Evaluation of immunologic profile in patients with nickel sensitivity due to use of fixed orthodontic appliances

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The aim of this study was to develop a new approach to testing the impact of nickel antigen on in vitro cell-proliferation assay, to identify adverse reactions to casting alloys among orthodontic patients. Cell-proliferation assay in vitro was used as the basic methodology to assess the influence of such variables as source of nickel antigen, type of serum used to supplement the culture medium, and number of cells in the culture. We selected 35 orthodontic patients who were classified as nickel sensitive and non–nickel sensitive, based on their clinical records. Our results showed that hexahydrated nickel sulfate at 10 μg/mL, 10% of autologous sera, and 2 × 10^5 cells was the best condition for inducing the most marked nickel proliferation response in vitro. This optimized method was able to distinguish nickel-sensitive from non–nickel-sensitive dental patients and also to discriminate those with positive skin tests. Our data suggest that continuous exposure to nickel casting alloys might lead to oral tolerance mechanisms that modulate nickel sensitivity, as evidenced by the lower cell proliferation index in patients undergoing orthodontic treatment over 24 months. Finally, our findings demonstrated a known nickel-induced type 2 immune response and a marked lack of type 1 immunity (interferon γ) as the hallmarks of nickel-sensitive patients. Further studies are needed to clarify the major cell phenotype associated with this type 2 immune response and the lack of type 1 immunity observed in nickel-sensitive people. (Am J Orthod Dentofacial Orthop 2003;123:000-00) (Am J Orthod Dentofacial Orthop 2003;124:46-52)

Many objects contain nickel and can cause nickel sensitivity, and many people are in continuous contact with items that contain nickel.1 Nickel-containing objects are common in dentistry, especially in dental implants and compounds used in crowns, bridgework, and orthodontic appliances. Nickel-titanium alloys are widely used in orthodontic treatment, and all these devices can induce skin and mucosal reactions, leading to tissue inflammation.2-4 An immune response induced by nickel appliances is generally called contact dermatitis and, from the immunologic standpoint, is considered type IV hypersensitivity. In this context, nickel binding to endogenous macromolecules can stimulate macrophages and cytotoxic cells, up-regulating the expression of adhesion molecules.5-7 It has also being reported that low-dose exposure can alter the metabolism of human monocytes.8 Additionally, nickel induces T lymphocytes to produce several cytokines, including interferon IFN-γ, interleukin IL-2, IL-5, and IL-10, and stimulates cellular proliferation.9 The pattern and level of cytokines secreted are critical to triggering differential immune responses, which can lead to different degrees of tissue damage. The temperature, microorganisms, enzymatic compounds, and ions normally present in the oral cavity particularly favor the breakdown and release of metallic elements from dental casting alloys.10 Contact dermatitis generally appears clinically as eczema. Reactions of the mucous membranes, such as stomatitis, also occur, and gum hyperplasias, cheilitis, labial desquamation, and multiform erythema are frequently noted. Nickel hypersensitivity has increased in the last 10 years, and it is estimated that 15% to 30% of
Europeans and Americans, in a proportion of 1 to 8, men to women, show symptoms.\textsuperscript{11} A prevalence of 7% to 28% of the general population has been also reported.\textsuperscript{12,13} Nickel sensitivity has been diagnosed through biocompatibility testing,\textsuperscript{14,15} including cutaneous sensitivity tests,\textsuperscript{13} and also through clinical observations and family history. However, major problems regarding specificity of the cutaneous tests are frequently reported. Therefore, a great challenges in the diagnosis of nickel sensitivity has been to discover and develop alternative in vitro assays to improve specificity and minimize false-positive results. Lymphocyte stimulation assays with peripheral blood mononuclear cells (PBMC) and different concentrations of nickel salts have contributed to advances and show promising results.\textsuperscript{16} In one study, lymphocytes from all suspected nickel-sensitive patients showed a significantly greater response than did those of healthy controls.\textsuperscript{17} Lymphocyte transformation, as measured by increased deoxyribonucleic acid (DNA) synthesis, seems to be an important tool for investigating the problems arising from false-positive or false-negative patch tests.\textsuperscript{18}

The purpose of this research was to develop a new approach to testing the effect of nickel antigen on lymphoproliferative response in vitro, by using PBMC from patients with orthodontic appliances. Using this strategy, we evaluated whether lymphoproliferative response is a useful method to discriminate nickel-sensitive from non–nickel-sensitive dental patients. We also studied patients wearing orthodontic appliances (either with or without clinical symptoms) to determine the influence of certain variables on in vitro lymphoproliferation assay, including source of antigen, type of serum used to supplement the medium, and the number of cells used.

