Efficacy of oral hyposensitization in allergic contact dermatitis caused by nickel

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Summary

Background. Nickel contact allergy remains common in Western countries, and the dermatitis may require prolonged treatment. The development of new strategies aimed at improving the quality of life of affected individuals is needed.

Objectives. To investigate the efficacy of oral hyposensitization in nickel-allergic individuals and how this affects *in vitro* T cell responsiveness to the metal.

Methods. Twenty-eight nickel-allergic patients received a daily dose of 50 μg of elemental nickel (given as $NiSO_4 \cdot 6H_2O$) in cellulose capsules for 3 months. Severity of clinical manifestations, *in vivo* nickel responsiveness and *in vitro* T cell responses to the metal were assessed after 1 and 3 months.

Results. Twenty-six patients finished the study. In these patients, oral hyposensitization ameliorated clinical manifestations despite continued nickel exposures, and increased the threshold of skin responsiveness to nickel. The 12 enrolled patients in the immunological study showed decreased *in vitro* T lymphocyte responsiveness to the metal, in terms of both cell proliferation and cytokine release. In the 1-year follow-up, 50% of the patients experienced relapses of the clinical manifestations at sites of topical exposure to nickel.

Conclusions. Our study suggested therapeutic efficacy of oral hyposensitization in allergic individuals. Placebo-controlled studies are required to confirm the results and determine the optimal therapeutic regimen for prolonged beneficial effects.

Key words: allergic contact dermatitis; nickel allergy; oral hyposensitization; regulatory T lymphocytes; T lymphocytes.

Nickel is the most common contact allergen in industrial countries. The prevalence of nickel allergy in the general population ranges between 8% and 17% in females and between 1% and 5% in males (1–4). This high frequency is related to its frequent use and contact with the metal in both occupational and non-occupational settings (5).

With a few exceptions, nickel allergy is a lifelong condition (6, 7). Interventions aimed at reducing nickel hypersensitivity represent an attractive alternative to current immunosuppressive strategies.

Oral tolerance is a mechanism that impedes the development of undesired immune responses towards dietary antigens (8, 9). Animal models have clearly shown that oral administration of haptens, including nickel, leads to a state of immunological unresponsiveness that prevents subsequent sensitization through the skin. Tolerance induced by oral feeding is long-lasting, is hapten-specific, and can be transferred into naïve

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animals with $CD4^+$ T lymphocytes (10, 11). Multiple mechanisms have been discussed to explain tolerance induction (12), including expansion of $CD4^+CD25^+$ T regulatory cells (Tregs) (13), augmented secretion of interleukin (IL)-10 in response to hapten challenge (14), induction of suppressive $CD8^+$ T cells (9, 15), apoptosis of effector T lymphocytes (16), intervention of natural killer T cells (17), and, finally, the suppressive function of plasmacytoid dendritic cells (18). Whether single or multiple mechanisms are simultaneously armed following antigen feeding is still debated. Possibly, the dose of antigen administered is critical for tolerance induction. In mice, oral tolerance can be induced either with a single administration of a high dose of antigen or with repeated low-dose exposures. The current view is that low-dose tolerance depends on the expansion of Tregs, whereas high-dose tolerance relies on induction of anergy/apoptosis of effector lymphocytes. However, the definition of 'low' or 'high' is somehow arbitrary, being highly dependent on the antigen considered, and on the characteristics of the recipient receiving the hyposensitization protocol.

Although *in vitro* evidence has been provided that human allergic contact dermatitis (ACD) caused by nickel is a highly regulated process (19, 20), the possibility of inducing specific tolerance *in vivo* has not been adequately investigated. Indirect evidence that nickel allergy can be modulated and/or prevented *in vivo* has been provided by epidemiological studies reporting a lower frequency of nickel allergy in children wearing orthodontic braces prior to ear piercing (21, 22). More direct evidence has been provided by attempts to induce specific oral tolerance to the metal in nickel-allergic individuals. Bagot et al. demonstrated, in a double-blind study, that oral administration of 5 mg of nickel sulfate once weekly reduced the *in vitro* response of T cells to the metal in allergic patients, but failed to improve the clinical expression of ACD (23). In contrast, other reports showed that oral administration of 3.5 mg or 5 mg, but not 0.5 mg, of nickel sulfate once weekly for 6 weeks, as well as sublingual administration, significantly improved cutaneous manifestations and nickel reactivity (24, 25). Altogether, these findings strongly suggest the possibility of inducing oral tolerance to nickel in allergic patients.

