Title	Splenocyte Cytokine Profile in Mouse with Oral Mucosa-sensitization and Oral-tolerization by NiSO4
Author(s)	Fujimoto, M; Ukichi, K; Okamura, T; Takada, A; Fukushima, D; Morimoto, M; Yamane, G; Takahashi, S
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Original Article

Splenocyte Cytokine Profile in Mouse with Oral Mucosa-sensitization and Oral-tolerization by NiSO$_4$

Masaru Fujimoto, Kenichirou Ukichi, Taito Okamura, Atsushi Takada, Daihei Fukushima, Mitsuaki Morimoto, Gen-yuki Yamane and Shin-ichi Takahashi*

Department of Oral Medicine, Oral and Maxillofacial Surgery, Tokyo Dental College, 5-11-13 Sugano, Ichikawa, Chiba 272-8513, Japan
* Division of Dermatology, Ichikawa General Hospital, Tokyo Dental College, 5-11-13 Sugano, Ichikawa, Chiba 272-8513, Japan

Abstract

Metals used in the oral cavity have been reported to cause various allergic diseases of the skin and mucosa. Skin manifestations due to dental restorations appear not only in the oral cavity, but also on the hands, feet or the whole body, as in the cases of pustulosis palmoplantaris and lichen planus. These phenomena implicate different pathogeneses from that of conventional skin sensitization and tolerance. Therefore, we compared skin and oral mucosa sensitization with nickel and oral tolerance for nickel in a mouse model. Female C57BL/6J mice were sensitized by injection of NiSO$_4$ into the skin or oral mucosa. Allergic reactions were evaluated by the mouse ear swelling test and splenocyte proliferation and cytokine profiles. Skin and oral mucosa sensitization succeeded in all mice. Ear swelling was significantly greater in the skin- than in the oral mucosa-sensitized mice at 48 hr after challenge. Ear swelling was also suppressed by single oral administration of NiSO$_4$ in both the skin- and oral mucosa-sensitized mice to the level of that in non-sensitized mice. Splenocytes from skin-sensitized mice proliferated similarly to those from oral mucosa-sensitized mice. Splenocytes from orally-tolerized mice also showed similar proliferation activity to those from skin and oral mucosa-sensitized mice. In the challenge phase, IL-2, IFN-γ, and IL-10 production was induced in splenocytes from both skin- and oral mucosa-sensitized mice. However, IL-4 was induced only in those from skin-sensitized mice. In addition, IL-4 in splenocytes from oral mucosa-sensitized mice was up-regulated to the level in those from skin-sensitized mice by oral tolerance. These results suggest that sensitization sites in mice influence not only the degree of excitation, but also Th-1 and Th-2 balance in the challenge phase and oral tolerance.

Key words: Oral mucosa—Nickel—Oral tolerance—Splenocytes—Cytokine profile

Introduction

Allergic contact dermatitis is known to be caused by nickel contained in metal accessories[8]. Nickel contained in dental materials in the oral cavity has been reported to cause various allergic disorders of the skin and mucosa[4]. Allergies such as conventional contact stomatitis occur in the oral mucosa near dental materials. However, dyshidrotic eczema appears in areas of skin distant from the contact site with dental materials[22]. This type of systemic allergic contact dermatitis is thought to be accompanied by systemic effects[7]. In contrast, patients who had oral contact with nickel-releasing orthodontic appliances when orthodontic treatment preceded ear piercing showed a reduced frequency of nickel hypersensitivity[18]. We believe that this may be explained by induction of oral tolerance through release of nickel ions.

Studies using peripheral blood mononuclear cells (PBMC) revealed that not only Th-1, but also Th-2 cytokines played an important role in sensitization and challenge with nickel[10]. Studies have also shown participation of IL-10 in tolerance for nickel[11]. However, digestive organs other than the oral mucosa are involved in sensitization and challenge by and oral tolerance to dentistry materials. Various clinical manifestations due to dental alloys other than skin sensitization implicate different pathogeneses from that of conventional skin sensitization and tolerance. As in the case of atopic and contact dermatitis, differences in Th-1 and Th-2 balance may be involved in these clinical variations[20]. In order to test this hypothesis, we need to compare skin and oral mucosa sensitization with nickel and oral tolerance for nickel in various models. Previous studies have shown that delayed-type hypersensitivity is induced in the oral mucosa by haptens such as 1-chloro-2, 4-dinitrobenzene (DNCB)[13]. However, a model for oral sensitization by metal ions such as those of nickel remains to be established.

