were determined as shown in Fig. 2.

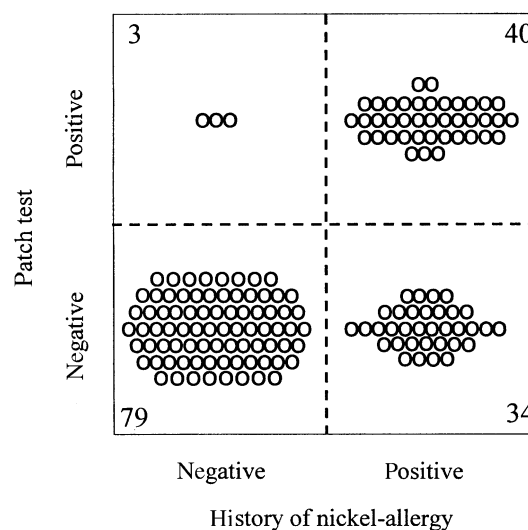


Fig. 1. Detection of nickel allergy by patch testing. Inserted numerals indicate the number of true positive (right upper), false positive (left upper), true negative (left lower) and false negative cases (right lower square).

Table 1. Clinical patients' data separated for the history of nickel allergy

Parameter	History of nickel allergy		Risk for nickel allergy (odds ratio)	
	positive (n = 74)	negative (n = 82)		Fisher's exact
Mean age (range)	38 [18–77]	37 [18–75]		
Female	71	43	21.5	< 0.0001
Atopic disorders	35	37	1.1	0.87
Long-lasting mucosal metal exposure before piercing	8	26	0.2	< 0.01
Body piercing	67	40	10.1	< 0.0001