The aim of our study was to investigate the therapeutic efficacy of 3 months of oral administration of 50 μg of nickel (given as $NiSO_4 \cdot 6H_2O$) in a population of nickel-allergic individuals, and to evaluate how this could modulate *in vitro* T cell responsiveness to the metal. The results showed that oral administration significantly reduced the severity of nickel allergy, and reduced specific T cell responses to the metal.

Material and Methods

Study population

Twenty-eight patients, 27 females and 1 male (age range 18–60 years, mean 39.5 years), with a documented history of contact dermatitis caused by metals and positive patch test reactions only to $NiSO_4 \cdot 6H_2O$ 5% in petrolatum were enrolled in the study. Nickel-allergic patients who reacted to other chemicals included in the Italian Society of Environmental, Occupational and Allergological Dermatology series and palladium were excluded from the study. Each patient answered a questionnaire regarding the extent and recurrence of their clinical manifestations, and was recommended to follow a diet with a low nickel content, in order not to interfere with the bioavailability of nickel therapeutically administered, and in an effort to standardize as much as possible within the protocol. Compliance with the diet was monitored by weekly telephone questionnaire and during visits. Topical immunosuppressive treatments were not allowed during the period of the study.

Patients included in the study gave informed written consent according to the Declaration of Helsinki with regard to scientific use, and under approval of the ethical committees of the three dermatological departments involved in the study.

Study design

This was an open multicentre study conducted in three dermatology clinics between September 2007 and May 2008. All patients enrolled presented a history of contact dermatitis caused by metals for at least 4 months (mean 14 years), confirmed by patch testing with $NiSO_4 \cdot 6H_2O$ 5% pet. (Table 1). Exclusion criteria were: concomitant sensitization to other chemicals; pregnancy; concomitant chronic dermatological diseases, including atopic dermatitis, and/or systemic diseases that could affect the outcome of the study; use of non-steroidal anti-inflammatory drugs, systemic corticosteroids or other systemic drugs in the previous 30 days; or topical immunosuppressive therapy in the previous 15 days.

The hyposensitizing protocol consisted of the daily oral administration of a single cellulose capsule containing 50 μg of nickel (given as $NiSO_4 \cdot 6H_2O$) (Capsugel®, Bornem, Belgium, obtained from Lofarma S.p.A., Milan, Italy) for 3 months. Each patient received six medical evaluations during the course of the study (Fig. 1). During the first visit (T0), each patient was carefully examined for evaluation of the affected body surface area (BSA). Eligible patients were tested with scalar dilutions of nickel sulfate ($NiSO_4 \cdot 6H_2O$), and blood samples were obtained

Table 1. Clinical characteristics of the study population

Patient no.	Age (years)	Sex	Time of onset of dermatitis	Localizations of lesions
1	27	F	7 y	Wrists, periumbilical region, neck
2	26	F	1.5 y	Ear lobes, neck, wrists, forearms
3	32	F	8 y	Ear lobes, wrists
4	37	F	20 y	Ear lobes, feet, legs
5	49	F	30 y	Ear lobes, neck, décolleté, periumbilicus region
6	27	F	2 y	Wrists, hands
7	41	F	22 y	Wrists, forearms, hands, feet, legs, thighs
8	60	M	2 y	Neck, wrists, forearms
9	60	F	10 y	Wrists, forearms
10	53	F	2 y	Ear lobes
11	32	F	18 y	Ear lobes, neck, periumbilicus region
12	55	F	30 y	Ear lobes, décolleté, wrists, feet, legs
13	41	F	24 y	Forearms, neck, periumbilicus region
14	29	F	18 y	Ear lobes, forearms, periumbilical region
15	39	F	28 y	Forearms, neck, periumbilical region, ear lobes
16	42	F	26 y	Neck, wrists, forearms, ear lobes
17	38	F	18 y	Ear lobes, wrists, hands
18	18	F	30 y	Forearms, neck, periumbilical region, ear lobes
19	54	F	36 y	Ear lobes, neck, periumbilicus region
20	39	F	16 y	Ear lobes, forearms, hands
21	41	F	20 y	Ear lobes, neck, wrists, periumbilical region
22	36	F	12 y	Ear lobes, neck, hands
23	60	F	3 y	Ear lobes, face (metallic frames), hands
24	26	F	1.8 y	Ear lobes, face (metallic frames), wrists, forearms
25	27	F	2.4 y	Face (metallic frames), hands
26	58	F	2 y	Face (metallic frames), hands, feet
27	21	F	4 m	Ear lobes, forearms, hands
28	39	F	1.8 y	Ear lobes, face (metallic frames), wrists, forearms

