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Immunopharmacology and Immunotoxicology

Publication details, including instructions for authors and subscription information:
<http://www.informaworld.com/smpp/title-content=t713597257>

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Online Publication Date: 01 June 2008

To cite this Article: Falsafi-Amin, Hale, Holst, Mikael and Lundeberg, Lena (2008) 'Early Expression of Interleukin-2 mRNA by Peripheral Blood Mononuclear Cells Isolated from Nickel-Allergic Subjects and Subsequently Exposed to Nickel *In Vitro*', Immunopharmacology and Immunotoxicology, 30:2, 227 — 234

To link to this article: DOI: 10.1080/08923970801948960

URL: <http://dx.doi.org/10.1080/08923970801948960>

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Early Expression of Interleukin-2 mRNA by Peripheral Blood Mononuclear Cells Isolated from Nickel-Allergic Subjects and Subsequently Exposed to Nickel *In Vitro*

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The aim of the present study was to characterize the time course of the synthesis of mRNA encoding interleukin-2 (IL-2) by peripheral blood mononuclear cells (PBMCs) isolated from nickel-allergic women and subsequently exposed to nickel sulphate *in vitro*. The level of this mRNA (determined by employing real-time polymerase chain reaction [PCR]) and of the corresponding protein (quantitated using an enzyme-linked immunosorbent assay [ELISA]) were significantly increased after only 10 and 24 h of incubation, respectively. The increased level of mRNA coding for IL-2 supports the hypothesis that this process is a key part of the response to nickel stimulation of PBMCs of hypersensitive subjects.

Keywords Contact Allergy, IL-2, Nickel, Peripheral Blood Mononuclear Cells.

INTRODUCTION

Ten percent to 15% of the female population in Europe suffers from nickel allergy.⁽¹⁾ The golden standard for diagnosing contact allergy is epicutaneous patch testing, in connection with which the skin is exposed to various substances

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of interest. Development of erythema, swelling, and vesicles after such contact with an antigen indicates the presence of an allergic reaction.

Nickel allergy is a delayed hypersensitivity reaction, the initial phase of which consists of a T cell-mediated antigen-specific immune response. This reaction is associated with a number of other responses at the cellular level, including DNA synthesis and release of cytokines. Accordingly, measurement of the rate of proliferation of peripheral blood lymphocytes *in vitro* in the presence of nickel has been considered as a possible alternative test for nickel allergy. The major problem in this connection has been nickel-induced stimulation of lymphocytes from control individuals without clinical symptoms of nickel allergy, i.e., false positives.⁽²⁾ Attempts have been made to explain such stimulation, at least in certain of these cases, by the presence of a latent allergy to nickel.

T-cell clones sensitized specifically to nickel, isolated from both nickel-allergic and nonallergic donors, appear to produce cytokines in a pattern characteristic of Th1 cells⁽³⁾ (i.e., interferon- γ [IFN- γ], interleukin-2 [IL-2], and tumor necrosis factor- β [TNF- β]). However, a predominance of Th2 cells (which release IL-4, IL-5, IL-6, IL-10, IL-13, and, possibly, IL-9) as well as Th0 cells (IL-2) has been observed in the skin of patients allergic to nickel.⁽⁴⁾ This indicates that the pattern of cytokines produced in response to nickel may vary with time, depending on when these various cell types become involved.

In a previous study,⁽⁵⁾ enhanced DNA synthesis and elevated levels of IL-2, IL-4, and to some extent, TNF- β were observed in peripheral blood mononuclear cells (PBMCs) following exposure of nickel-allergic patients to this allergen. The primary cytokine response appeared to involve IL-2, the serum level of which was elevated in all of the nickel-allergic subjects examined. Furthermore, employing the ELISpot technique, Lindeman et al.⁽⁶⁾ demonstrated enhanced secretion of IL-2 by PBMCs isolated from nickel-allergic patients challenged with nickel sulphate. Thus, IL-2 appears to be a critical cytokine released by PBMCs of nickel-allergic patients at an early stage in their response to nickel.