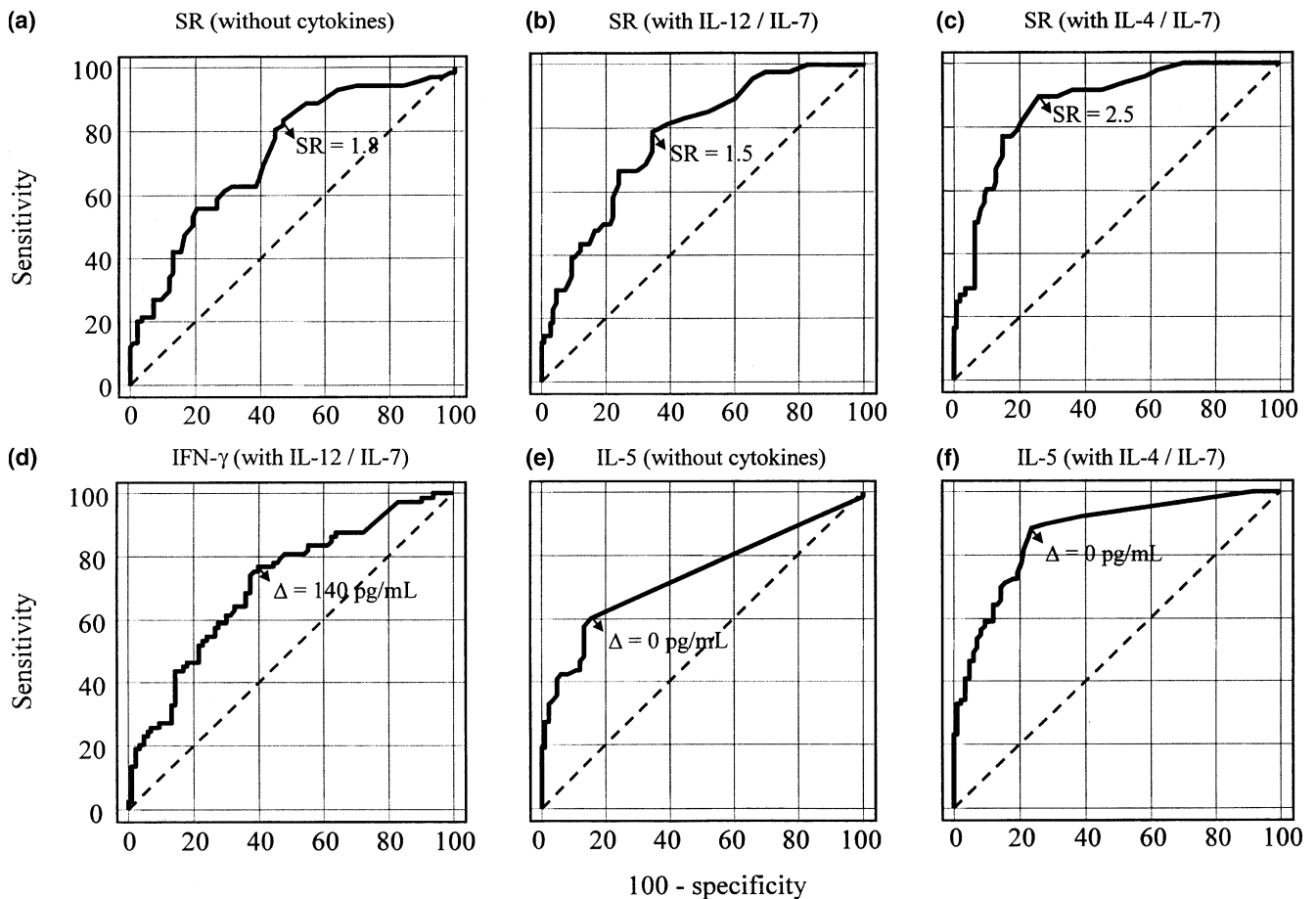


Fig. 2. Establishment of optimal cut-off values. By receiver operator characteristics-curve analysis, ranking sensitivity and specificity equivalently important, optimal threshold values were determined for nickel-specific proliferation cultures (a–c): without supplementation of cytokines (a), with supplementation of IL-12/IL-7 (b) and IL-4/IL-7 (c), and for nickel-specific cytokine production (d–f): IFN- γ cytokine production was analysed in cultures supplemented with IL-12/IL-7 (d), IL-5 cytokine production was studied in cultures without cytokine supplementation (e) and with supplementation of IL-4/IL-7 (f).

Applying the newly defined SR cut-off (1.8) for non-cytokine supplemented cell cultures, numbers of false positives and negatives were found to be 39 and 12, respectively (Fig. 3). Supplementation of cell cultures with both IL-12/IL-7 and IL-4/IL-7 combinations further improved the numbers of correctly diagnosed patients. Although supplementation of IL-12/IL-7 resulted in a lower number of falsely positives (29 out of 82), stimulation of T cells by IL-4/IL-7 supplementation reduced both the numbers of false positives (23 out of 82) and false negatives (seven out of 74) (Fig. 3). These results underline the possibility to improve *in vitro* diagnosing nickel allergy by introducing cytokine supplementation such as IL-12/IL-7 and, in particular, of IL-4/IL-7 by enhancing the accuracy of the

common LPT from 68% to 72% and 82%, respectively (Table 2).

Nickel allergy can be detected by both interferon- γ and interleukin-5 cytokine production

Next to proliferation, cytokine secretion can provide an *in vitro* detection method of antigen-specific T cell activation. Therefore, we analysed nickel-specific secretion of both type-1, i.e. IFN- γ , and type-2 cytokines, i.e. IL-5, in lymphocyte cultures. IFN- γ production was only detectable when IL-12 was added to the cell cultures, and found to be further stimulated in the presence of IL-7 (data not shown). Thus, IFN- γ was measured routinely in IL-12/IL-7 supplemented