F, female; M, male; m, months; y, years.

for immunological investigations in 12 of them. Two days later, patch test reactivity was evaluated. At 1 month of treatment (T1) BSA was calculated, a second patch test with scalar dilutions of nickel sulfate was applied, and skin responsiveness was evaluated after 48 h. At 90 days (T3), BSA was again measured; a patch test with serial concentrations of nickel sulfate was applied, and read after 48 h, and blood samples for immunological study were obtained in the same 12 patients.

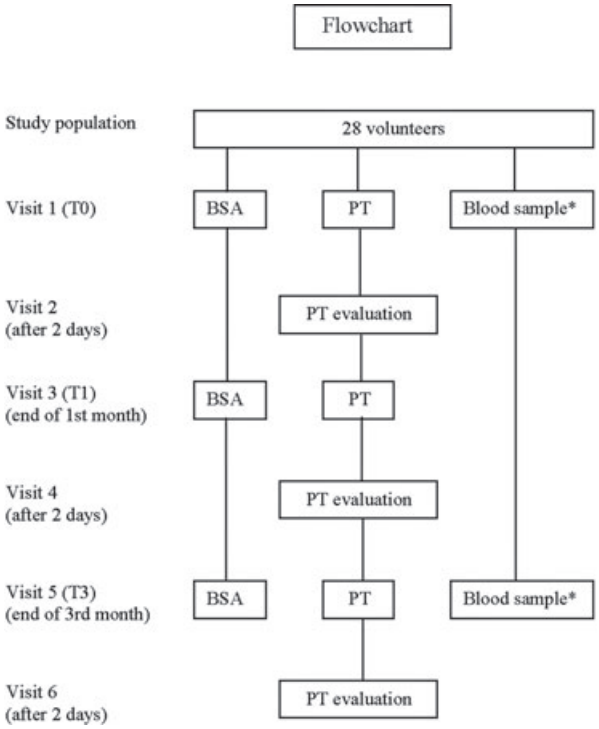

Fig. 1. Flowchart of the study. PT, patch test; BSA, body surface area. *Blood samples for immunological studies were collected from 12 patients.

Patch testing

Patch tests with scalar concentrations (2.5%, 1%, 0.5%, 0.1% and 0.05% wt/vol) of $NiSO_4 \cdot 6H_2O$ (Sigma Chemicals, St Louis, MO, USA) in water were performed at T0, T1, and T3.

The standard technique was used with Finn Chambers® on Scanpor® tape (8 mm in diameter; Epitest, Tuusula, Finland). In the Finn Chambers®, 15 µl of each nickel sulfate dilution was applied with a micropipette on a filter paper disc. The test chambers were left on the back for 2 days, and readings were taken 0.5–3 hr after removal and on day 4, and scored according to International Contact Dermatitis Research Group criteria (26), with two classifications added to the three usual positive gradings: strong + and ++ reactions were graded as +± and ++±, respectively. The different steps, including the intermediate steps, were defined as follows:−, negative reaction; +, erythema with papules; +±, erythema with papules and a few vesicles; ++, erythema with papules and vesicles; ++±, erythema with papules and spreading vesicles; and +++, erythema with papules and bullae.

To enable statistical calculations, the various scores were given values as follows:− = 0; + = 1; +± = 1.5; ++ = 2; ++± = 2.5; and +++ = 3.

The lowest concentration of nickel sulfate eliciting at least a positive reaction was ascertained for each patient, and registered as the minimal eliciting concentration (MEC). The scores for all reactions were summed to obtain a variable denominated as the summarized test score (STS).

In each of the three dermatology clinics, the same person tested the patients, whereas evaluation was performed by a second doctor who did not know the patients and the study design.