**MATERIAL AND METHODS**

Thirty-five patients, aged 10 to 21 years (mean 14.3), all undergoing orthodontic treatment with fixed appliances, were selected based on their clinical health records and classified into 2 groups. The first group comprised 26 patients (8 males, 18 females, aged 10 to 21 years) with clinical manifestations of contact dermatitis, including angular cheilitis, labial desquamation, or gum hyperplasia resulting from metallic compounds, especially earrings, bracelets, and other jewelry. The second group comprised 9 patients (3 males, 6 females, aged 11 to 21 years) who did not have clinical signs or histories of nickel sensitivity. All patients underwent cutaneous nickel sensitivity tests, and those selected for the control group had negative results. We could not use all 26 patients for all tests owing to the limited quantity of PBMC we were able to extract from their blood samples. Informed consent was obtained from all subjects and their parents. The studies were approved by the Ethical Committee of Universidade Estadual de Campinas and Universidade Vale do Rio Doce.

The cutaneous sensitivity test was performed as described by Carvalho.\textsuperscript{19} Briefly, a 5% nickel sulfate and petroleum jelly substrate (nickel sulfate 5%, Alergofar, Rio de Janeiro, Brazil) was kept in direct contact with the skin of the forearm for 48 to 72 hours. The results were classified as positive or negative, based on the reaction.

Nickel sources used as stimulating agents for the cellular proliferation assays in vitro were obtained from solutions containing nickel from in vitro corrosion of orthodontic appliances (nickel extract) and from hexahydrated nickel sulfate solutions (NiSO$_4$.6H$_2$O, Sigma Chemical, St. Louis, Mo). The nickel extracts were obtained from 4 different brands of orthodontic appliances. Alloys were kept in a sterile 0.85% saline solution at 37°C for 7, 30, and 60 days. After centrifugation to remove the particles produced during the corrosion process, the solutions were sterilized by filtration (0.2 μm, Filter Millex-HA Millipore Products Division, Bedford, Mass). Then the dosage of nickel in this solution was measured with an atomic absorption spectrophotometer (Hitachi Z-8200, Tokyo, Japan). The extracts thus obtained were diluted in culture media (RPMI-1640, Gibco BRL, Grand Island, NY) containing a 3% solution of antibiotic-antimycotic (stock solution: 10,000 IU penicillin, 10,000 IU streptomycin/mL, and 25 μg amphotericin B/mL, Sigma Chemical) and 1.6% L-glutamine (stock solution: 200 mMol/L, Gibco), labeled incomplete RPMI. Extracts containing nickel at concentrations of 1.25, 2.5, and 5 μg/mL were used in the cellular proliferation assays. The spectrophotometer readings of saline extract from the 4 appliances (A, B, C, and D) showed similar levels of nickel after 60 days of spontaneous corrosion. No significant differences on the yield of nickel between the 4 different brands were observed (A, 40.23 μg/mL; B, 39.58 μg/mL; C, 40.03 μg/mL, and D, 40.23 μg/mL). Solution A was used in cell culture. The nickel sulfate (NiSO$_4$.6H$_2$O, Sigma) was also diluted in the above-described incomplete RPMI medium and used at concentrations of 2.5, 5, and 10 μg/mL in the cellular proliferation assays.