There have been reports that mice splenocytes from sensitized donors can transfer nickel sensitization and that those from orally-tolerized donors can transfer nickel tolerance[2,9,16]. These results suggest that the spleen plays an important role in sensitization and orally-induced immunologic tolerance.

Therefore, we established skin-sensitized, oral mucosa-sensitized, and orally-tolerized mice by $NiSO_4$. We then compared challenge reactions, splenocyte proliferation, and splenocyte cytokine profiles between these mice.

Materials and Methods

1. Animals

A total of 42 animals were used in total, divided into 7 groups of 6 animals each. Ten-week-old, female, inbred, C57BL/6J mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Animal experiments were performed according to The Guidelines for the Treatment of Experimental Animals at Tokyo Dental College.

2. Antigen

We used $1\,\mu M$ $NiSO_4$ (Wako Pure Chemicals Inc., Osaka, Japan) in 0.9% NaCl (Otsuka Pharmaceutical, Tokushima, Japan) mixed 1:1 with Complete Freund's Adjuvant (CFA), (Difco Laboratory, Detroit, MI).

3. Oral mucosa and skin sensitization

We performed oral mucosa and skin sensitization experiments with C57BL/6J mice using an adjuvant-based method. The administration sites were both flanks or the bilateral buccal mucosa. The mice were divided into groups of 6 for sensitization. In the skin-sensitized mice, flank hair was shaved using electric hair clippers on the day before initiation of the test. On the day of initiation (Day 0), the mice were injected intradermally in both flanks with $50\,\mu l$ solution containing $1\,\mu M$ $NiSO_4$ in saline mixed 1:1 with CFA for sensitization[3]. Eleven days later, the mice were challenged by injection with $0.2\,\mu M$ $NiSO_4$ in $20\,\mu l$ saline into the pinna of each ear[19]. Ear thickness was measured at 24, 48, 72, and 96 hr after challenge using a digimatic thickness gauge® (0.01–10 mm, Mitutoyo,

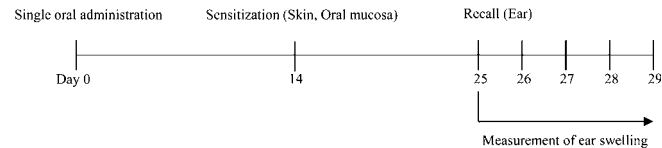## A: Sensitization of naive mice

B: Tolerization of naive mice

Fig. 1 Schematic of experiments
A: Naive mice were sensitized in both flanks or bilateral oral mucosa by injection of NiSO$_4$ with CFA. Eleven days later, they were challenged for recall at ears, and ear-swelling response as an indicator of nickel-allergic reaction determined.
B: Naive mice were tolerized by single oral administration of 1μM NiSO$_4$. After treatment-free interval of 2 weeks, mice were sensitized in both flanks or bilateral oral mucosa. Eleven days later, mice were challenged for recall at ears. Ear-swelling was determined after excitation.

Kanagawa, Japan). The difference in ear thickness between before and after challenge was presented as the mean $(10^{-2}\,\text{mm}) \pm \text{SD}$ of ear swelling. Allergic reactions were evaluated with the mouse ear swelling test (MEST)[19].

In the oral mucosa-sensitized mice, 50 μl of 1 μmol NiSO$_4$ was injected submucosally in the bilateral buccal regions for sensitization on Day 0. As in the skin-sensitized mice, a reaction was induced 11 days after sensitization. The evaluation was the same as in the skin-sensitized mice. The experimental design is shown in Fig. 1.

In the control mice, 50 μl CFA was injected into both flanks or the bilateral buccal regions on Day 0. As in the skin-and oral-sensitized mice, a reaction was induced 11 days after sensitization.

4. Single oral administration

For oral tolerance, mice were fed 50 μl of 1 μM NiSO$_4$ once before sensitization, while avoiding contact with the oral mucosa by use of a pipette. After a treatment-free interval of 2 weeks, the mice were sensitized at skin- or oral-mucosal sites. The orally-tolerized mice

were divided into groups of 6 animals each. The experimental design is shown in Fig. 1.

