

## Short communication

# Molecular analysis of T-cell clonality with concomitant specific T-cell proliferation *in vitro* in nickel-allergic individuals

**Background:** The peripheral blood mononuclear cells (PBMC) of individuals with nickel contact allergy are reported to proliferate to a varying degree upon nickel stimulation *in vitro*. Different phenotypes of the T cells involved are described. With regard to preferential use of the T-cell receptor (TCR), analysis of the several families of the TCR- $\gamma$  gene allows rearrangement evaluation of all T cells regardless of predominant surface expression of TCR  $\alpha/\beta$ .

**Methods:** The PBMC of 10 nickel-allergic and five nonallergic individuals were cultured for 4 days in the presence of either medium, PHA, NiSO<sub>4</sub>, or tetanus toxoid (TT). Proliferation was measured by radioactive thymidine uptake and expressed as stimulation index (SI). T-cell clonality was assessed by analysis of the TCR- $\gamma$  chain gene, including the use of PCR with a primer combination covering the four main groups (V $\gamma$ 1-8, V $\gamma$ 9, V $\gamma$ 10, and V $\gamma$ 11) of the variable region of the TCR- $\beta$  chain gene.

**Results:** In the allergic individuals, proliferation to NiSO<sub>4</sub> was significantly ( $P < 0.05$ ) higher than in nonallergics (mean SI: 18.05/17.87 vs 0.67/2.27). In unstimulated and PHA-stimulated cultures, there was a random TCR spectrum in both groups. In contrast, in nickel-allergic individuals or individuals with recent TT-booster, oligoclonality could be observed in the correspondingly stimulated cultures.

**Conclusions:** In addition to proliferation assay, analysis of T-cell clonality may be a further means to characterize clinical hypersensitivity reactions on the basis of antigen-dependent oligoclonal T-cell expansion, as in the case of tissue-infiltrating lymphocytes.

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Key words: allergy; clonality; nickel; PCR;  
proliferation; T-cell receptor- $\gamma$ ; T cells.

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Accepted for publication 22 March 2001

Nickel is one of the most frequent elicitors of contact allergy in the general population. The sensitization rate is in the range 2–12%, depending on age, sex, and living habits (1–3). Besides typical clinical findings such as hand eczema, there are also unusual manifestations, such as pseudolymphoma formation, airborne contact eczema, or implant-associated intolerance reactions (1–7). Nickel allergy as a delayed-type hypersensitivity is reflected in eczematous reactions upon patch testing and by an antigen-specific T-cell proliferation *in vitro* upon appropriate stimulation (8–11). Here we examined to what extent the proliferative response of PBMC from nickel-allergic and nonallergic individuals to nickel stimulation *in vitro* would be paralleled by clonal T-cell expansion. The potentially restricted use of the T-cell receptor (TCR) and clonal expansion of specifically reacting T cells was examined by PCR analysis using individual primers for each of the four main groups of variable genes of the TCR- $\gamma$ .

## Material and methods

### Patients

Ten individuals allergic to nickel (eight female, two male, aged 21–60 years; with history of eczema and patch test reaction to nickel sulfate, nos. 1–10 in Table 1) and five control persons (three female, two male, aged 35–72 years; with history of eczema due to other proven contact allergy, nos. 11–15 in Table 1) volunteered as blood donors.

### Isolation of cells and cultures

PBMC were obtained from heparinized venous blood (30 ml) by Ficoll-Hypaque density gradient centrifugation. The cells were resuspended at  $1 \times 10^6/\text{ml}$  in 10% AB serum containing RPMI 1640 medium supplemented with HEPES (25 mM), L-Glutamin, antibiotic-antimycotic solution (Gibco International), and NEAA and MEM vitamins (Biochrom, Germany). Subsequent cultures were done in quadruplicate in 24-well, polystyrene culture plates (Nalge Nunc International, Denmark).