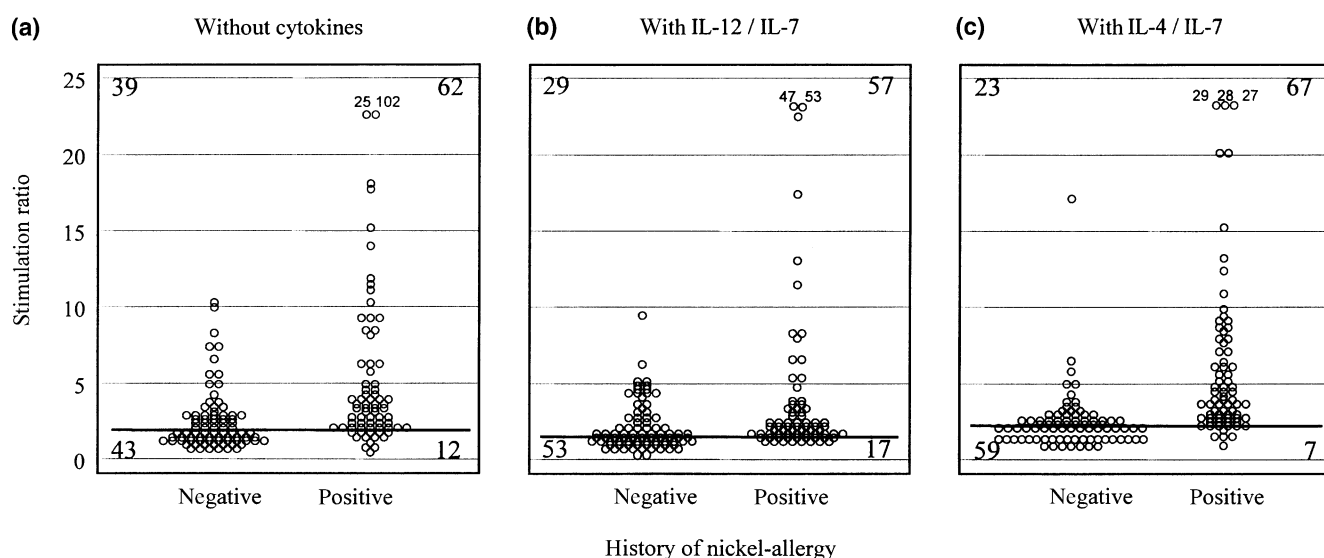


Fig. 3. Nickel-specific proliferation in the group of patients with a positive and negative history of nickel allergy. Nickel-specific proliferation (expressed as SI) was studied in lymphocyte cultures without cytokine supplementation (a), with supplementation of IL-12/IL-7 (b) and IL-4/IL-7 (c). Inserted numerals give the number of true positive (right upper), false positive (left upper), true negative (left lower) and false negative cases (right lower square). The bold line indicates the respective cut-off value as determined by receiver operator characteristics-curve analysis.

Table 2. Statistical characteristics of patch testing and *in vitro* tests for diagnosing nickel allergy

Test	Test condition	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
Patch testing		54	96	93	72	76
Lymphocyte proliferation	Without cytokines	63	75	77	61	68
	With IL-12/IL-7	77	64	58	71	72
	With IL-4/IL-7	91	72	85	75	82
IFN- γ production	With IL-12/IL-7	63	75	77	61	70
IL-5 production	Without cytokine	61	84	77	71	75
IL-5 production	With IL-4/IL-7	92	75	79	90	83

