Influence of metals on cytokines production in connection with successful implantation therapy in dentistry

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Abstract

OBJECTIVES: In most of patients in need of implantation treatment in the oral cavity, implants heal well, nevertheless, there are some individuals, in whom titanium implants fail for reasons, which remain unclear.
DESIGN: The aim of our study was to determine if there is a difference between metal influenced IL-1β, IL-4, IL-6, TNF-α and IFN-γ cytokines production in patients with successfully healed implants compared to those, whose implant therapy was unsuccessful.
SETTING: The two study groups included 12 patients with failed dental titanium implants and 9 patients with successfully healed implants. In the subjects, cytokine production was established after lymphocyte cultivation with mercury, nickel and titanium antigens.
RESULTS: IL-1β levels were significantly increased in all patients after stimulation with titanium and in patients with accepted implants compared to patients with failed implants after the stimulation with mercury and titanium. Titanium caused significantly increased IL-6 production in all patients. TNF-α and IFN-γ levels were also significantly increased after the stimulation with titanium. Significantly increased TNF-α levels were found in patients with accepted implants as compared to patients with failed implants.
CONCLUSIONS: Increased production of IL-1β a IL-6 cytokines in reaction to titanium and increased production of TNF-α and IFN-γ cytokines in reaction to mercury, which is very often present in the form of amalgam in the oral cavity of persons in need of implant therapy, can play an important role in immune reactions during implant healing process. In patients with failed titanium implants, decreased production of these cytokines may participate in implant failure.

Abbreviations:

CR	- Czech Republic
GUH	- General University Hospital
IGA	- Internal Grant Agency
IKEM	- Institute of Clinical and Experimental Medicine
MAPTM	- multianalyte profiling
MH	- Ministry of Health
MHC	- major histocompatibility complex
OPG	- orthopantomogram
RVG	- radiovisiography

ORIGINAL ARTICLE

INTRODUCTION

Continuously increasing demand to treat missing teeth by dental implants results in an increasing demand for excellent mechanical properties and biocompatibility of materials used in dental implants.

The most commonly used dental implants are made of titanium. These implants have very good price/properties ratio and they heal well in most of patients. Nevertheless, there are some persons in whom implants fail for reasons, which remain unclear.

Studies on titanium implants biocompatibility and titanium cytotoxicity show different results. There are reports of high amount of titanium particles (Lalor *et al.* 1991) and pigmentations (Dupre *et al.* 1985) detected in the environ of failed implants, on the other hand there are reports of high stability and biocompatibility of titanium implants (Kanematu *et al.* 1990; Wever *et al.* 1997).

Metals, including titanium in ionized form, bind easily to the body proteins, thus constituting haptens, which activate the immune system (Stejskal *et al.* 1999). Long-term exposition to titanium implants may decrease a population of functional osteoprogenitor cells and may play a role in poor bone quality in the implant environ and in implant failure (Wang *et al.* 2003). Similarly, T cell proliferation significantly decreases as a result of titanium influence, which may participate in an increased risk of infection in patients with titanium implants (Wang *et al.* 1996).

Important factors of immune reaction include cytokines, soluble substances with biological effects produced by different types of immuno-competent cells, through which the cells influence each other. Metals, including titanium, may cause an increased production of pro-inflammatory cytokines and thus an activation of antigen-presenting cells and/or neo-antigen induction. Thereby the immune reaction of Th0 lymphocytes activation and differentiation to Th1 or Th2 lymphocyte clones develops (Pieters *et al.* 2003). Metals participate in antibacterial immune reactions through the macrophage activation. Therefore, metals take part in inflammatory reaction and they are responsible for delayed type hypersensitivity – allergic reaction type IV. Activated Th1 lymphocyte clones produce IFN-γ and IL-2, cytokines participating in target cell elimination. IFN-γ induces expression of class II MHC molecules on immuno-competent and non-lymphatic cells and it inhibits B lymphocytes proliferation. IL-2 is one of the cytokines signalling T lymphocytes activation, released proportionally to DNA synthesis. IL-4 is pro-inflammatory cytokine produced by Th2 lymphocyte clones (Krejsek & Kopecky 2004).

