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Original Article

## Th1/Th2 Balance in Mouse Delayed-type Hypersensitivity Model with Mercuric Chloride *via* Skin and Oral Mucosa

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### Abstract

In order to compare delayed-type hypersensitivity (DTH) among different exposure sites, we evaluated the sensitization potency of mercuric chloride ( $\text{HgCl}_2$ ) *via* exposure to the skin, or oral or esophageal mucosa using the mouse ear swelling test. Furthermore, we investigated *in vitro* splenocyte proliferation reaction and cytokine profile in  $\text{HgCl}_2$ -exposed and control mice. Sensitization with  $\text{HgCl}_2$  was established *via* the skin and oral mucosa but not *via* the esophageal mucosa. The splenocyte proliferation reaction was significantly enhanced to a similar degree in skin and oral mucosa-sensitized mice compared with in the control mice. IL-10 levels from cultured splenocytes were significantly increased in skin and oral mucosa-sensitized mice compared with those in control mice, whilst IFN- $\gamma$  significantly increased only in splenocytes from skin-sensitized mice. These results suggest that exposure of the skin or oral mucosa to  $\text{HgCl}_2$  can induce DTH, but that Th1/Th2 balance differs according to the site of antigen exposure.

Key words: Mercury— $\text{HgCl}_2$ —Oral mucosa—DTH—Mice

### Introduction

Despite the fact that skin contact with mercury can cause contact dermatitis, this metal is often used in dental restoration as an amalgam<sup>4,8)</sup>. Dental restoration can induce oral mucosal diseases such as lichen planus and allergic stomatitis from direct exposure to metal<sup>7,18)</sup>. In addition, metals other than mer-

cury used in dental restoration have also been reported to induce skin lesions at distant sites from the oral cavity, including palmoplantar pustulosis<sup>20)</sup>.

Diagnosis of mercury allergy is usually based on patch tests. However, a history of allergic reaction is sometimes present, even when negative results are obtained with patch testing<sup>5)</sup>. Patch tests need to be carried out on a

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frequent basis and interpreting the results can be problematic in the summer months. Furthermore, this procedure carries the risks of patient sensitization to new antigens and excited skin syndrome, and specialized training is necessary to interpret the results. Therefore, the lymphocyte transformation test (LTT) has attracted attention as a potential new option in testing for metal allergy<sup>12,17</sup>. However, accurate interpretation is difficult, as the LTT in humans can result in non-specific lymphocyte proliferation and false negative results<sup>6,10,15</sup>. Therefore, the establishment of a new diagnostic method is required.

As described above, some metals used in dental restoration have been reported to induce oral mucosal lesions. Oral contact sensitivity may be associated not only with local exposure, but also with exposure of the esophageal and lower digestive tract mucosa to metals released from dental amalgam. Therefore, it is important to compare delayed-type hypersensitivity (DTH) reactions from mercury exposure at different sites in order to clarify the pathogenesis of mercury allergy.

The successful establishment of a mouse skin sensitization model using mercuric chloride (HgCl<sub>2</sub>) as an antigen has been reported<sup>19</sup>. In mouse models using oxazolone or picryl chloride as an antigen, the oral mucosa can serve as the sensitization and/or expression site of DTH<sup>1</sup>. To our knowledge, no reports describing the sensitizing effect of mercury-containing metals on the oral, esophageal and lower digestive tract mucosa have been published to date.

Therefore, we established a mouse sensitization model using BALB/cA Jcl mice (BALB/c mice hereafter) using the mouse ear swelling test without the use of adjuvants, based on the method of Natsuaki *et al.* and Okamura *et al.*<sup>14,16</sup>. HgCl<sub>2</sub> applied to the oral mucosa may flow into the esophageal and lower digestive tract mucosa. To clarify its action, we compared HgCl<sub>2</sub> applied to the oral mucosa alone with HgCl<sub>2</sub> applied solely to the esophageal and lower digestive tract mucosa (whilst avoiding contact with the oral mucosa) and determined whether sensitiza-

tion was established.

To compare DTH resulting from HgCl<sub>2</sub> sensitization of the skin, oral mucosa, and esophageal/lower digestive tract mucosa, we examined ear swelling response, splenocyte proliferation *in vitro*, and cytokine profile in HgCl<sub>2</sub>-exposed and control mice.