The aim of the present investigation has been to elucidate the early responses of the PBMCs of nickel-allergic individuals to nickel in greater detail. To this end, the level of IL-2 mRNA in these cells has been quantitated by polymerase chain reaction (PCR), and their secretion of IL-2 *in vitro* determined using an enzyme-linked immunosorbent assay (ELISA).

SUBJECTS AND METHODS

Subjects

Seven women (28 to 75 years of age, with a mean age of 50 years) with a history of nickel allergy (confirmed by a patch test reaction scored as ++ [erythema, edema, papules] or +++ [erythema, edema, papules, and vesicles] according to the recommendations of the International Contact Dermatitis

Table 1: Characteristics of patients.

Subject	Age (years)	Patch test reactivity to NiSO ₄	Year patch test	Year blood sample	Other contact allergies
1	51	+++	1979	2000	Neg
2	47	++	1999	2000	Neg
3	44	+++	1990	2000	Neg
4	60	+++	1998	2000	Neg
5	77	+++	>10 years ago	2000	Neg
6	27	++	2000	2000	Neg
7	43	++	2000	2000	Neg

Research Group [ICDRG]), and seven control women (age-matched, with negative patch tests, and no history of nickel allergy) participated in this study (Table 1). The patch tests had been applied on the backs of the patients and controls for 48 h. The results were evaluated after 72 h and after 6 days. This study was approved by the local Medical Ethics Committee at the Karolinska University Hospital (Stockholm, Sweden), and all subjects gave their informed consent to participate.

Preparation of PBMCs

Blood samples obtained by venous puncture were collected in heparinized tubes and subjected to density gradient centrifugation on a Ficoll-Paque (Pharmacia-Upjohn, Uppsala, Sweden) gradient.⁽⁷⁾ The mononuclear cells thus separated were washed with phosphate-buffered saline (PBS) and subsequently incubated (2×10^6 cells/ml) in RPMI 1640 culture medium (Seromed Biochrom KG, Berlin, Germany) supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), and 15 % heat-inactivated human AB serum. From this stock culture, 1-ml aliquots were removed and preincubated for 30 min, following which 200 µl nickel sulphate (3.8×10^{-5} M) was added to each of half of these cell suspensions and the same volume of 0.9% saline to the remainder. These cultures were then incubated for 5, 10, 24 and 48 h at 37°C under an atmosphere containing 5% CO₂.

RNA Isolation

RNA was isolated from the cells in suspensions by employing a solution of phenol and guanidine isothiocyanate (TRIZOL Reagent; Life Technologies, Gaithersburg, MD) in accordance with the instructions of the manufacturer. In brief, the cell suspensions were lysed with the TRIZOL Reagent, after which chloroform was added and the aqueous phase containing the RNA was separated by centrifugation. The RNA was then precipitated with isopropyl

alcohol, washed with 75% ethanol by centrifugation, redissolved in RNase-free water and stored at -80°C until use.

Analysis of IL-2 mRNA by Real-Time Polymerase Chain Reaction

To synthesize cDNA, the isolated RNA was processed further using the Superscript cDNA kit (Life Technologies) and Oligo(dT) Primer (Invitrogen Life Technologies, Sweden), in accordance with the manufacturers' protocols. For this purpose, two nucleotide primers based on the sequence of the human IL-2 mRNA published by Devos et al.⁽⁸⁾ were prepared as templates. The 20-nucleotide upstream primer (5'-TTAAGTTTACATGCCCAAG-3') corresponded to positions 292 to 311 in this mRNA and the 23-nucleotide downstream primer (5'-AGATCCATATTTATAGTTTAAAG-3') to positions 707 to 685. These primers were carefully chosen to encompass two introns in order to avoid amplification of IL-2 DNA. The level of expression of mRNA encoding the housekeeping protein β -actin in the lymphocytes was determined as an internal standard.