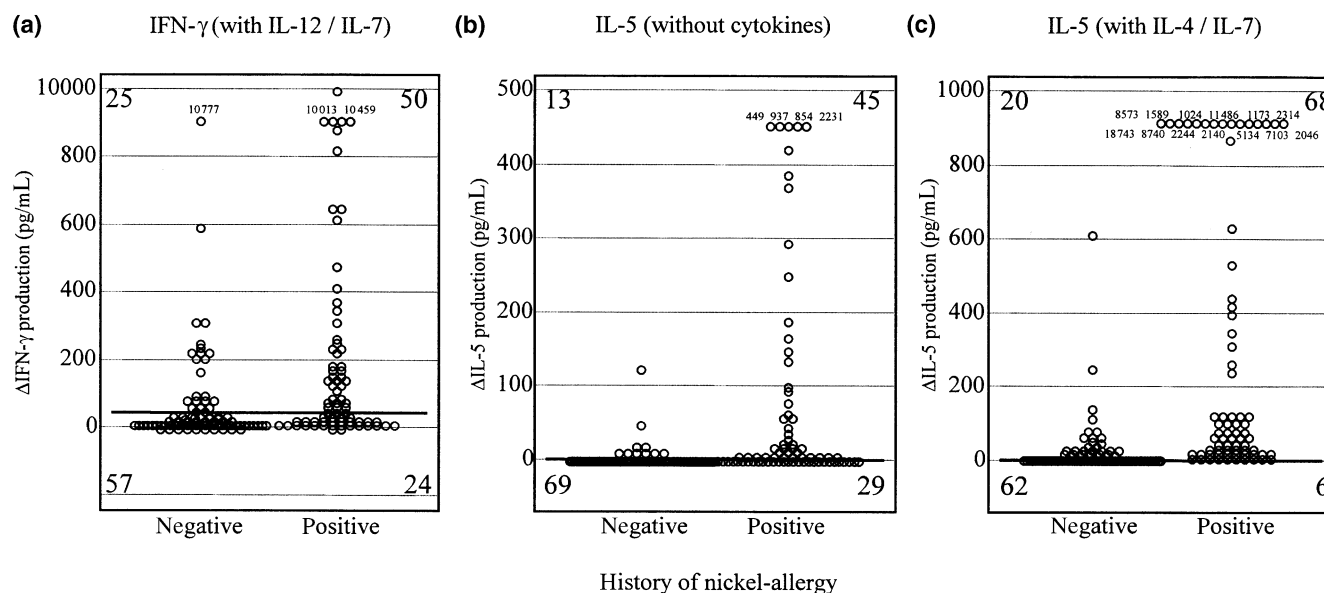


Fig. 4. Nickel-specific cytokine production in the group of patients with a positive and negative history of nickel allergy. Nickel-specific release of cytokines (expressed as Δ) was analysed in cultures supplemented with IL-12/IL-7 for IFN- γ production (a), IL-5 cytokine production in cultures without cytokine supplementation (b) and with supplementation of IL-4/IL-7 (c). Inserted numerals indicate the number of true positive (right upper), false positive (left upper), true negative (left lower), and false negative cases (right lower square). The bold line gives the respective cut-off value as determined by receiver operator characteristics-curve analysis.