Lymphocyte isolation and culture

Twelve patients gave their consent for being enrolled in the immunological study: 50-ml blood samples were obtained at T0, before the initiation of the clinical trial, and at the end of the hyposensitization protocol (T3). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Lymphoprep (Nycomed-Pharmacia, Oslo, Norway), suspended in RPMI-1640 culture medium plus 40% fetal bovine serum (Hyclone, Logan, UT, USA) and 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA), and finally stored in liquid nitrogen.

Culture medium, reagents, and antibodies

Lymphocytes were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Lonza, Basel, Switzerland) (complete RPMI), plus 5% human serum (HS) (Sigma-Aldrich), as previously described (27).

Mouse allophycocyanin (APC)-conjugated and phycoerythrin (PE)-conjugated monoclonal anti-CD4 (SK2) and CD8 (SK1) and PE-conjugated anti-CD25 were obtained from BD Biosciences (San José, CA, USA). Mouse IgG isotype controls were purchased from BD Biosciences.

Assessment of nickel reactivity in vitro and fluorescence-activated cell sorting (FACS) staining

Proliferation assays were performed in flat 96-well plates (Corning Costar, Cambridge, MA, USA) by incubating thawed PBMCs (2×10^5 cells/well) in RPMI-1640 with 5% HS, in the presence or absence of $NiSO_4$ (20 μg/ml). After 5 days, wells were pulsed with 0.5 μCi of [^{3}H]thymidine for 8–10 hr, and the radioactivity incorporated was measured in a beta-counter. Results are given as stimulation index (SI), defined as the mean c.p.m. of the antigen-stimulated cells divided by the mean of the response in the absence of antigen. In selected experiments, results were confirmed by incubating PBMCs with 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) for 4 min at room temperature, as previously reported (28), prior to nickel exposure, and CFSE fluorescence was evaluated at day 7. Cell surface markers were detected upon staining with APC-conjugated and PE-conjugated monoclonal antibody or isotype controls, using a FACS Aria equipped with DIVA software (BD Biosciences).

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of PBMCs activated with $NiSO_4$ were collected at day 5. Concentrations of interferon (IFN)-γ, tumour necrosis factor (TNF)-α and IL-10 in cell-free supernatants were measured by using a commercial DuoSet ELISA system (R&D Systems, Minneapolis, MN, USA).

Evaluation of efficacy

Main efficacy outcomes were defined by: (i) total subjective and objective symptoms recorded by the patient from baseline to the end of treatment; (ii) the values for the variables investigated (MEC and STS); (iii) BSA; and (iv) results provided by the *in vitro* T cell responsiveness to nickel. Patients were evaluated for the severity of both subjective and objective dermatological symptoms and signs with a visual analogue scale (VAS) from 0 to 10, where 0 corresponded to the highest intensity of clinical signs and symptoms, and 10 was the complete absence of signs and symptoms. A patient's compliance with treatment was calculated as the mean of tablets counted on treatment days 30 and 90, with the following formula: [(tablets dispensed – tablets returned)/(date of final visit – date of initial visit) + 1].

Follow-up

Follow-up was performed every 3 months up to 1 year after the end of the study.

Statistical analysis

Statistical analysis was performed with Student's *t*-test.

Results

Patient baseline characteristics

Twenty-six of the 28 enrolled patients finished the study. Two patients discontinued the protocol because of adverse effects: one patient, a 55-year-old female, complained of

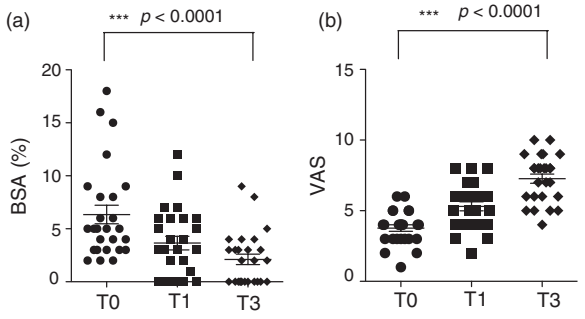

Fig. 2. The extent of skin involvement decreased and the visual analogue scale (VAS) score progressively increased upon hyposensitization therapy. (a) The percentage of body surface area (BSA) affected was measured before the initiation of the protocol (T0), and at 1 month (T1) and 3 months (T3) of oral nickel administration. (b) Patients evaluated the severity of both subjective and objective dermatological symptoms using a VAS from 0 to 10, where 0 corresponded to the highest intensity of clinical signs and symptoms, and 10 was the complete absence of signs and symptoms.