## Materials and Methods

### 1. Animals

We purchased 7-week-old female inbred BALB/c mice from CLEA Japan (Tokyo, Japan). Experiments were performed in accordance with the Tokyo Dental College ethical guidelines for animal experiments. We performed the experiments in an animal room of the Research Building at Ichikawa General Hospital, Tokyo Dental College. The body weight of the animals was uniform at about 20g each.

### 2. Antigen

HgCl<sub>2</sub> (Wako Pure Chemicals, Osaka, Japan) was dissolved in a 4:1 mixture of acetone and olive oil (A-O solution)<sup>14</sup>. The concentration of HgCl<sub>2</sub> was set to 0.5, 1.0, 2.0, 5.0 or 10.0 mg/ml.

### 3. Irritation dose response

Delayed-type hypersensitivity reaction was determined based on ear thickness, so the reaction needed to be induced at a non-irritating concentration. Therefore, we selected the highest non-irritation concentration of HgCl<sub>2</sub> as the optimum challenge concentration (Table 1). HgCl<sub>2</sub> (in a 20  $\mu$ l volume) was applied to the front and reverse sides of the right auricle at concentrations of 0.5, 1.0, 2.0, 5.0 or 10.0 mg/ml and ear thickness measured at 12, 24, 48 and 72 hr. We divided the experimental groups according to concentration of HgCl<sub>2</sub> administered with 5 animals per group (total number of animals was 25). Difference in thickness from the pre-challenge thickness is presented as the mean  $\pm$  standard error (S.E.M.) (10<sup>-2</sup> mm).

Table 1 Irritation dose response results for HgCl<sub>2</sub>

Irritation dose (HgCl <sub>2</sub> mg/ml)	Mean ear thickness increase ( $\times 10^{-2}$ mm $\pm$ S.E.M.)	Maximal ear thickness increase ( $\times 10^{-2}$ mm)
0.5	0.2 $\pm$ 0.45	1
1.0	1.0 $\pm$ 0.71	1
2.0	0.4 $\pm$ 0.55	1
5.0	0.6 $\pm$ 0.55	2
10.0	3.4 $\pm$ 1.23	5

Ear thickness increase was computed by averaging change from pre-challenge value at 24hr after exposure.

#### 4. Mouse ear swelling test

We performed the sensitization experiments on BALB/c mice using a method which required no use of adjuvants<sup>14,16</sup>. The administration sites were the dorsal skin, bilateral buccal mucosa and esophageal/lower digestive tract mucosa. We divided the experimental groups into 3 groups according to site of administration (skin, oral mucosa and esophagus). The same was also done in the control group. Within each group of mice that were exposed to HgCl<sub>2</sub>, there were 5 animals per concentration, with a total of 25 animals in each group.

In the skin group, we shaved dorsal hair using electric hair clippers on the day before antigen application. On the day of sensitization (day 0), we applied 20  $\mu$ l HgCl<sub>2</sub> to the dorsal skin at a concentration of 2.0, 5.0, 10.0, 15.0 or 20.0 mg/ml (applied area was about 2 cm<sup>2</sup>). Five days later, the right auricle was challenged with 20  $\mu$ l HgCl<sub>2</sub> solution. We measured ear thickness at 12, 24, 48 and 72 hr after the challenge using the Digimatic thickness gauge (0.01–10 mm, Mitutoyo, Kanagawa, Japan). The difference in ear thickness between before and after application was presented as the mean  $\pm$  S.E.M. ( $10^{-2}$  mm). When ear swelling was greater than the maximum ear swelling at the optimum challenge concentration, the reaction was regarded as positive. We selected the minimum concentration for obtaining the highest sensitization rate as the optimum sensitizing concentration.

In the oral mucosa group, we applied 20  $\mu$ l HgCl<sub>2</sub> to the dried bilateral buccal mucosa at

concentrations of 2.0, 5.0, 10.0, 15.0 or 20.0 mg/ml on the day of sensitization (day 0). The buccal mucosa was dried with a drier before and after application. The applied area was approximately 2 cm<sup>2</sup>.

In the esophagus group, we applied 20  $\mu$ l HgCl<sub>2</sub> to the esophageal mucosa at concentrations of 2.0, 5.0, 10.0, 15.0 or 20.0 mg/ml on the day of sensitization (day 0). Administration of HgCl<sub>2</sub> was performed using a pipette to avoid contact with the oral mucosa. The reagent was poured from the pharyngeal region. As with the skin group, we challenged the ears at 5 days after HgCl<sub>2</sub> administration in the oral mucosa and esophagus groups.