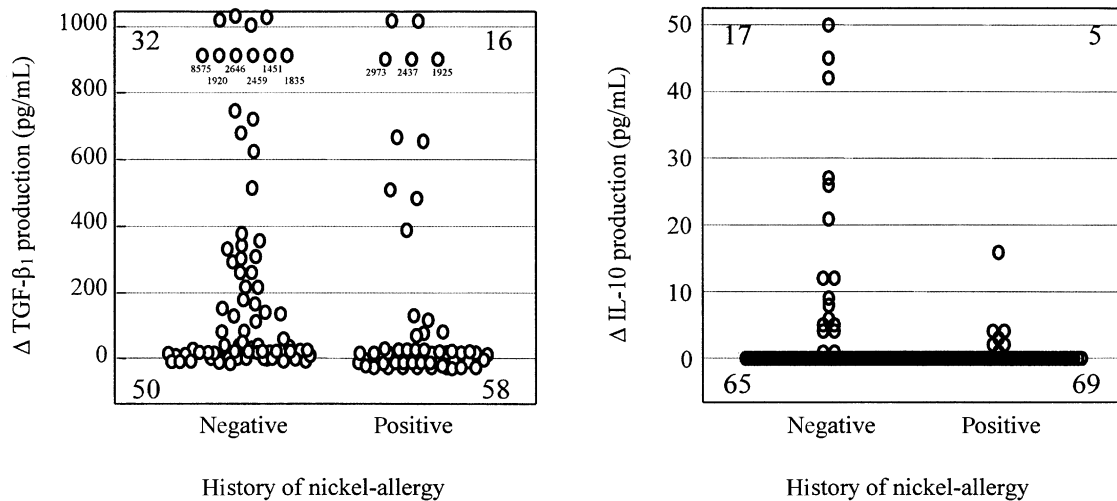


Fig. 5. Detection of immunoregulatory cytokine IL-10 and TGF- β_1 production. Nickel-specific release of IL-10 and TGF- β_1 (expressed as Δ) was analysed in non-cytokine supplemented cell cultures.

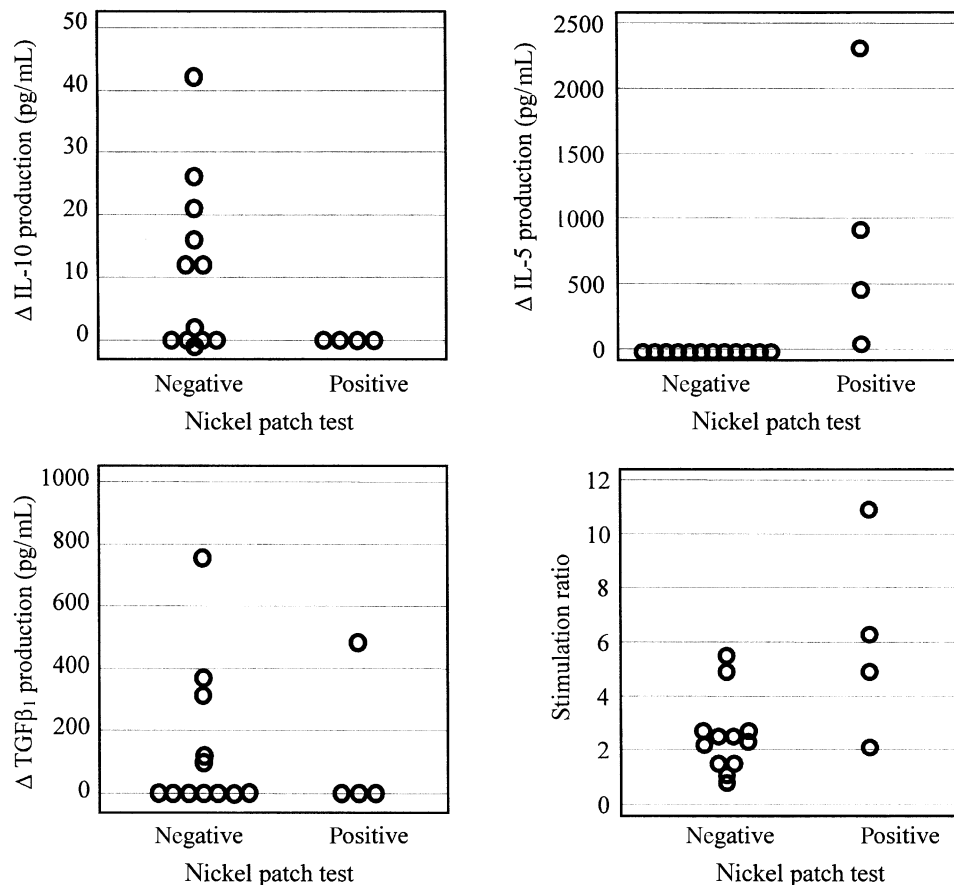


Fig. 6. Nickel-specific tolerized individuals are characterized by IL-10 and the absence of IL-5 production. For patients with dental braces preceding skin piercing, nickel-specific production of the cytokines (expressed as Δ) IL-10 and IL-5, and nickel-specific proliferation (expressed as SI) were studied in lymphocyte cultures without cytokine supplementation.

cultures. Using optimized cut-off values (Fig. 2), IFN- γ allowed for correctly identifying 57 out of 82 negative and 50 out of 74 positive patients (accuracy: 70%; Fig. 4).