5. Preparation of splenocytes

All mice were sacrificed at 48 hr after challenge. The spleen was excised from all mice. A splenocyte suspension in phosphate-buffered saline (PBS) was prepared by crushing the spleen with a frosted glass slide. The suspension was filtered through a nylon mesh and centrifuged at 1,500 rpm for 5 min. After removal of the supernatant, a hemolysis solution (0.83% NH$_4$Cl: Tris HCl, pH 7.4, 9:1v/v) was added to hemolyze red blood cells. The cells were filtered through a nylon mesh, washed twice with RPMI1640® (Lonza Walkersville, MD, USA) and a splenocyte suspension at 5×10^6 cells/ml prepared[14].
1) Splenocyte proliferation
Splenocyte suspension was distributed onto a 96-well microplate at 200 μl/well. The splenocytes were then cultured at 37°C for 72 hr in a 5% CO$_2$ incubator. Finally, ^{3}H-thymidine (0.2 μCi/20 μl) was added to each well. The cells were then cultured for 16 hr and ^{3}H-thymidine uptake measured using a cell

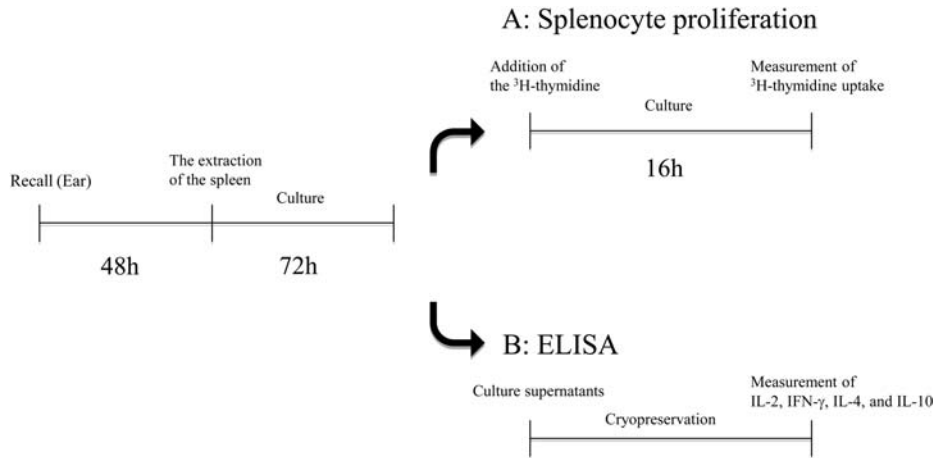## A: Splenocyte proliferation

Fig. 2 Schematic of experiments

All mice sacrificed at 48 hr after challenge. Spleen was excised from all mice. Splenocyte suspension in PBS was prepared by crushing spleen with frosted slide glass. Suspension was filtered through nylon mesh and centrifuged at 1,500 rpm for 5 min. After removal of supernatant, hemolysis solution (0.83% NH_4Cl:Tris HCl, pH 7.4, 9:1v/v) was added to hemolyze red blood cells. Cells were filtered through nylon mesh, washed twice with RPMI1640® (Lonza Walkersville, MD, USA), and splenocyte suspension at 5×10^6 cells/ml prepared.

A: Splenocyte proliferation
    Splenocyte suspension was distributed onto a 96-well microplate at $200\mu l$/well. Splenocytes were then cultured at $37°C$ for 72 hr in 5% CO_2 incubator. Finally, ^{3}H-thymidine ($0.2\mu Ci/20\mu l$) was added to each well, cells were cultured for 16 hr, and ^{3}H-thymidine uptake was measured using cell harvester and liquid scintillation counter.

B: ELISA
    Splenocytes were then cultured at $37°C$ for 72 hr in 5% CO_2 incubator. Culture supernatants were sorted at $-20°C$ prior to ELISA. In culture supernatants, IL-2, IFN-γ, IL-4, and IL-10 were measured using commercially available ELISA kit (R&D systems, Minneapolis, MN, USA).

harvester and liquid scintillation counter. The experimental design is shown in Fig. 2.