#### 5. Splenocyte proliferation reaction

We excised the spleen from mice in the control, skin, oral mucosa and esophagus groups. Splenocyte suspension in phosphate-buffered saline (PBS) was prepared by crushing the spleen with a frosted slide glass. The splenocyte suspension was filtered through a nylon mesh and centrifuged at 1,500 rpm for 5 minutes. After removal of the supernatant, hemolysis solution (0.83% NH<sub>4</sub>Cl in Tris-HCl, pH 7.4, 9:1, v/v) was added to hemolyze red blood cells. The cells were filtered through a nylon mesh and washed with RPMI1640 twice, and a splenocyte suspension at a concentration of  $5 \times 10^6$  cells/ml was prepared<sup>6</sup>. We distributed the splenocyte suspension into a 96-well microplate at 200  $\mu$ l/well. The splenocytes were then cultured at 37°C for 72 hr in a 5% CO<sub>2</sub> incubator. Finally, we added <sup>3</sup>H-thymidine (0.2  $\mu$  Ci/20  $\mu$ l) into each well.

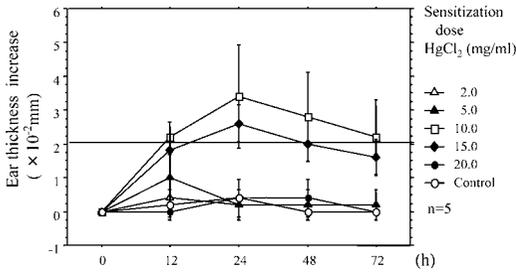


Fig. 1 Time-course of ear swelling in HgCl<sub>2</sub>-sensitized mice (skin sensitization, 5.0 mg/ml challenged)

Responses upon induction to 5 different sensitization doses were evaluated, in addition to response in control group (0 mg/ml), which was given acetone/olive oil (n=5). All groups of mice were challenged with 5.0 mg/ml HgCl<sub>2</sub> on day 5. Sensitization application was given on back skin. Each value represents mean  $\pm$  standard error (S.E.M.).

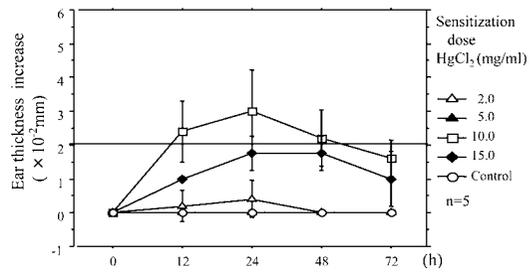


Fig. 2 Time-course of ear swelling in HgCl<sub>2</sub>-sensitized mice (oral mucosa sensitization, 5.0 mg/ml challenged)

Responses upon induction to 5 different sensitization doses were evaluated, in addition to response in control group (0 mg/ml), which was given acetone/olive oil (n=5). All groups of mice were challenged with 5.0 mg/ml HgCl<sub>2</sub> on day 5. Sensitization application was given on buccal mucosa. Each value represents mean  $\pm$  standard error (S.E.M.).

Cells were cultured for 16 hr and <sup>3</sup>H-thymidine uptake measured using a cell harvester and liquid scintillation counter. Each group comprised 5 animals, giving a total number of 20 animals.

## 6. Enzyme-Linked Immunosorbent Assay (ELISA)

We cultured the splenocytes at 37°C for 72 hr in a 5% CO<sub>2</sub> incubator. The culture supernatants were stored at -20°C prior to ELISA. We measured the cytokines IL-2, IFN- $\gamma$ , IL-4 and IL-10 in culture supernatants using commercially available ELISA kits (R&D systems, Minneapolis, MN, USA).

## 7. Statistical analysis

Statistical significance with respect to change in splenocyte proliferation and cytokine profile for each group was analyzed using the Mann-Whitney U-test.