PCR was performed on a Perkin-Elmer GeneAmp PCR System 2400 in a 50- μl reaction volume containing 0.5 μM primers, 0.2 mM dNTP, 1.5 mM MgCl_2 , and 1.75 U Expand High Fidelity (Roche Diagnostics Scandinavia, Sweden). The reaction steps included a time delay (at 96°C for 5 min), the step cycle (96°C for 30 s, 52°C for 2 min, and 72°C for 2 min, repeated altogether 35 times), a subsequent 5-min time delay at 72°C , and, finally, a soak at 4°C for at least 5 min. Thereafter, aliquots (10 μl) of all samples were separated on a 2% agarose gel containing ethidium bromide (15 $\mu\text{g}/100$ ml gel). The gel was run for 1.5 h at 75 V and photographed under ultraviolet (UV) light. The levels of mRNA coding for IL-2 and the housekeeping protein were analyzed utilizing an HP Scanjet 7400c flatbed scanner (Hewlett-Packard, USA) and the ImageJ 1.29x image-processing program (National Institutes of Health, USA).

Quantitation of the IL-2 Protein

Following incubation (see above), the supernatants from the nickel-treated and control cell cultures were analyzed for IL-2 by an ELISA (Duo Set ELISA; Genzyme Diagnostics, Cambridge, MA, USA), in accordance with the protocol supplied by the manufacturer. In brief, flat-bottomed, high-binding plates (Costar, Cambridge, MA, USA) were coated with the capture antibody (diluted in 0.1 M sodium carbonate, pH 9.5; 100 μl per well) by incubation overnight at 4°C . Thereafter, these plates were blocked with PBS (0.01 M, pH 7.3) containing 4% bovine serum albumin (BSA) (250 μl per well) by incubation for 2 h at 37°C . Next, standards and samples were added in duplicate to separate wells (100 μl per well) and incubated for 1 h at 37°C , followed by addition of 100 μl of a solution of the diluted second antibody and incubation for 1 h at 37°C .

After this step, 100 μl of the detection reagent was added to each well and incubation continued for 15 min at 37°C . Between steps, the plates were routinely

washed extensively with PBS/0.05% Tween 20. Finally, tetramethyl benzidine (TMB) (Sigma, St Louis, MO, USA) and hydrogen peroxidase (100 μ l per well) were added, and, following incubation for 10 min at room temperature, stop solution (2 N H_2SO_4) was added and the absorbance of the solution in the wells at 450 nm was determined within 30 min using an ELISA reader (Vmax; Molecular Device, CA, USA).

Statistical Analysis

The nonparametric Mann-Whitney test was employed to compare the values obtained with the nickel- and saline-treated cultures, a p value of <0.05 being regarded as statistically significant.

RESULTS

The level of mRNA coding for IL-2 in nickel-stimulated cultures of PBMCs isolated from the nickel-allergic subjects increased distinctly after 10 and 24 h of incubation, to decrease again somewhat after 48 h (Figure 1). The difference between the IL-2 mRNA levels in nickel-stimulated and control cells was statistically significant ($p < 0.05$) at all time points investigated.

Although the level of IL-2-protein in the medium of nickel-stimulated cultures of PBMCs isolated from nickel-allergic patients also increased, most obviously after 24 and 48 h of incubation (Figure 2), these levels were not significantly different than those observed for the control (saline) cultures.