### Stimulation

The cells were cultured for 4 days with complete medium alone (control) or with addition of PHA (2.4  $\mu\text{g}/\text{ml}$ , Biochrom, Germany),

Table 1. Preferential expression of TCR- $\gamma$  families in PBMC of nickel-allergic subjects (nos. 1–10) and individuals with other contact allergy (nos. 11–15) upon culture with medium alone or after addition of following stimuli: PHA (phytohemagglutinin), TT (tetanus toxoid), NiSO<sub>4</sub> (nickel sulfate)

| Patient no. | Medium | PHA | TT   | NiSO <sub>4</sub> 10 <sup>-4</sup> M | NiSO <sub>4</sub> 10 <sup>-5</sup> M | NiSO <sub>4</sub> 10 <sup>-6</sup> M |
|-------------|--------|-----|------|--------------------------------------|--------------------------------------|--------------------------------------|
| 1           | –      | –   | Vg11 | –                                    | –                                    | Vg2                                  |
| 2           | –      | –   | Vg11 | Vg10                                 | –                                    | –                                    |
| 3           | –      | –   | Vg11 | Vg10                                 | Vg10                                 | –                                    |
| 4           | –      | –   | Vg9  | Vg9                                  | Vg9                                  | –                                    |
| 5           | –      | –   | Vg11 | Vg2, Vg11                            | –                                    | Vg10                                 |
| 6           | Vg9    | Vg9 | Vg9  | Vg9                                  | Vg9                                  | Vg9                                  |
| 7           | –      | –   | –    | Vg10                                 | –                                    | Vg11                                 |
| 8           | –      | –   | Vg11 | Vg10                                 | Vg10                                 | –                                    |
| 9           | –      | –   | –    | –                                    | –                                    | –                                    |
| 10          | –      | –   | Vg9  | Vg2                                  | Vg2                                  | Vg2                                  |
| 11          | –      | –   | Vg11 | –                                    | Vg11                                 | Vg11                                 |
| 12          | –      | –   | Vg9  | Vg2, Vg9                             | Vg9                                  | Vg9                                  |
| 13          | –      | –   | Vg9  | –                                    | –                                    | Vg10                                 |
| 14          | Vg10   | Vg2 | Vg10 | Vg2                                  | –                                    | Vg11                                 |
| 15          | –      | –   | –    | Vg11                                 | Vg10                                 | Vg9                                  |

TT (5  $\mu$ g/ml), or NiSO<sub>4</sub> (10<sup>-4</sup> M, 10<sup>-5</sup> M, 10<sup>-6</sup> M, Sigma, Germany). On day 5, <sup>3</sup>H-thymidine was added to half of the cultures. Overnight, incorporated radioactivity was assessed by liquid scintillation after harvesting homogenized cells. The stimulation index (SI) was defined by the ratio of mean cpm of stimulated to unstimulated cultures. In parallel, the nonradioactive cultures were used for DNA analysis.

#### DNA isolation

DNA was isolated by the Fast Prep Cells and Tissue DNA Isolation Kit® (Pharmacia, Germany). According to standard procedures (12), a substitution of organic solvents for the salts in the deproteinization step was done, and the cells were lysed by anionic detergent in the presence of the DNA preservative. Contaminating RNA was removed by RNase A, and cytoplasmic and nuclear proteins by salt precipitation. Genomic DNA was then isolated by precipitation with alcohol.