In vitro cellular proliferation assays were performed with the procedure described by Gazzinelli et al.\textsuperscript{20} In 96-well, flat-bottom, tissue-culture plates, 2 different concentrations of PBMC (2.0 $\times 10^5$ and 3.0 $\times 10^5$ cells per well) were incubated with 100 μL of 2 different
culture mediums (RPMI supplemented with 5% heat-inactivated AB+ human serum and with 5% autologous serum) with several concentrations of the antigenic preparations, including nickel extract (1.25, 2.5, and 5 μg/mL) and nickel sulfate (2.5, 5, and 10 μg/mL). Triplicate cultures were incubated at 37°C (Queue Systems, Parkersburg, WV), 95% humidity, 5% CO₂ for 6 days. Six hours before harvesting the cells, 1.0 μCi of tritiated thymidine (³H) was added to each well. After this stage, the cells were collected on glass fiber paper (Whatman, Clifton, NJ) with an automatic cell harvester (Skatron Instruments, Sterling, VA). The incorporated radioactivity was determined with a Beckman LS 100 C scintillator (Beckman, Scientific Instruments Division, Irvine, Calif). The results obtained from the proliferation assays were expressed in counts per minute. The cellular stimulation index was calculated by taking the average value of the triplicate series of the stimulated cultures (E) divided by the average values from the triplicate series of controls (C). We chose E/C values greater than 2.0 to indicate significant enhanced PBMC proliferation, based on values described in previous studies.17,21-23

The analysis of cytokines, IFN-γ, and IL-5 in the supernatants from nickel-stimulated and control cultures were assayed with enzyme-linked immunosorbent assays, as described by Lunde et al.24 Briefly, a 1 × 10⁶ PBMC sample from each patient undergoing orthodontic treatment was stimulated with nickel sulfate (10 μg/mL) for 24 hours in medium containing autologous sera. Controls of nonstimulated cells were tested. For cytokine analysis in the supernatant, initially, 60 μL per well of anti-IL-5 monoclonal antibodies (5 μg/mL; DNAX, Palo Alto, Calif), and 60 μL per well of anti-IFN-γ (2 μg/mL; R&D Systems, Minneapolis, Minn) were added to the 96-well plates (Imunolon 2, Dynatech Laboratories, Alexandria, Va) for 12 hours at room temperature. After washing, the plates were blocked with phosphate-buffered saline (PBS) solution supplemented with 1% of bovine serum albumin, 5% of sucrose, and 0.05% of sodium azide. After blockage, 50 μL of culture supernatants were added to the test wells. Blank and standard wells were prepared with 50 μL of PBS, recombinant IFN-γ (25-0.78 ng/mL; R&D Systems), and recombinant IL-5 (50-1.56 ng/mL; DNAX), respectively. The plates were then incubated for 2 hours at room temperature. After washing with PBS 0.05% of Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma), 60 μL per well of biotinylated anti-IFN-γ polyclonal antibody (0.3 μg/mL; R&D Systems) and anti-IL-5 (0.3 μg/mL; DNAX) were added and the plates incubated for 1 hour at room temperature. After incubation, the plates were washed with PBS 0.05% of Tween 20 and 100 μL of peroxidase-conjugated streptavidin (1 μg/mL, Sigma) and reincubated for 20 minutes at room temperature. After this step, 60 μL of the substrate (2,2 azino-bis 3 ethylbenz-thiazolidine-6-sulfonic acid, Sigma, 1 mg/mL) were added to all wells. After color development, the reaction was stopped by adding 50 μL of 1 M sulfuric acid. The optical density was read with an automatic reader (Benchmark Microplate Reader, Bio-Rad Laboratories, Hercules, Calif) with a 405-nm filter.

Data analysis was performed by one-way analysis of variance followed by Student t tests. Significance was set at P < .05.

RESULTS

To improve specificity of nickel sensitivity diagnosis, we tested the effect of nickel antigen source on the in vitro lymphoproliferative response by using PBMC from 22 patients wearing orthodontic appliances. Using the basic blastogenesis method described by Gazzinelli et al,20 we tested 3 different concentrations of nickel extract and nickel sulfate (Fig 1). Data analysis demonstrated that exposure of PBMC to the nickel extract did not lead to significant proliferation (E/C < 2.0). Concentrations of 2.5 and 5.0 μg/mL of nickel sulfate did not give significant results. On the other hand, a concentration of 10 μg/mL of nickel sulfate significantly stimulated cell proliferation (E/C > 2.0), measured by ³H-thymidine incorporation. Therefore, the antigen source chosen for further study was nickel sulfate.