Regarding IL-5, used as a marker of activated type-2 T cells, substantial production was found under both non-

cytokine and IL-4/IL-7-supplemented conditions. In the first, analysis of IL-5 allowed for reliable distinction between negative (69 out of 82 correctly identified) and positive (45 out of 74) patients (accuracy: 75%). Supplementing with IL-4/IL-7, however, led to superior identification of positive

patients (68 out of 74) and good identification of negative patients (62 out of 82) (accuracy: 83%; Fig. 4). These results, therefore, demonstrate that both IFN- γ and IL-5 can serve as reliable markers for the *in vitro* detection of nickel allergy, but highest sensitivity and specificity can be obtained by assessment of IL-5 production under culture conditions favouring type-2 T cell activation. Combination of different *in vitro* parameters did not result in further increased accuracy (data not shown).

Identification of tolerized individuals by nickel-specific production of immunomodulatory cytokines

We studied, next, the possibility to detect nickel-specific production of immunomodulatory cytokines, in particular IL-10 and TGF- β_1 , in cell culture supernatants. As shown in Fig. 5, significantly more individuals with a negative history of nickel allergy were found to produce nickel-specific TGF- β_1 (32 out of 82; allergic patients: 16 out of 74; $P < 0.02$). Also substantial IL-10 production was found, in particular, among non-nickel-allergic patients (17 out of 82; allergic patients: five out of 74; $P < 0.02$). To evaluate the potency of these cytokine parameters to identify tolerized individuals, we then selected a subgroup of patients with potentially tolerogenic oral metal contacts by wearing dental braces before skin piercing (Fig. 6). Within this group we compared individuals with negative nickel skin tests ('successfully tolerized') with individuals with positive skin tests ('sensitized' despite oral contact). Detectable IL-10 production was only present in the successfully tolerized group, whereas detectable IL-5 production was only seen in the sensitized group. Differences between these groups were, however, less pronounced when analysing proliferation or TGF- β_1 production (Fig. 6).

Discussion

In the studies reported here, using novel insights into immune-effector functioning, we aimed at the improvement of *in vitro* methodology for the detection of nickel allergy in a clinical setting. We could demonstrate that supplementation of cell cultures with recombinant cytokines, in particular IL-4 plus IL-7, resulted in a significantly improved detection of nickel allergy (82% vs. 68% accuracy without cytokine supplementation). This increase can be ascribed, first, to the capacity of IL-7 to promote proliferation and cytokine production of recently activated T cells [24, 29]. Next, the addition of IL-4 could enhance these effects by improving antigen presentation and expression of co-stimulatory molecules, stimulating T cell proliferation and, in particular, release of type-2 cytokines like IL-5 [18, 21]. Indeed, detection of nickel-specific IL-5 production, especially in IL-4/IL-7 supplemented cultures, provided a highly sensitive and specific marker for the identification of nickel-allergic patients. Thus, analysis of type-2 cytokines can serve as a reliable parameter for the detection of nickel-specific effector T cells in peripheral blood. Also for the detection of drug allergy, [45] the type-2 cytokine IL-5 was a highly sensitive parameter [44]. Very recently, Lindemann et al. showed that also IL-4 production was positively correlated with the presence of nickel allergy. However, IL-5 is of particular

interest in this regard, since this cytokine is exclusively produced by T cells and, in contrast to IL-4, hardly consumed by PBMC. The IL-5 receptor CD125 is among PBMC solely expressed by few activated B cells, whereas all PBMC types, in particular stimulated T cells, express the IL-4 receptor CD124 and can consume IL-4 [46].