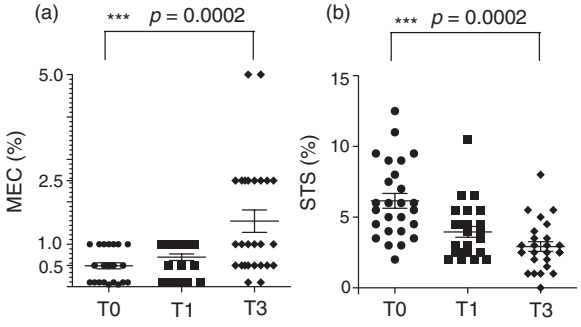

Fig. 3. The hyposensitization protocol decreased skin reactivity to nickel. Patch tests with scalar concentrations (2.5%, 1%, 0.5%, 0.1% and 0.05% wt/vol) of nickel sulfate hexahydrate (Sigma Chemicals, St Louis, MO, USA) in water were performed at T0, T1, and T3. (a) The lowest concentration of nickel sulfate eliciting at least a positive (+) reaction was ascertained for each patient, and registered as the minimal eliciting concentration (MEC). (b) The scores for all positive reactions were summed to obtain a variable called the summarized test score (STS).

itching, abdominal distension, dyspnoea and flushing after 3 days of treatment; and a 54-year-old female complained of worsening of skin changes at day 20 of treatment. In both cases, symptoms rapidly disappeared after discontinuation of the hyposensitization therapy. In the remaining cases, patient compliance with the protocol, in particular taking the daily doses of nickel, was good. We did not observe a flare-up at the site of a previous positive patch test with nickel sulfate 5% in any of these patients.

Effect of treatment on symptom scores

Oral hyposensitizing treatment progressively improved symptoms in all patients. BSA decreased from 6.34% (range 2–18%) to 3.65% (range 0–12%) at T1, and to 2.11% (range 0–9%) at T3 ($p < 0.0001$) (Fig. 2a). In aggregate, VAS increased from 3.76 (basal value, range 1–5) to 5.3 (range 2–8) at 1 month, and to 7.26 (range 4–10) at 3 months ($p < 0.0001$) (Fig. 2b). MEC progressively increased from 0.49% (range 0.05–1%) to 0.69% (range 0.1 to 1%) at T1, and to to 1.54% (range 0.1 to 5%) at T3 ($p = 0.0002$) (Fig. 3a). STS decreased from 6.15 (range 2–12.5) to 4.13 (range 2–10.5) at 1 month, and to 2.92 (range 0–5.5) at 3 months ($p = 0.0002$) (Fig. 3b).

In vitro nickel responsiveness of peripheral blood T cells is decreased upon hyposensitization

The immunological effects of oral hyposensitization were studied in 12 patients at T0 and at T3. The results showed a substantial decrease of *in vitro* T cell responsiveness to the metal, with an average reduction of the SI from 19.9 ± 5.7 [mean $\pm$ standard error (SE)] to 5.04 ± 0.98 (mean $\pm$ SE) ($p = 0.02$) (Fig. 4a). In particular, 11 of 12 patients had a strong decrease in SI in the presence of the metal (Fig. 4b).

To confirm these results, in 4 selected patients we investigated nickel responses of $CD4^+$ and $CD8^+$ T cells, using CFSE intravital staining. The results showed that, upon oral hyposensitization, the numbers of both $CD4^+$ and $CD8^+$ proliferating T cells and the magnitude of the cell division in the two subsets were strongly reduced (Fig. 5a–d).

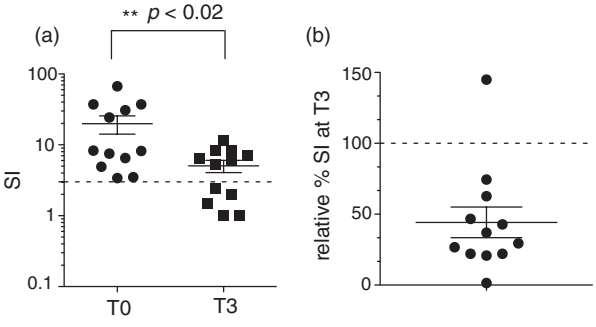

Fig. 4. *In vitro* nickel-specific T cell responsiveness decreased upon hyposensitization. (a) Increases in the numbers of peripheral blood mononuclear cells in the presence of nickel are given as stimulation index (SI) at T0 and T3. (b) Variation in the percentage of SI at T3 as compared with T0 in each patient is shown.