2) ELISA

Splenocytes were cultured at $37°C$ for 72 hr in a 5% CO_2 incubator. The culture supernatants were sorted at $-20°C$ prior to ELISA. In the culture supernatants, IL-2, IFN-γ, IL-4, and IL-10 were measured using a commercially available ELISA kit (R&D systems, Minneapolis, MN, USA). The experimental design is shown in Fig. 2.

The lowest detection limit in ELISA was 7.8 pg/ml.

6. Statistical analysis

Statistical significance was determined with an ANOVA followed by the Mann-Whitney U-test. The level of significance was set at $p < 0.05$.

Results

1. Time course of ear swelling after challenge by skin and oral mucosal sensitization

Ear swelling peaked in all mice at 48 hr after challenge. Ear swelling was significantly greater in both skin- and oral mucosa-sensitized mice than in non-sensitized mice (*: $p < 0.01$, **: $p < 0.001$, Fig. 3; $p < 0.001$, Fig. 4, respectively). Sensitization *via* the skin and oral mucosa was successful in all mice. Ear swelling was significantly greater in skin- than in oral mucosa-sensitized mice at 48 hr after challenge ($p < 0.01$, Table. 1).

2. Time course of ear swelling after challenge by single oral administration

Ear swelling was significantly suppressed by single oral administration of $NiSO_4$ in both
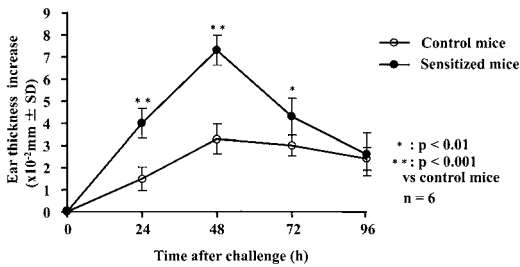
Fig. 3 Time-course of ear swelling in NiSO$_4$-skin sensitized mice

Mice were sensitized to nickel as indicated in Materials and Methods (●). Control mice received emulsified CFA only (○). Eleven days later, mice were challenged with nickel in pinna of each ear. Data are expressed as mean ear-swelling response ($\times 10^{-2}$ mm) $\pm$ SD of 6 mice at 24, 48, 72, or 96 hr after challenge.
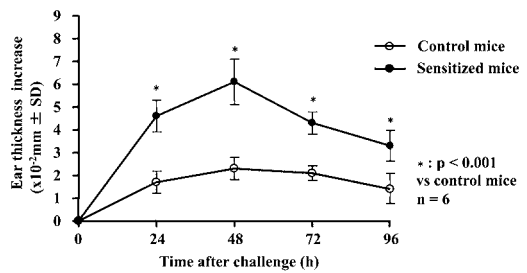

Fig. 4 Time-course of ear swelling in NiSO$_4$-oral sensitized mice

Mice were sensitized to nickel as indicated in Materials and Methods (●). Control mice received emulsified CFA only (○). Eleven days later, mice were challenged with nickel in pinna of each ear. Data are expressed as mean ear-swelling response ($\times 10^{-2}$ mm) $\pm$ SD of 6 mice at 24, 48, 72, or 96 hr after challenge.

Table 1 Ear swelling in NiSO$_4$-skin and oral sensitized mice

	n	Ear thickness increase ($\times 10^{-2}$ mm $\pm$ SD) 48 hr after challenge
Skin sensitized mice	6	7.3 $\pm$ 0.67 ⎤
Oral sensitized mice	6	6.1 $\pm$ 0.99 ⎦ *

*: p<0.01

Data are expressed as mean ear-swelling response ($\times 10^{-2}$ mm) $\pm$ SD in 6 mice at 48 hr after challenge.
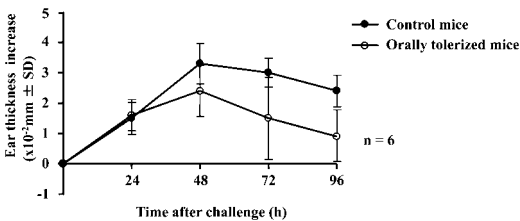

Fig. 5 Time-course of ear swelling in NiSO$_4$-skin sensitized mice by single oral administration