The responses of PBMC from 21 subjects to nickel sulfate 10 μg/mL was evaluated in the presence of autologous and AB+ type sera with 2 × 10⁵ and 3 × 10⁵ cells per well (Fig 2). The in vitro responses of 2 ×
10^5 PBMC in the presence of autologous serum differed significantly (E/C > 2.0) from 3 x 10^5 cells in autologous serum and AB+ serum. Additional comparative studies were performed with 2 x 10^5 PBMC in the presence of autologous serum to derive E/C values.

The PBMC responses of 21 subjects, 12 nickel-sensitive and 9 non–nickel-sensitive, were measured after in vitro stimulation with nickel sulfate in medium containing autologous serum (Fig 3). The nickel-sensitive group had significantly higher cell proliferation indexes than did the non–nickel-sensitive group. No differences were observed when nickel-sensitive patients were subdivided into groups based on morbidity of oral disease (data not shown).

It has been proposed that long-term exposure to nickel-containing devices can induce immunologic tolerance. To investigate this hypothesis, PBMC from 21 dental patients with different times of exposure to orthodontic appliances were submitted to the lymphoproliferation protocol described above. Evidence of cell proliferation index inhibition due to long-term exposure to orthodontic appliances was found when patients were divided into 2 groups based on the time of exposure to nickel-containing devices (Fig 4). Patients with less than 24 months of exposure (n = 10) showed significant cell proliferation indexes (E/C > 2.0) in contrast to those with more than 24 months of oral nickel exposure (n = 11).

To validate the use of cell proliferation assay in vitro in clinical trials, we conducted a parallel study of PBMC nickel sulfate–induced proliferation in vitro with skin tests (patch test) for nickel sensitivity. Our findings demonstrated that a positive skin test is associated with a higher proliferation index, leading to significant results compared with patients with a negative patch test (Fig 5).

In our system, the variables of sex and allergic reaction were also evaluated, but they had no impact on cell proliferation index (data not shown).

To further investigate the impact of in vitro nickel stimulation on PBMC from 19 subjects (13 nickel-sensitive and 6 non–nickel-sensitive), IFN-γ and IL-5 were measured after 24 hours of stimulation with nickel sulfate in medium containing autologous sera (Fig 6). Our data demonstrated a significant difference in IL-5 production between nickel-stimulated and nonstimulated cultures from nickel-sensitive patients. This difference was not detected in control cultures. On the other hand, nickel stimuli significantly inhibit in vitro IFN-γ production by PBMC from nickel-sensitive patients but not non–nickel-sensitive subjects (Fig 6).
DISCUSSION

Induction of nickel hypersensitivity has been studied extensively. Orthodontic appliance therapy could enhance the liberation of nickel directly into the oral cavity, and therefore it is a potential source of antigenic stimulation for the human immune system. Orthodontic treatment is available to many more people. In general, the appliances and archwires used in treatment are composed of metal alloys that are more than 55% nickel, representing a potential source of this heavy metal in the buccal cavity. The biodegradation of orthodontic appliances has been studied by various authors, and the allergic response to nickel-containing dental alloys has been reported in several publications. Because nickel can sensitize certain people and cause severe allergic reactions, the safe use of these alloys is still under study.