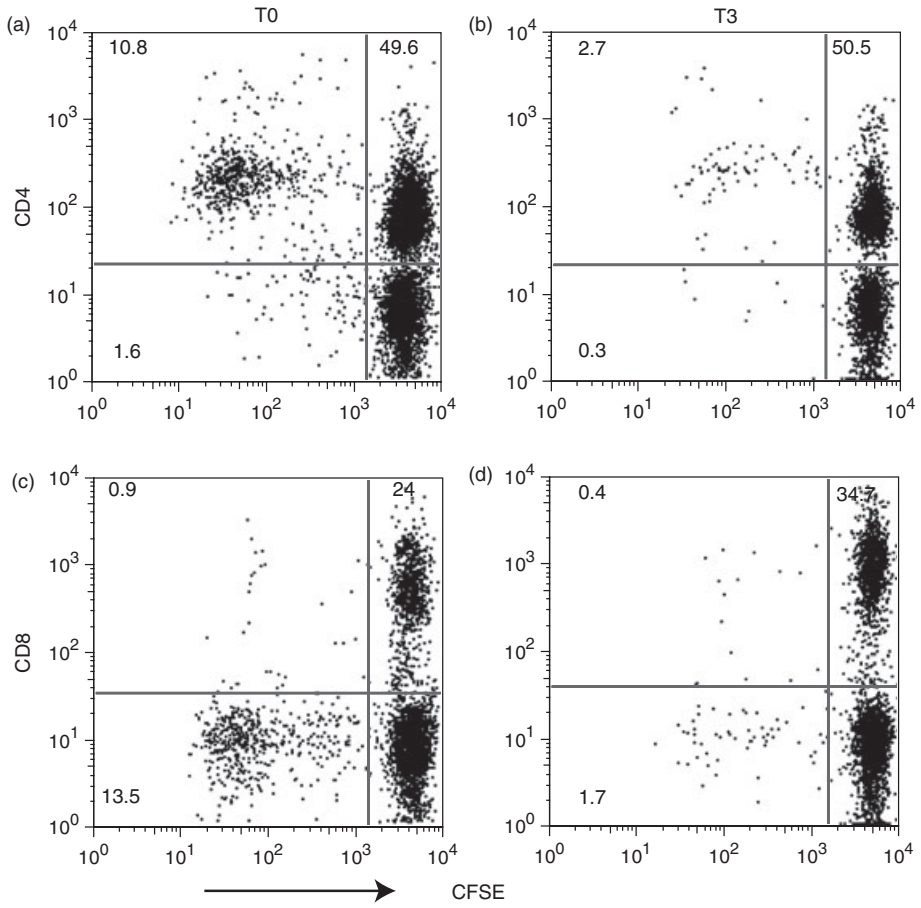

Fig. 5. Nickel-specific responsiveness of both $CD4^+$ and $CD8^+$ T cells is reduced after oral hyposensitization. Peripheral blood mononuclear cells obtained from 12 patients were incubated at a final concentration of 10^7 cells/ml in 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), incubated with nickel for 7 days, and finally incubated with monoclonal antibodies against CD4 or CD8. CD4+ (a, b) and CD8+ (c, d) dividing cells were measured by two-colour cytofluorimetric analysis. The figure shows the results obtained in one representative experiment of 4 patients investigated.

Oral hyposensitization decreases the amounts of IFN-γ and TNF-α, but not the amount of IL-10, released by peripheral blood T lymphocytes

To evaluate whether the decreased T cell proliferation was paralleled by modifications in the production of T cell cytokines upon nickel exposure, PBMCs were incubated for 5 days with nickel, and the supernatant was collected for measurement of cytokine content by ELISA. Figure 6 shows that nickel-stimulated T cells obtained from patients at the end of the oral hyposensitization protocol produced lower amounts of IFN-γ (Fig. 6a) and TNF-α (Fig. 6b) than T cells isolated prior to therapy. However, the level of IL-10 (Fig. 6c), a cytokine that has been linked in animal models with oral tolerance, was not significantly affected by the induction of oral tolerance.