Mice were fed 1 μmol NiSO$_4$ before sensitization. Mice were sensitized to nickel as indicated in Materials and Methods (○). Control mice received emulsified CFA only (●). Eleven days later, mice were challenged with nickel in pinna of each ear. Data are expressed as mean ear-swelling response ($\times 10^{-2}$ mm) $\pm$ SD of 6 mice at 24, 48, 72, or 96 hr after challenge.
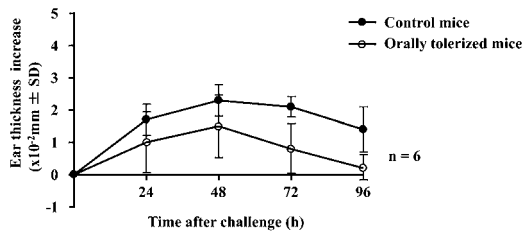

Fig. 6 Time-course of ear swelling in NiSO$_4$-oral sensitized mice by single oral administration

Mice were fed 1 μmol NiSO$_4$ before sensitization. Mice were sensitized to nickel as indicated in Materials and Methods (○). Control mice received emulsified CFA only (●). Eleven days later, mice were challenged with nickel in pinna of each ear. Data are expressed as mean ear-swelling response ($\times 10^{-2}$ mm) $\pm$ SD of 6 mice at 24, 48, 72, or 96 hr after challenge.

skin- and oral mucosa-sensitized mice to the level in control mice (Figs. 5 and 6).

3. Proliferation activity of splenocytes

The proliferation activity of splenocytes from all sensitized mice with or without single oral administration was significantly higher than that in untreated mice (*: p<0.05, **: p<0.001, respectively, Fig. 7). No significant difference was noted between the sensitized
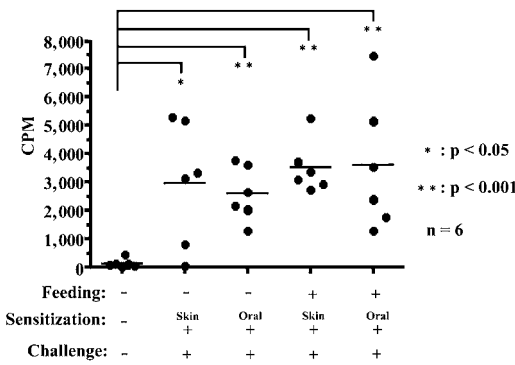
Fig. 7   Splenocyte proliferation response

Splenocyte suspension was distributed onto 96-well microplate at $200\mu l$/well. Splenocytes were then cultured at 37°C for 72 hr in 5% CO_2 incubator. Finally, ^3H-thymidine $(0.2\mu Ci/20\mu l)$ was added to each well, cells were cultured for 16 hr, and ^3H-thymidine uptake was measured using cell harvester and liquid scintillation counter.
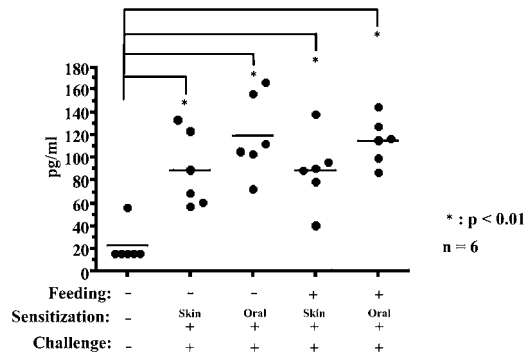

Fig. 8   Effects of $NiSO_4$ treatment on IL-2 production by splenic cells

Splenic cells were harvested and cultured at 1×10^6 cells/$200\mu l$ per well in 96-well plates. After 3-day incubation period, culture supernatant was collected and assayed for IL-2 production. Each value represents mean ± SD.
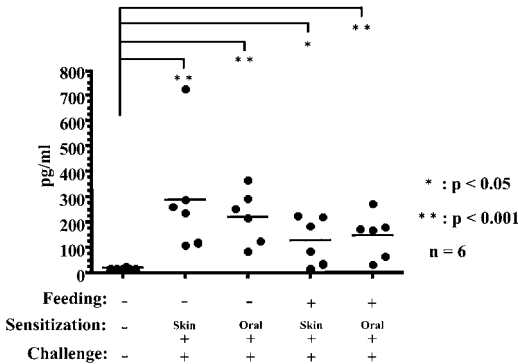
Fig. 9   Effect of $NiSO_4$ treatment on IFN-γ production by splenic cells

Splenic cells were harvested and cultured at 1×10^6 cells/$200\mu l$ per well in 96-well plates. After 3-day incubation period, culture supernatant was collected and assayed for IFN-γ production. Each value represents mean ± SD.