Metals, including titanium, influence T lymphocytes function in the sense of stimulation as well as in the sense of inhibition, they change Th1/Th2 ratio to the benefit of Th2 and therefore they increase IL-4 production to the prejudice of IFN-γ production in animal experiment as well as in human T lymphocytes *in vitro* (Jiang & Möller, 1995; Sharma & Dugyala 1996; Shenker *et al.* 1992).

Cytokines can be determined by multiplex analysis MAP™, belonging to the most recent immuno-analytical methods. It is used to quickly and accurately determine the concentration of a large number of cytokines (maximally 96 samples in a small volume of sample liquid (50 μL) – supernatant, plasma or serum).

This method uses flow cytometry principle. It is based on quantitative multiplex analysis using labelled microparticles. Each microparticle is labelled by the colour using specific proportion of two fluorescent colours and primary specific antibody against definite cytokine is bound to the microparticle. Supernatant, biotinyled secondary specific antibody against the cytokine and conjugate with streptavidin-PE are added to the microparticle. Thereafter the analysis is performed on LUMINEX analyser using two lasers. One laser is specific for microparticles and it determines which analyte will be detected. Second laser determines the range of the secondary phycoerythrin signal, which is in direct relationship to the amount of monitored analyte (Khan *et al.* 2006; Prabhaker *et al.* 2002).

The aim of our study was to monitor production of selected pro-inflammatory and anti-inflammatory cytokines in patients with failed implants and to compare the results with values obtained by examination of patients with successfully healed implants.

MATERIALS AND METHODS

Examined groups of patients

Based on the Informed consent form and in accordance with the Helsinki declaration a group of 21 patients, 12 men and 9 women with the mean age of 54 years was examined at our Institute.

Twelve patients, 9 men and 3 women with the mean age of 52 years, were referred for the examination at our Institute from all regions of the Czech Republic, because they rejected one or more dental titanium implants or their dental implant was found to be lost during a clinical check-up and a diagnosis of peri-implantitis was consequently confirmed by radiovisiography (RVG). These individuals constituted a group of patients with failed implants (FI).

The remaining examined individuals constituted a control group with accepted implants (AI). These subjects, patients of the Institute of Dental Research in Prague, had successfully healed dental titanium implants at least for 5 years, they had metal alloys in the oral cavity comparable with the failed implant group and they were compliant to participate in the study. This group included 9 patients with the mean age of 57 years, 3 men and 6 women.

Clinical examination

In all the study subjects we have recorded a detailed personal and family history focused on the exposition

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to metals. For these purposes, target-compiled questionnaire was used.

We performed a detailed examination of hard dental tissues and soft tissues of the oral cavity including panoramic dental X-ray picture (OPG) and radiovisiography (RVG) of the oral cavity. The examination was focused on the identification of dental metals inserted in the oral cavity and morphologic changes – inflammatory and lichenoid changes of oral mucosa, tongue and gingiva. The presence of metallic pigmentations was also evaluated.

An evaluation of dental implants by the clinical examination, by OPG and RVG was an integral part of the examination. At the time of examination, 12 patients did not have any dental implant in their oral cavity, because of implant failure 3–6 months prior to the examination.

Cytokines production in lymphocyte tissue cultures

From the study subjects we collected 16 mL of venous blood using two heparinized tubes of Vacuette system (Greiner, USA) and 8 mL of venous blood for serum separation using one tube of Vacuette system (Greiner, USA). Lymphocytes were obtained using separation on Ficoll-Paque (Sigma-Aldrich, USA) gradient by 600 g centrifugation for 25 minutes, then cells were washed twice by RPMI medium and incubated for 40 minutes in 37 °C and in 5% CO_2 atmosphere in RPMI medium with 10 % of autologous serum to remove redundant monocytes, lymphocytes were diluted in RPMI medium with 2% of glutamine (Sevac, CZ) to the concentration of 10^6 lymphocytes/mL and they were cultivated in 37 °C with presented metal antigens as tissue cultures for 5 days.