The prominent release of IL-5 is remarkable, since contact allergy has been regarded as a classical model of type-1 T cell-mediated hypersensitivity reaction [12, 13]. In line with the latter view, nickel-specific T cell responsiveness under type-1 T cell activity promoting conditions, thus upon IL-12 and IL-7 supplementation, was also augmented (with accuracies increasing from 68% to 72%; Table 2). But, in one-third of the nickel-allergic patients, IFN- γ was absent, while in most of these patients (22 out of 24) nickel-specific IL-5 production was found. Interestingly, a considerable number of individuals with a negative history of nickel allergy produced IFN- γ in a nickel-specific fashion (25 out of 82). During the development of immunological responses, in general, type-1 immune reactivity precedes type-2 immunity [47–49]. Thus, nickel-specific IFN- γ production in non-allergic patients might reveal nickel sensitization preceding the clinical manifestation of the disease and may, therefore, serve as a risk-assessment marker. Anyhow, the present results support the view that nickel allergy is not exclusively mediated by IFN- γ secreting type-1 T cells. Recent studies on nickel-specific cell lines and clones demonstrated the presence of type-0, type-1 and type-2 T cells in nickel-allergic patients [15, 17, 50]. Here, using freshly isolated PBMC, we could show the actual relevance of distinct nickel-specific T cell cytokine secretion types in the blood of allergic patients. Of note, nickel allergic patients with different cytokine profiles were clinically undistinguishable. Recently, we obtained evidence for local release of both type-1 and type-2 cytokines in contact allergic skin sites. In early punch biopsies of nickel re-challenge reactions, IL-4-releasing T cells could be detected, whereas production of IFN- γ was revealed indirectly by the local up-regulation of the IFN- γ -dependent chemokines CXCL-9, -10 and -11 (M. Jonker et al., unpublished data). Certainly, the precise interactions of distinct T cell types in contact allergy, and their possible capacity to repolarize under the influence of local mediator conditions still have to be unravelled. Detailed knowledge of these inflammatory processes might help to develop intervention strategies in clinical allergic contact dermatitis.

Hereto, insights in nickel-specific regulatory T cell mechanisms are also of critical importance. In this study we were able to detect significant production of nickel-specific immunoregulatory cytokines, i.e. TGF- β_1 and IL-10, in nickel-stimulated PBMC cultures of patch test negative individuals with a history of wearing dental braces before getting skin pierced. This confirms earlier epidemiological studies revealing that long lasting oral metal contacts preceding skin piercing can cause lower frequencies of nickel allergy [37, 51]. The present results indicate that successfully tolerized individuals can be characterized by nickel-specific production of IL-10 in the absence of IL-5, with patch test positive patients showing substantial IL-5 and no IL-10 production (Fig. 6). These results are in line with the view that long-lasting oral nickel exposure can induce a state of active immunological tolerance mediated by regulatory/suppressor

T cells that prevent subsequent sensitization. As compared with nickel-allergic patients, nickel-tolerized individuals may, apparently, be identified on the basis of their nickel-specific cytokine production profile rather than on regular proliferation assays. In fact, the presence of nickel-specific proliferation in these tolerized individuals lacking any history of nickel allergy most likely contributes to the relatively low specificity of proliferation tests for diagnosing nickel allergy *in vitro*.

In conclusion, the newly developed *in vitro* methodology for assessment of nickel-specific T cell functions offers promising improvements in diagnosing nickel allergy and –nickel tolerance. Furthermore, detailed information on distinct T cell subsets involved in nickel-immune reactivity can be obtained. This can provide relevant information not only in routine diagnostic procedures but also when repeated immunomonitoring is desirable, e.g. in case of possible occupational allergen exposure. Further studies addressing the interactions of distinct nickel-reactive T effector cell types should help improve the knowledge on nickel-induced pathologies and contribute to immunomodulatory prevention and therapy approaches.

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