Number of circulating $CD4^+$ $CD25^{high}$ T cells is not affected by oral hyposensitization

To investigate whether oral hyposensitization was followed by an increase in the frequency of Tregs, the percentage of circulating $CD4^+$ $CD25^{high}$ T cells was determined before and after treatment in 4 responsive patients. FACS staining failed to demonstrate an increase in the percentage of Tregs in all cases investigated (Fig. 7).

Clinical follow-up

Follow-up at 12 months from the end of the treatment was possible in 24 of 26 patients who finished the study. Twelve (50%) patients remained clear of dermatitis despite the continuation of exposure to metal. The remaining patients had a deterioration of the dermatitis: in 4 (16.7%) this occurred soon after the termination of the treatment, whereas in the remaining 8 patients (33.3%), relapse was observed after 2–6 months upon exposure to metal.

Discussion

Our results show that oral hyposensitization is a promising approach for the management of nickel allergy.

In all 26 patients who completed the study, we observed a significant improvement of the severity of clinical

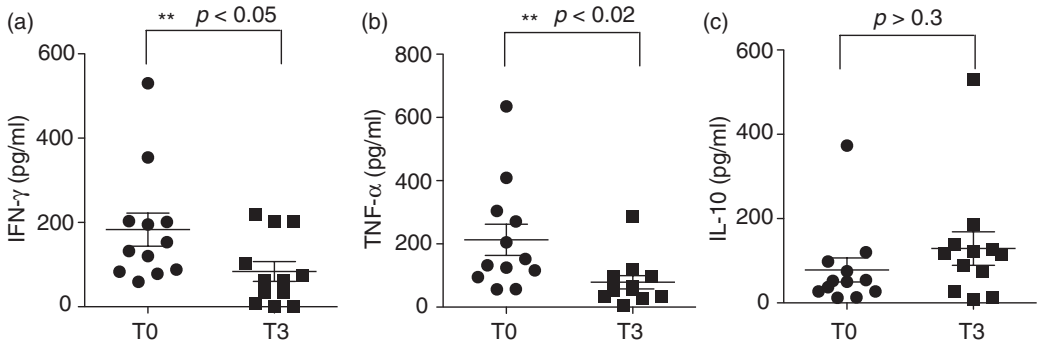

Fig. 6. Nickel-specific T lymphocytes released less interferon (IFN)-γ and tumour necrosis factor (TNF)-α after oral hyposensitization. Supernatants of peripheral blood mononuclear cells obtained from each patient at T0 and T3 were activated for 5 days with $NiSO_4$ and filtered, and the concentrations of IFN-γ (a), TNF-α (b) and interleukin (IL)-10 (c) were evaluated by enzyme-linked immunosorbent assay.

manifestations, with rapid reduction of itching and of extension of the eczematous dermatitis. The reduced clinical severity of the dermatitis was accompanied by a decreased responsiveness to nickel skin challenge, as MEC increased from the average values of 0.49–1.54% and STS decreased from values of 6.15–2.92. Accordingly, patient subjective evaluation, measured as VAS, confirmed the efficacy of the therapeutic regimen. Importantly, clinical improvement was accompanied by a significant reduction of *in vitro* nickel responsiveness of both $CD4^+$ and $CD8^+$ T lymphocytes in all but one patient. Average SI decreased consistently from 19.9 ± 5.7 at T0 to 5.04 ± 0.98 at T3. All but 1 patient showed a significant reduction of T cell proliferation *in vitro* (ranging from 28% to 95%). Decreased T cell proliferation was paralleled by impaired secretion of IFN-γ and TNF-α, whereas secretion of IL-10 remained unchanged.