The establishment of cytokines IL-1β, IL-4, IL-6, TNF-α and IFN-γ production was performed in supernatants of lymphocytes tissue cultures after the cultivation with metal antigens of mercury ($HgCl_2$, Sigma-Aldrich, USA), nickel ($NiCl_2 \times 6H_2O$, Sigma-Aldrich, USA) and titanium (TiO_2, Sigma-Aldrich, USA) and in non-stimulated negative control culture of lymphocytes by multiplex analysis LUMINEX method using standard kits Human MultiAnalyte Profiling Human Base Kit A (R&D Systems Fluorokine®MAP, USA).

The test was performed in HLA laboratory of IKEM, Prague. Fluorokine® MAP Human MultiAnalyte Profiling Human Base Kit A (R&D Systems Fluorokine®MAP, USA) was used for the establishment of the following cytokine concentrations: IL-1β, IL-4, IL-6, TNF-α and IFN-γ. The test was performed according to the instructions for use provided with this commercial kit.

Before the measurement a protocol was created – programming of Luminex 100 IS Software programme. The protocol included information about the standard, number of measured cytokines, regions of individual cytokines detection and their concentration. The measurement of each well in plate lasted for a maximum of 94 seconds. During that time labelled microparticles with bonded cytokines were harvested and analysed.

Statistics

The collected data were statistically processed using the ANOVA test in the SGStatFolio programme.

RESULTS

IL-1β levels were significantly increased after the stimulation by titanium antigen compared to non-stimulated culture in all patients, increased levels of this cytokine were found also after the stimulation by mercury and nickel antigen (Figure 1).

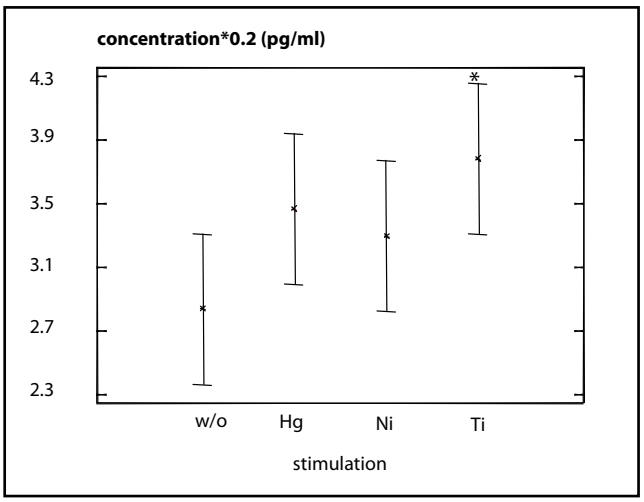

Fig. 1. Determination of IL-1 beta in tissue culture of lymphocytes after stimulation with mercury, nickel and titanium – Means and 95-percentile intervals of logaritmic standard deviation (* -significant difference compared to cell culture without stimulation)

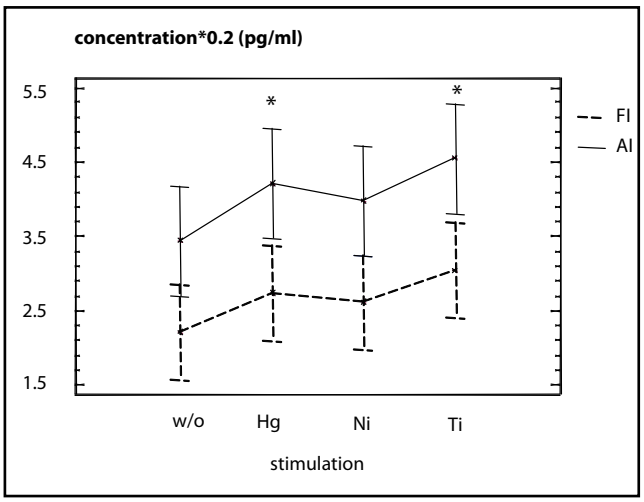

Fig. 2. Determination of IL-1 beta in tissue culture of lymphocytes in patients with failed implants (FI) and in patients with accepted implants (AI) after stimulation with mercury, nickel and titanium - Interactions and 95-percentile intervals of logaritmic standard deviation (* - significant difference between groups of FI and AI)