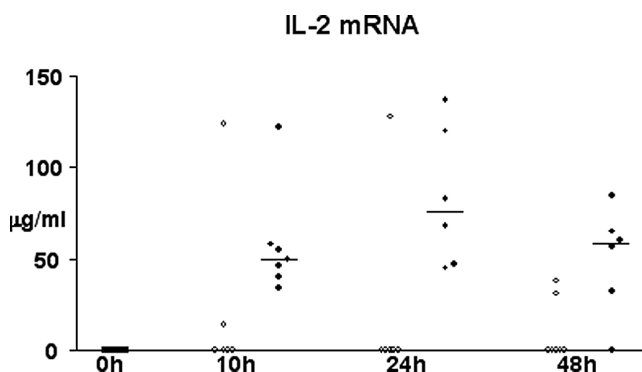


Figure 1: The time course of the IL-2 mRNA expression by peripheral blood mononuclear cells (PBMCs) isolated from seven nickel-allergic patients and subsequently incubated for 5, 10, 24, and 48 h with nickel sulphate (filled circles) or saline (open circles). The levels of IL-2 mRNA (determined by RT-PCR) in the nickel-stimulated cultures increased distinctly after 10 and 24 h of incubation, and decreased again somewhat after 48 h. The difference between the IL-2 mRNA levels in nickel-stimulated and control cells was statistically significant ($p < 0.05$) at all time points investigated ($p < 0.05$ compared to the values at the beginning of the incubation, i.e., at time 0).

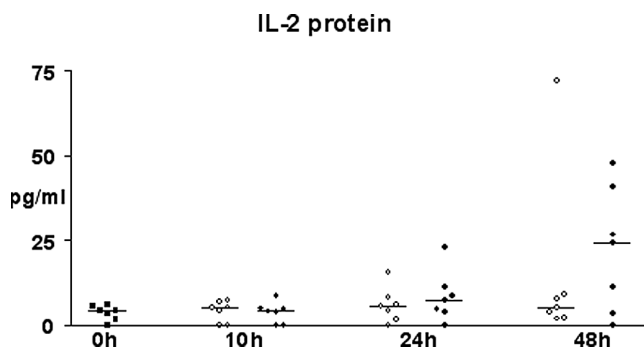


Figure 2: The time course of the synthesis and expression of the IL-2 protein by peripheral blood mononuclear cells (PBMCs) isolated from seven nickel-allergic patients and subsequently incubated with nickel sulphate (filled circles) or saline (open circles). The level of IL-2 protein (quantitated employing an ELISA procedure) in the supernatant from the cell cultures exposed to nickel increased with time, most obviously after 24 and 48 h of incubation ($p < 0.05$ compared to the values at the beginning of the incubation, i.e., at time 0). However, at none of the time points examined was this level significantly different from that associated with the control (saline-treated) cell cultures.

There was no change in the level of either IL-2 protein or mRNA in cultures of PBMCs isolated from the control subjects and incubated with nickel or saline under the same conditions (not shown).

DISCUSSION

In addition to its growth-promoting effect on T lymphocytes, IL-2, produced by Th0 and Th1 cells,⁽⁹⁾ is involved in the activation of various types of immune cells, including B lymphocytes, natural killer cells, and T killer cells, as well as in the release of other cytokines, e.g., IL-5.⁽¹⁰⁾ IL-2 is released into the medium of cell cultures engaged in allogeneic mixed-lymphocyte reactions and of cultures of peripheral blood lymphocytes stimulated with T cell-specific mitogens.⁽¹¹⁾ Quantitation of IL-2 mRNA following stimulation of whole blood with allogeneic T cell-depleted PBMCs has previously been made by real-time PCR.⁽¹²⁾ The induction of the expression of IL-2, IL-4, and IFN- γ mRNA is shown to be associated with delayed-type hypersensitivity reactions to β -lactam antibiotics, where the level of IL-2 mRNA increases as soon as after 48 h of culturing.⁽¹³⁾

In the present investigation, the level of IL-2 mRNA in PBMCs isolated from nickel-allergic subjects and exposed in vitro to nickel was shown to increase significantly within 10 h. Later, a tendency (although not statistically significant) towards an increased level of the IL-2 protein in the culture medium was also observed. The increased level of mRNA coding for IL-2 supports the hypothesis that this process is a key part of the response of the PBMCs of

hypersensitive subjects to nickel stimulation. Future studies are required to determine both the pathophysiological significance and possible clinical usefulness of these findings.

ACKNOWLEDGMENTS

The technical assistance of Ms. Gunnie Westerholm, Yvonne Löfgren, and Wiveca Ring-Persson is gratefully acknowledged. This study was supported financially by grants from the Swedish Asthma and Allergy Association, the Swedish Council for Working Life and Social Research, the Samariten Society, and the Society for Child Care.

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