The design of a hyposensitization protocol must take into consideration several important aspects that may influence the efficacy and safety of the therapeutic approach. Of major importance are the dose and the duration of treatment. In mice, induction of high-dose and low-dose tolerance appears to be mediated by distinct immunological mechanisms, the first involving mostly an increase in the number of T cells with regulatory functions, and the second involving the induction of

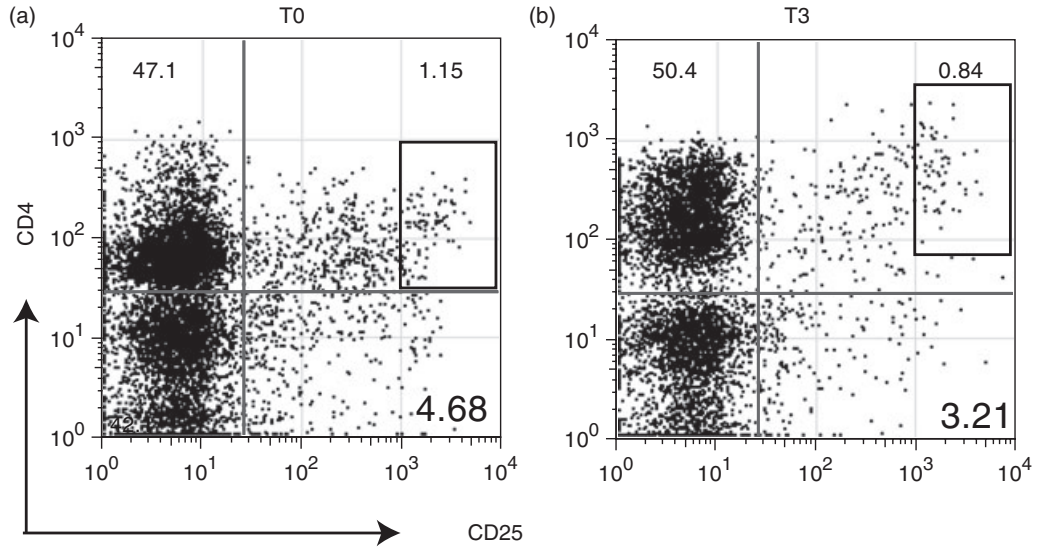

Fig. 7. The number of $CD4^+CD25^{high}$ T lymphocytes is not increased after oral hyposensitization. Peripheral blood mononuclear cells obtained from 4 patients at T0 (a) and T3 (b) were washed with phosphate-buffered saline (PBS) added with 1% human serum and 0.01% NaN_3, and stained with phycoerythrin-conjugated and APC-conjugated monoclonal antibodies. Staining with matched isotype control IgG was included. The results shown are from one representative experiment of four performed.

functional anergy or apoptosis (14, 29). However, these findings cannot be immediately translated to humans. Indeed, high and low antigen concentrations may depend on the route of administration, on the biological availability of the compound, and on the capacity of resident antigen-presenting cells to capture and then to present the antigen to naïve T lymphocytes in regional lymph nodes. Previous observations indicated that substantial amelioration of nickel allergy could be achieved with 5 or 3.5 mg, but not 0.5 mg, of nickel sulfate administered once weekly (21, 24, 30). In contrast, the results obtained in our trial show that clinical manifestation are significantly improved, and *in vitro* T cell responsiveness to nickel decreased with a daily dose of 50 μg of nickel.

One additional critical aspect concerns the necessity for a maintenance protocol aimed at reducing the risk of relapses. Our results indicate that this may be the case. Indeed, upon termination of the oral administration, 50% of the patients relapsed during the 12-month follow-up period, although with decreased severity of the clinical manifestations.

Although immunological data showed reduced nickel responsiveness and impaired IFN-γ and TNF-α responses to the metal in responding patients, neither the release of IL-10 upon nickel exposure nor the relative percentage of circulating $CD4^+CD25^{high}$ cells was modified during the desensitization protocol. Despite these findings, we could not exclude a role of $CD4^+CD25^{high}$ Tregs in the tolerogenic mechanism, as the mere presence of these cells does not necessarily correlate with the presence of nickel-specific tolerance. Owing to the limited amount of human material available, it was not possible to investigate in detail the mechanisms responsible for the observed reduction in nickel responsiveness; further functional studies are required to investigate the mechanisms underlying the induction of nickel oral tolerance.

In conclusion, our study suggests the clinical efficacy of oral hyposensitization in nickel-allergic individuals. Further studies designed with proper placebo controlled groups are required to confirm the observation and determine the optimal concentration and administration intervals for efficacy, safety and persistence of clinical improvement.

Acknowledgements

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