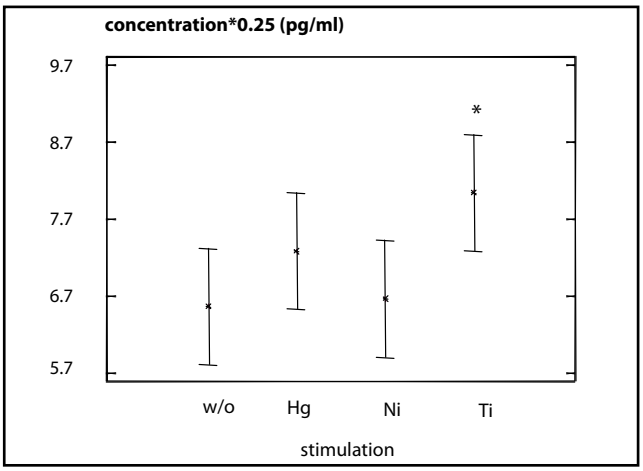

Fig. 3. Determination of IL-6 in tissue culture of lymphocytes after stimulation with mercury, nickel and titanium – Means and 95-percentile intervals of logaritmic standard deviation (* - significant difference compared to cell culture without stimulation)

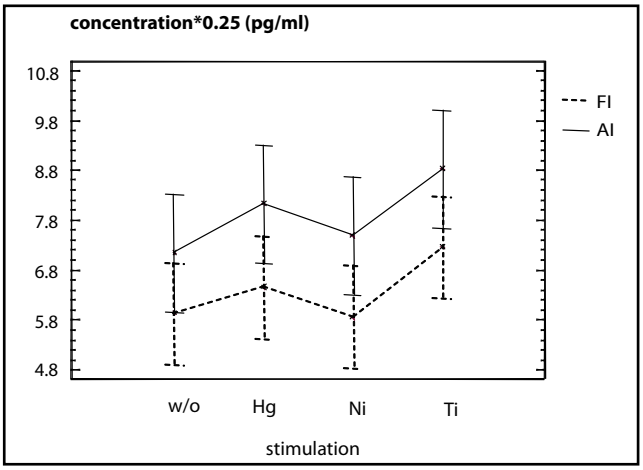

Fig. 4. Determination of IL-6 in tissue culture of lymphocytes in patients with failed implants (FI) and in patients with accepted implants (AI) after stimulation with mercury, nickel and titanium - Interactions and 95-percentile intervals of logaritmic standard deviation

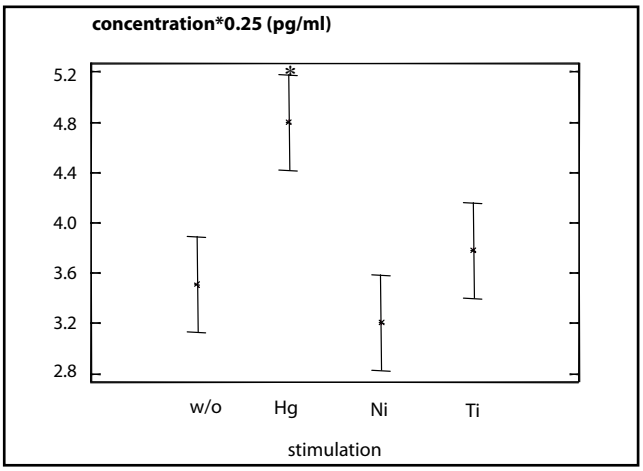

Fig. 5. Determination of TNF-alpha in tissue culture of lymphocytes after stimulation with mercury, nickel and titanium – Means and 95-percentile intervals of logaritmic standard deviation (* - significant difference compared to cell culture without stimulation)

In the cell culture stimulated by mercury and titanium antigens we have found significantly increased levels of IL-1β in patients with accepted implants (AI) compared to patients with failed implants (FI). Also in non-stimulated cell culture and in culture stimulated by nickel antigen we have found increased levels of IL-1β in patients with accepted implants (AI) compared to results in patients with failed implants (FI). However, the difference was not significant (Figure 2).