Fig. 10   Effect of $NiSO_4$ treatment on IL-4 production by splenic cells

Splenic cells were harvested and cultured at 1×10^6 cells/$200\mu l$ per well in 96-well plates. After 3-day incubation period, culture supernatant was collected and assayed for IL-4 production. Each value represents mean ± SD.

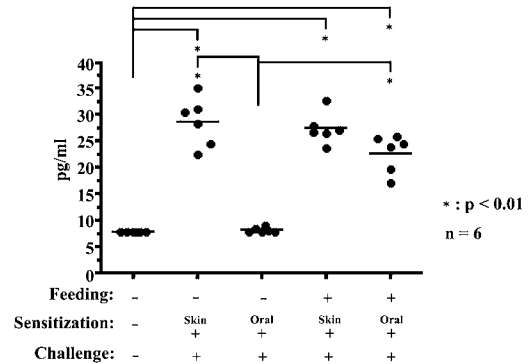mice with or without single oral administration (Fig. 7).

4. IL-2, IFN-γ, IL-4, and IL-10 production in cultured splenocytes

IL-2, IFN-γ, and IL-10 production was significantly increased in skin- and oral mucosa-sensitized mice and orally-tolerized mice compared with in untreated mice ($p<0.01$, Fig. 8, 11; *: $p<0.05$, **: $p<0.001$, Fig. 9,

respectively). However, IL-4 production was induced in skin-sensitized mice and orally-tolerized mice (Fig. 10).

No difference was noted in IL-2, IFN-γ, or IL-10 production with single oral administration between skin- and oral mucosa-sensitized mice (Fig. 8, 9, 11).

IL-4 production in skin-sensitized mice was significantly higher than that in oral mucosa-sensitized mice ($p<0.01$, Fig. 10). IL-4 pro-
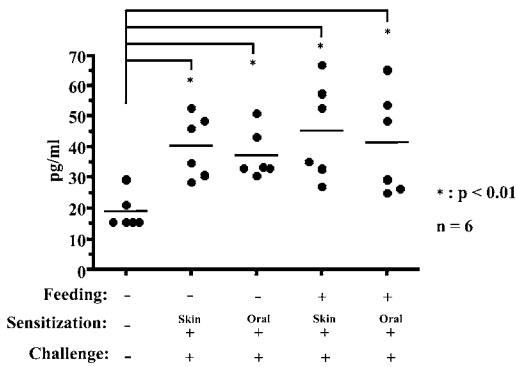
Fig. 11 Effect of NiSO$_4$ treatment on IL-10 production by splenic cells

Splenic cells were harvested and cultured at 1×10^6 cells/200μl per well in 96-well plates. After 3-day incubation period, culture supernatant was collected and assayed for IL-10 production. Each value represents mean ± SD.

duction in oral mucosa-sensitized mice was also significantly increased to the level of that in skin-sensitized mice by single oral administration (p<0.01, Fig. 10). However, no change was observed in IL-4 level by single oral administration in cutaneously sensitized mice.

Discussion

It has been reported that mice can be sensitized by percutaneous injection with NiSO$_4$[3]. However, there have been no previous reports on sensitization with NiSO$_4$ *via* the oral mucosa. In our study, all animals were sensitized either by percutaneous or submucosal administration. These results clearly indicate that NiSO$_4$ can sensitize *via* the oral mucosa similarly to other antigens such as DNCB[13], although submucosal injection of an antigen does not completely simulate contact with dental materials as seen in a clinical setting. Furthermore, ear swelling was significantly greater in the skin- than in the oral mucosa-sensitized mice at 48 hr after challenge with NiSO$_4$. Weaker swelling in the oral- sensitization mice may be due to the lower number of Langerhans' cells, as in the case of DNCB[13].

In previous reports, oral tolerance was suc-

cessfully induced in mice by drinking water containing 9μM NiSO$_4$ for 1 week or 10 mM NiCl$_2$ for 4 weeks[12,15,19]. We found that immunologic tolerance to nickel could only be induced by single oral administration.