#### PCR

To cover the range of the TCR  $\gamma$ -chain gene (12–14), we used a combination of primers of the four main groups of the variable region (V $\gamma$ 1–8, V $\gamma$ 9, V $\gamma$ 10, and V $\gamma$ 11) and a primer mix containing four joining primers (J $\gamma$ 2, J $\gamma$ p, J $\gamma$ p1, and J $\gamma$ p2). The TCR region V $\gamma$ 1–8 was detected with the consensus primer V $\gamma$ 2 as follows. V $\gamma$ 2: TACATCCACTGG TACCTACACCAG; V $\gamma$ 9: GAAAGGAATCTGGCATTCGTCAG; V $\gamma$ 10: AAGCAACAAAGTGGAGGC-AAGAAAG; V $\gamma$ 11: AGTAAAAATGCTCACACTTCCACTTC; J $\gamma$ 2: TACCTGTGACAA CAAGTGTGTGTC; J $\gamma$ p: AAGCTTTGTTCCGGGACCAATAC; J $\gamma$ p1: GAAGTTA CTATGAGCTT-AGTCCCTT; J $\gamma$ p2: GAAGTTACTATGAGCCTAGTCCCTT. As positive controls, DNA of Jurkat, a T-cell lymphoma cell line, was used for the amplification of V $\gamma$ 1–8 and V $\gamma$ 11, and HSB2, also a T-cell lymphoma line, for the amplification of V $\gamma$ 9 and V $\gamma$ 10. The volume of one PCR assay was 50  $\mu$ l. It consisted of 1  $\mu$ l DNA, 10  $\mu$ l primer mix (1 mmol/l of the V $\gamma$  primer and 0.6 mmol/l of each of the J $\gamma$  primers), 10  $\mu$ l 10x buffer (20 mmol/l Tris-HCl, pH 8.4, 1.5 mmol/l MgCl<sub>2</sub>, 50 mmol/l KCl, and 0.01% gelatin), 24.5  $\mu$ l H<sub>2</sub>O, 4  $\mu$ l dNTP-mix (10 mM), and 0.5  $\mu$ l Taq-polymerase (all Pharmacia). First,  $\beta$ -actin-specific DNA was amplified as a quality control. Then four PCR steps for the TCR- $\gamma$  chain were done. The PCR started with an 8-min

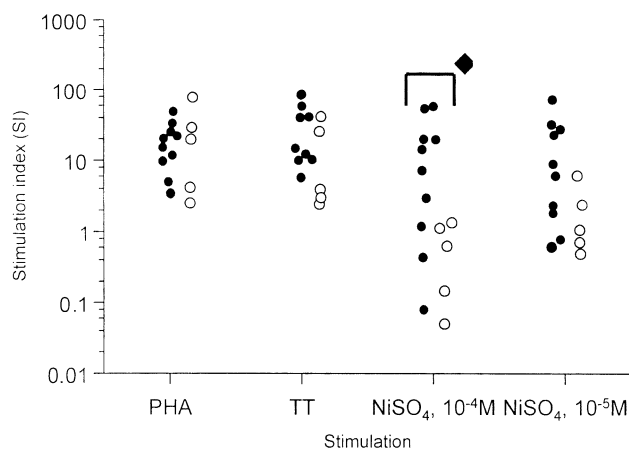


Figure 1. Proliferative response (stimulation index) from PBMC of 10 nickel-allergic (●) and five nonallergic (○) individuals; ◆:  $P < 0.05$ .

denaturing step at 95°C, followed by 45 cycles (1 min 95°C, 1 min 62°C, and 1 min 72°C), and ended with a final extension at 72°C for 7 min to fill the gaps in the DNA.

#### Detection

The PCR products were separated on a 6% polyacrylamid-gel and stained with ethidium bromide. The gel was photographed under UV light and screened for distinct bands reflecting clonal expansion of the few specifically reacting T-cell populations.

#### Results

##### Lymphocyte proliferation test

As shown in Fig. 1, a specific proliferation of PBMC could be detected in nickel-allergic patients upon addition of NiSO<sub>4</sub>. The mean SI was  $18.05 \pm 10.95$  for NiSO<sub>4</sub> 10<sup>-4</sup> M and  $17.87 \pm 11.74$  for NiSO<sub>4</sub> 10<sup>-5</sup> M ( $P < 0.05$  when compared to the nonallergic patients by Mann-Whitney U-test). In the controls, only marginal effects were seen ( $0.67 \pm 0.65$  for NiSO<sub>4</sub> 10<sup>-4</sup> M and  $2.27 \pm 3.03$  for NiSO<sub>4</sub> 10<sup>-5</sup> M).

##### Analysis of TCR- $\gamma$ gene rearrangement

TCR- $\gamma$  chain gene rearrangement could be found by the use of PCR (Table 1). Most of the nickel-allergic patients showed proliferation of T cells with the V $\gamma$ 10 specificity. One patient showed an oligoclonal rearrangement in three of the four families. Two patients had only T-cell clones with V $\gamma$ 9, four with V $\gamma$ 2 and two with V $\gamma$ 11 specificity. However, V $\gamma$ 2 was represented in two of the five control patients. Four control patients had more than one clonal rearrangement or no specific rearrangement was detectable. For further study of the clonal rearrangement of the TCR- $\gamma$  gene in nickel-stimulated cultures, unstimulated medium cultures were used as comparison. Our finding of clonal rearrange-

ment in the PCR pattern in some control cultures was interpreted to reflect active eczema in these patients or other actual T-cell-stimulating exposure to antigen.