No significant differences were found in IL-4 levels of the tested cell cultures.

In all patients, IL-6 levels after the stimulation by titanium antigen were significantly increased compared to non-stimulated culture, higher levels of this cytokine were also measured after the stimulation by mercury antigen (Figure 3).

We also found increased levels of IL-6 in patients with accepted implants (AI) compared to patients with failed implants (FI), namely in non-stimulated cell culture as well as in cultures stimulated by mercury, nickel and titanium antigens (Figure 4).

In all patients, TNF-α levels were significantly increased after the stimulation by mercury antigen compared to non-stimulated culture, increased levels of this cytokine were also measured after the stimulation by titanium antigen, on the contrary non-significant decrease was found in reaction to nickel antigen (Figure 5).

When comparing values in patients with accepted (AI) and failed (FI) implants, TNF-α levels were significantly increased in non-stimulated cell culture of FI group, on the contrary in cell cultures stimulated by mercury, nickel and titanium antigens significantly increased levels were found in AI group (Figure 6).

In all patients, IFN-γ levels were significantly increased after the stimulation by mercury antigen compared to non-stimulated culture, on the contrary in cultures stimulated by titanium a significant decrease of this cytokine was found (Figure 7).

Increased levels of IFN-γ were measured in patients with accepted implants (AI) compared to patients with failed implants (FI) in non-stimulated cell culture and in culture stimulated by mercury and titanium antigens. On the contrary, decreased levels of IFN-γ were found in cell culture stimulated by nickel antigen in patients with accepted implants (AI) compared to patients with failed implants (FI) (Figure 8).

DISCUSSION

In the Czech population, reaction to metals is most often detected to nickel and mercury (Prochazkova *et al.* 2004; Sterzl *et al.* 1999a; Sterzl *et al.* 1999b). In patients with failed implants, most frequently we have found an increased proliferation activity of lymphocytes to mercury and nickel (Prochazkova *et al.* 2006). Titanium is a basic metal component of dental implants. Therefore monitoring of immune reaction parameters influenced

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by these three metals appears to be significant for the healing processes in implantology.

In the past it was proven that cytokines are important factors influencing immune reactions (Striz, 1999).

IL-1β plays role in stress reactions and has an anti-inflammatory action similar to TNF-α, it supports the expression of genes participating in inflammatory processes. IL-4 is important for the induction of lymphocyte Th2 phenotype and it has anti-inflammatory activity. IL-6 is the activation factor of T lymphocytes. IFN-γ is anti-inflammatory cytokine, it is produced by activated T lymphocytes and it stimulates cytotoxic activity of T lymphocytes (Krejsek & Kopecky 2004). In this study, we decided to monitor these cytokines, because they represent specific type of immune reaction and play a crucial role in inflammatory reactions typical for implant failure.

The influence of metals on the cytokine production has already been proven in the past (Venclikova *et al.* 2006). In this study, we have tested *in vitro* cytokine production in supernatants of lymphocyte tissue cultures stimulated by metal antigens. We have found that mercury antigen stimulates the production of IFN-γ as well as the production of TNF-α, both anti-inflammatory cytokines in patients with failed implants. On the contrary, the production of IL-6 and IL-1β can be influenced by titanium antigen; however, according to our findings its production in patients with failed implants does not differ from patients, in whom implant therapy was successful.

We can conclude that increased production of IL-1β and IL-6 cytokines by lymphocytes in reaction to titanium antigen and increased production of TNF-α and IFN-γ cytokines by lymphocytes in reaction to antigen of mercury, which is very often present in the form of amalgam in the oral cavity of persons in need of implant therapy, can play an important role in the immune reactions during implant healing. In the patients with failed titanium implants, decreased production of these cytokines may participate in implant failure.