## Results

### 1. Determination of challenge concentration

Table 1 shows the data of the primary irritation reaction to HgCl<sub>2</sub>. The irritancy of HgCl<sub>2</sub> was investigated at 5 concentrations: 0.5, 1.0, 2.0, 5.0 or 10.0 mg/ml. The mean ear swellings at 24 hr after application of HgCl<sub>2</sub> solu-

tion at the 5 concentrations were  $0.2 \pm 0.45$ ,  $1.0 \pm 0.71$ ,  $0.4 \pm 0.55$ ,  $0.6 \pm 0.55$  and  $3.4 \pm 1.23 \times 10^{-2}$  mm, in order of increasing concentration. No ear swelling was noted at concentrations of up to 5.0 mg/ml. Therefore, 5.0 mg/ml was selected as the challenge concentration, and values higher than the maximum ear swelling at 5.0 mg/ml, *i.e.*  $2.0 \times 10^{-2}$  mm, were judged as positive for sensitization.

### 2. Time-course changes in ear swelling sensitized through different sites

#### 1) Skin group

Figure 1 shows the time-course changes with HgCl<sub>2</sub>-induced ear swelling. In mice sensitized with 10.0 mg/ml or 15.0 mg/ml HgCl<sub>2</sub>, ear swelling peaked at 24 hr after challenge. The sensitization rate was 100%. No ear swelling was noted at any time point in mice sensitized with less than 10.0 mg/ml HgCl<sub>2</sub>. The optimum skin sensitization concentration was 10.0 mg/ml.

#### 2) Oral mucosa group

Figure 2 shows time-course changes in HgCl<sub>2</sub>-induced ear swelling. Ear swelling peaked at 24 hr after challenge in mice sensitized with 10.0 mg/ml HgCl<sub>2</sub>, and the sensitization rate was 100%. All mice sensitized with 20.0 mg/ml HgCl<sub>2</sub> died within 1–2 days. As in the skin group, no ear swelling was noted in mice sensitized with less than 10.0 mg/ml

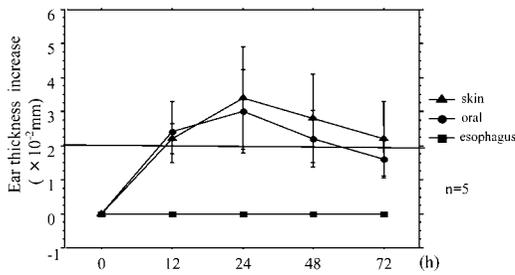


Fig. 3 Comparison of time-course of ear swelling in HgCl<sub>2</sub>-sensitized mice *via* skin, oral mucosa and esophagus (10.0 mg/ml sensitization 5.0 mg/ml challenged)

Each value represents mean  $\pm$  standard error (S.E.M.).

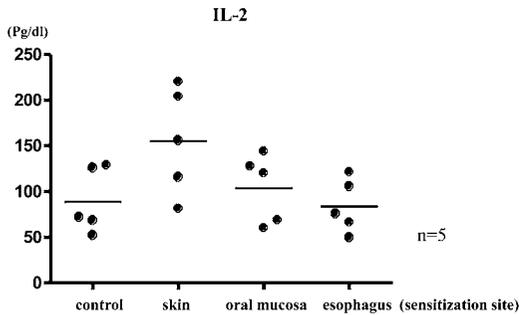


Fig. 5 Effects of HgCl<sub>2</sub> treatment on IL-2 production by splenic cells

Splenic cells were harvested and cultured at  $1 \times 10^6$  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  $\pm$  SD.

HgCl<sub>2</sub>. The optimum concentration for oral mucosal sensitization was 10.0 mg/ml. No significant difference in increase in ear thickness was noted between the oral group and the skin group after challenge (Fig. 3).

### 3) Esophagus group

No ear swelling was noted at any concentration (data not shown).

### 3. Proliferation of splenocytes

Compared to in the controls, splenocyte proliferation was significantly greater in splenocytes from the skin ( $p < 0.01$ ) and oral mucosa ( $p < 0.05$ ) groups. No significant dif-

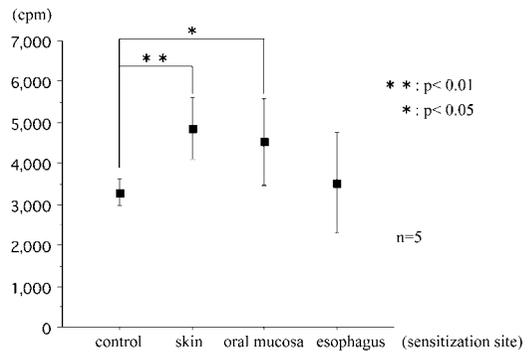


Fig. 4 Splenocyte proliferative response Splenocyte suspension was distributed to 96-well microplate at 200  $\mu$ l/well.