## Discussion

Cell-culture studies and tissue reactions in the proximity of metallic devices suggest that high nickel or cobalt load may be both immunosuppressive and toxic (15–19), and that low exposure may directly induce adhesion molecules such as ICAM-1 on vascular endothelium, thus favoring proinflammatory cell recruitment (20). In allergic individuals, small amounts of nickel can provoke contact eczema (1, 2), and specific T-cell reactivity may be shown by lymphocyte transformation assays using PBMC (21–23) or cells recovered from patch test sites (24). Upon hematogenous nickel exposure, patients who develop eczema also seem to have a higher number of circulating nickel-specific T cells (25). A MHC class II-restricted nickel recognition has been described (26, 27). However, nickel-specific T cells have been shown to be either CD4<sup>+</sup> or CD8<sup>+</sup> (21, 22, 28) and to produce T helper 1 (Th1) cytokines (IL-2, IFN- $\gamma$ ), Th2 (IL-4, IL-5) cytokines, or even predominantly IL-10 (21, 24, 26). Nickel-allergic individuals have also been reported to express a limited TCR- $\beta$  repertoire (24). Furthermore, amino-acid sequences in the TCR BV17 chain were found to be crucial for nickel recognition (29).

With a different approach, we examined whether restricted use of the TCR in nickel-stimulated PBMC would result in an oligoclonal T-cell expansion made visible by gene rearrangement analysis. Although 98–99% of human T cells show the  $\alpha\beta$  phenotype and about 1–2% are  $\gamma\delta$  T cells, the  $\gamma$  locus, independently of the TCR expression, is more frequently rearranged than the  $\beta$  locus, which often remains in germline configuration (30). MacDonald & Wilson (31) suggested a modified separate lineage model with commitment of either the  $\alpha\beta$  or the  $\gamma\delta$  lineage prior to the TCR gene rearrangement. During maturation,  $\alpha\beta$  or  $\gamma\delta$  TCR are considered to rescue appropriate lineage-committed

cells. The TCR  $\beta$ ,  $\gamma$ , and  $\delta$  genes rearrange in the same fashion in committed precursor cells of both lineages. Rearranged  $\gamma\delta$  genes are therefore also found in  $\alpha\beta$  T cells and vice versa.

In contrast to nonallergic donors, clonal expansion as reflected by overrepresentation of TCR V $\beta$  elements has been found not only among nickel-reactive individuals (24, 29, 32), but also in paraphenylenediamine-allergic persons (33). By T-cell clonotypic analysis, oligoclonal accumulation of T cells was seen in atopic eczema and interpreted as an antigen/allergen-driven process (34). In the case of nickel-reactive clones, Vollmer et al. excluded superantigen-like recognition, since the specific combination of  $\alpha$ - and  $\beta$ -chains was required for reactivity to nickel (35). We used the TCR- $\gamma$  chain, which has a less complicated genome organization, for clonality detection assays.

We found mono- or oligoclonal T-cell expansion in the nickel-stimulated PBMC of allergic donors. The few cases of different TCR- $\gamma$ -specificity for different NiSO<sub>4</sub> concentrations may be due to incomplete equivalence between TCR $\alpha/\beta$  surface specificity and TCR- $\gamma$  gene rearrangement. If clonal bands in nonallergic patients were detected, it was a broad array covering the series of TCR- $\gamma$  specifications. In accordance with other reports (24, 29), we interpreted our findings as indicating that nickel-stimulated T cells have a heterogeneous but selective usage of the TCR. However, interpretation of the results should always include the clinical picture. Thus, the combined analysis of T-cell clonality and mediator production, as in the investigation of suspected adverse reactions to metallic implants by analysis of peri-implantar tissue (15), may offer further help in identifying specific cellular immune reactions.

## Acknowledgments

We thank Mrs S. Barnstorf and Mrs U. Puchta for skillful technical assistance. We also thank the Bavarian Ministry of Science, Research and Art for financial support.

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