A total of 1.1 mM nickel was taken up by mice drinking 10 mM NiCl$_2$-containing water over 4 weeks in a previous report[12,15]. In our experiment, only a total of 1μM nickel induced tolerance by single oral administration, indicating that oral tolerance to nickel can be achieved at a lower concentration than suggested in earlier reports[12,15,19].

In a sensitization study using painting with picryl chloride, ear swelling was suppressed by single oral administration in skin-sensitized mice[1]. However, it was not suppressed in oral mucosa-sensitized mice[1]. In this study, using NiSO$_4$, ear swelling was suppressed by single oral administration in both skin- and oral mucosa-sensitized mice. These discrepancies might to be related to differences in antigen and/or administration method.

Splenocyte proliferation was observed not only in NiSO$_4$-sensitized mice, but also in orally-tolerized mice. We suspect that effecter Th-1 cells, Th-2 cells, Th-3 cells, or CD4$^+$CD25$^+$ regulatory T cells proliferate in the spleen of NiSO$_4$-sensitized and orally tolerized mice[6,10,11,21]. We believe that the splenocyte proliferation seen in this study may reflect oral tolerance, as well as sensitization.

IL-2, IFN-γ, IL-4, and IL-10 production was increased in splenocytes from skin-sensitized mice. Nickel induces *in vitro* production of both Th1- and Th2-type cytokines in PBMC from allergic subjects[10,11]. As in human, a mixed Th-1, Th-2-type cytokine response may occur in the splenocytes of the skin in mice sensitized with NiSO$_4$. Ear swelling was reported to be suppressed by administration of anti-IL-4 antibody in mice sensitized with trinitrochlorobenzene *via* the skin. IL-4 is considered to be essential for ear swelling in skin-sensitized mice[17]. Our report supports this hypothesis. With oral mucosa sensitization, we showed ear swelling without increase in the IL-4 production of splenocytes. Previous studies[5,17] have shown the essential role

of IL-4 among hapten-specific T cell lines from lymph node cells in passive transfer to recipient mice. We believe that this discrepancy with the current results may be due to the investigation of different organs, sensitization with different haptens, or sensitization at different sites. Further study is necessary, however, to elucidate this point.

IL-2, IFN-γ, and IL-10, but not IL-4, production by splenocytes was increased in oral mucosa-sensitized mice. These results suggest that the reaction was Th-1-dominant. IL-2 production was slightly higher than that in skin-sensitized mice. On the other hand, IL-4 was significantly lower than in skin-sensitized mice. These results suggest that IL-4 production in splenocytes is affected by sensitization site.

By inducing oral tolerance, IFN-γ production was slightly reduced only in skin-sensitized mice. This may indicate inhibition of Th-1 response by oral tolerance. In oral mucosa-sensitized mice, IL-4 production was significantly increased to the same level as that in skin-sensitized mice when oral tolerance was induced, indicating a mixed Th-1 and Th-2 response. Oral tolerance was reported to be accompanied by upregulation of IL-4 production in orally tolerized mice[6]. Therefore, these changes in cytokine production are considered to be associated with orally-induced immunologic tolerance. These results on challenge and oral tolerance suggest that Th-1 and Th-2 balance can also be influenced by sensitization site.

In conclusion, this MEST-based study demonstrated that mice can be sensitized with NiSO$_4$ *via* the oral mucosa, as well as the skin. In addition, we have shown that immunologic tolerance can be induced by single oral administration of an antigen. The splenocyte cytokine profile study indicated a mixed Th-1, Th-2-type cytokine response in skin-sensitized mice, but a Th-1-type cytokine response in oral mucosa-sensitized mice. By oral tolerance, Th-1 response was inhibited in skin sensitization. On the other hand, by oral tolerance, Th-2-type cytokine response was induced in oral sensitization. These results suggest that skin and oral mucosa sensitization induces different Th-1 and Th-2 balances in excitation and oral tolerance.

Acknowledgements

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Reprint requests to:
    Dr. Masaru Fujimoto
    Department of Oral Medicine,
    Oral and Maxillofacial Surgery,
    Tokyo Dental College,
    5-11-13 Sugano, Ichikawa,
    Chiba 272-8513, Japan
    E-mail: masarufujimoto@tdc.ac.jp