The assay most commonly reported to evaluate cellular immune response in vitro is the measure of lymphoproliferative activity of PBMC in the presence of specific antigenic materials of interest. From the dental care standpoint, it has been demonstrated that lymphocytes from nickel-sensitive dental patients showed significantly greater responses compared with those of controls. Several methods are available to assess cell proliferation in vitro, including protocols with whole blood and purified PBMC. Despite its potential use for evaluating immunologic status, the in vitro lymphocyte transformation test is not standardized for nickel sensitivity studies. Questions regarding nickel source and concentration for antigenic stimulation are still topics of research. In attempting to evaluate whether the lymphoproliferative response in vitro could be a useful method for discriminating nickel-sensitive from non–nickel-sensitive patients, we performed a parallel study of lymphocyte transformation in vitro with different concentrations of 2 distinct sources of nickel: nickel released from orthodontic appliances and nickel sulfate. Our data demonstrated that nickel sulfate at 10 µg/mL was the best solution to induce lymphocyte transformation in vitro. We observed that nickel extract in 3 different concentrations (1.25, 2.5, and 5.0 µg/mL) could not induce significant lymphocyte proliferation, and high doses of nickel extract were cytotoxic for PBMC cultures in vitro (data not shown). These data agree with those reported by Silvennoinen-Kassinen, showing that higher concentrations of nickel can induce lymphocyte death in vitro. Moreover, we found that optimization of lymphoproliferation assay in vitro can be achieved by substituting autologous serum for AB+ and by using fewer cells per culture (2.0 × 10^6 cells per well). We believe that several factors, such as the cytokines' microenvironment in cultures with autologous sera, were responsible for the differences observed. By using this improved methodology, it was possible to discriminate nickel-sensitive from non–nickel-sensitive patients (Fig 3). Our study also found a correlation between nickel-induced proliferation in vitro and nickel cutaneous sensitivity test results (Fig 5). Because skin tests have been routinely used not just to evaluate nickel hypersensitivity, but also for diagnostic purposes, lymphocyte activation and proliferation measured by the DNA synthesis test is a method for diagnosing nickel sensitivity that does not expose patients to the hazards of patch testing.

A question that remained without a clear answer is whether long-term intraoral exposure to nickel from orthodontic appliances might result in a higher incidence of allergic reactions or lead to oral tolerance. In this study, we found that patients who had been undergoing orthodontic treatment for more than 24 months showed lower nickel-induced cell proliferation indexes than those with less than 24 months of exposure (Fig 4). These data suggest that the longer the treatment continues, the lower the nickel-induced PBMC proliferation index; this in turn suggests that mechanisms of oral tolerance might develop in this context. Immunologic tolerance to nickel was described by Vreebur et al in 1984, when oral administration of nickel induced partial tolerance in guinea pigs with a splint fixed to their teeth or receiving nickel in their food. According to these authors, this state of partial tolerance could contribute to reducing the incidence of allergic reactions in patients undergoing orthodontic treatment in which nickel alloys are used. In agreement with this hypothesis, it has also been reported that
treatment with nickel-containing appliances before sensitization to nickel (ear piercing) can lead to reduced frequency of nickel hypersensitivity.30 Tolerance induction might be a possible benefit of using intraorally placed alloys.3

We have also begun to investigate the role of type 1 and type 2 immune responses in the pathogenesis of nickel sensitivity. It seems that nickel stimuli can elicit IL-5 production in nickel-sensitive patients. There was a significant difference between their cytokine levels and those observed in the control cultures. Moreover, we showed that our optimized method for lymphocyte transformation in vitro is a useful tool for identifying the lack of type 1 (IFN-γ) immune response in nickel-sensitive compared with non–nickel-sensitive patients (Fig 6). Further studies are needed to elucidate the major cell phenotype associated with the type 2 immune response, as well as the lack of type 1 immunity observed in nickel-sensitive people. Moreover, phenotypic studies will help define the target cells for nickel-specific activation that could lead to immunologic interventions in dental patients having orthodontic appliances.

CONCLUSIONS

- Optimization of lymphoproliferation assay in vitro was achieved by using fewer cells per culture (2.0 × 10⁵ cells per well), substituting autologous for AB+ sera, and stimulating cells with 10 μg/mL of nickel sulfate. With this ideal culture, it was possible to assess the nickel-induced cell proliferation index and thus distinguish nickel-sensitive from non–nickel-sensitive patients.

- This optimized methodology for assessing cellular immune status could enable diagnostic testing of nickel sensitivity without exposing patients to the hazards of patch testing.

- The exposure to nickel alloy castings for more than 24 months resulted in lower cell proliferation indexes, suggesting that the development of oral tolerance mechanisms might play a role in modulating the cellular response to nickel.

- A known nickel-induced type 2 immune response and a marked lack of type 1 immunity (IFN-γ) were the hallmarks of nickel-sensitive patients.

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REFERENCES