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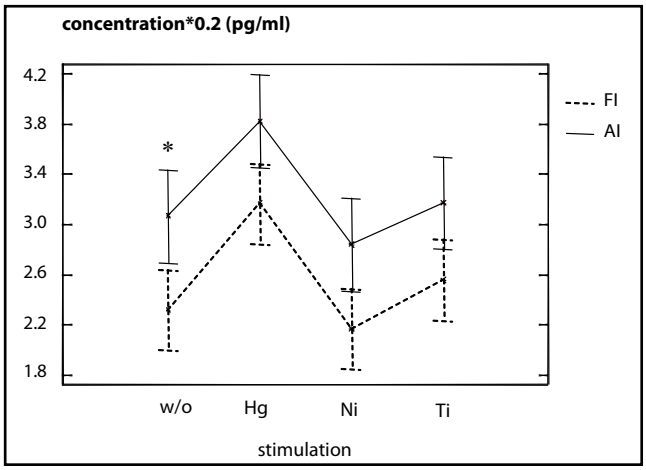

Fig. 6. Determination of TNF-alpha in tissue culture of lymphocytes in patients with failed implants (FI) and in patients with accepted implants (AI) after stimulation with mercury, nickel and titanium - Interactions and 95-percentile intervals of logaritmic standard deviation (* - significant difference between groups of FI and AI)

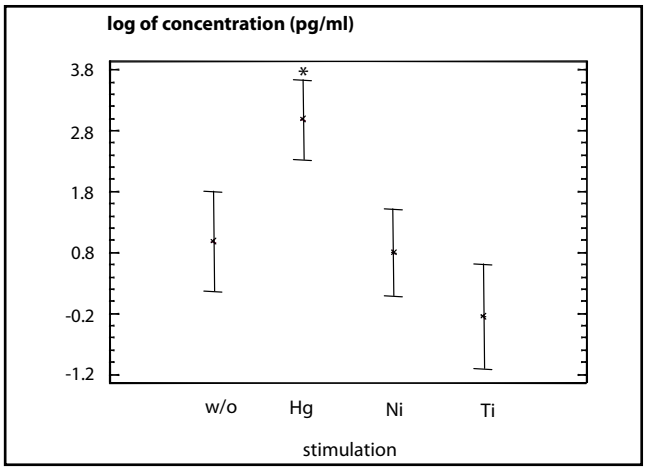

Fig. 7. Determination of IFN-gamma in tissue culture of lymphocytes after stimulation with mercury, nickel and titanium – Means and 95-percentile intervals of logaritmic standard deviation (* - significant difference compared to cell culture without stimulation)

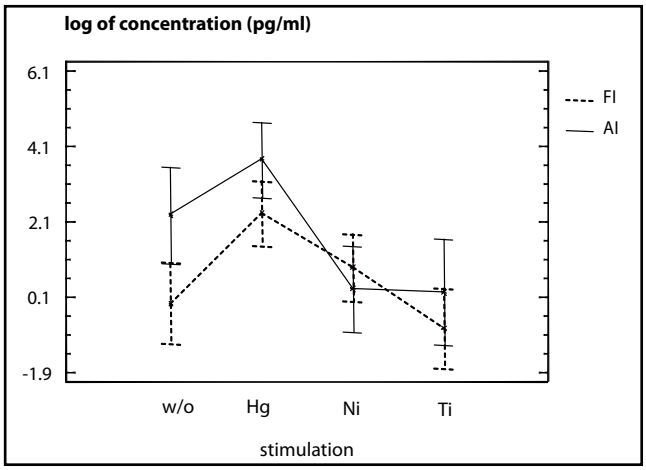

Fig. 8. Determination of IFN-gamma in tissue culture of lymphocytes in patients with failed implants (FI) and in patients with accepted implants (AI) after stimulation with mercury, nickel and titanium - Interactions and 95-percentile intervals of logaritmic standard deviation

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