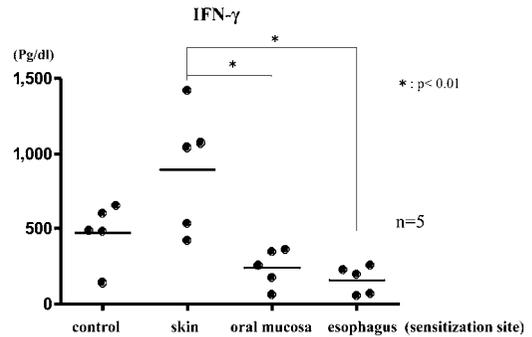


Fig. 6 Effects of HgCl<sub>2</sub> treatment on IFN- $\gamma$  production by splenic cells

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

ference was observed in splenocyte proliferation between the esophagus and control groups (Fig. 4).

### 4. Cytokine production by cultured splenocytes

The IL-2 level tended to increase in the skin group compared with in the other groups (Fig. 5). The IFN- $\gamma$  level was significantly higher in the skin group compared with in the oral mucosa and esophagus groups ( $p < 0.01$ , Fig. 6). No significant differences were observed in IL-4 production among the groups (Fig. 7). The level of IL-10 production

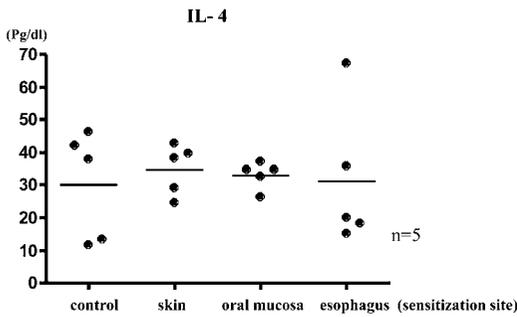


Fig. 7 Effects of  $\text{HgCl}_2$  treatment on IL-4 production by splenic cells

Splenic cells were harvested and cultured at  $1 \times 10^6$  cells/ $200 \mu\text{l}$  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  $\pm$  SD.

was significantly increased in the skin and oral mucosa groups compared with in the control group (Fig. 8).

## Discussion

We confirmed the establishment of sensitization with  $\text{HgCl}_2$  *via* skin and oral mucosa but not the esophageal or lower digestive tract mucosa. Previous studies have shown that mice were sensitized by application of the haptens DNCB or oxazolone to the oral mucosa, as well as the skin<sup>1-3,16</sup>. The present results are consistent with the results of these earlier studies. The non-sensitization of the mice in the esophagus group indicates that the oral mucosa mice were sensitized *via* only the oral mucosa, and not *via* the lower digestive tract, including the esophagus.

The optimal  $\text{HgCl}_2$  sensitization concentration was 10.0 mg/ml in both the skin and oral mucosa groups. To our knowledge, no previous studies have reported induction of sensitization in the oral mucosa by metals containing mercury. Vreeburg *et al.* reported that the optimal concentration for skin sensitization was 5.0%<sup>19</sup>. The difference in the optimal concentration for skin sensitization between this and our study may be due to the different solvents used (A-O solution vs. Vaseline).

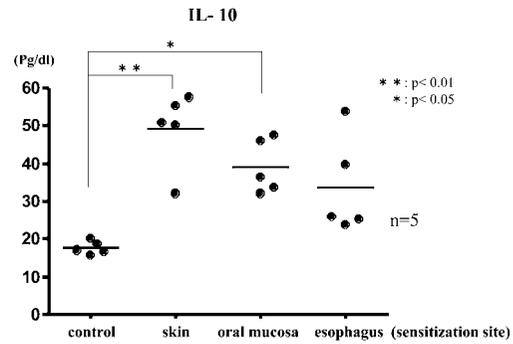


Fig. 8 Effects of  $\text{HgCl}_2$  treatment on IL-10 production by splenic cells

Splenic cells were harvested and cultured at  $1 \times 10^6$  cells/ $200 \mu\text{l}$  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  $\pm$  SD.

The splenocyte proliferation reaction significantly increased in the skin ( $p < 0.01$ ) and oral mucosa ( $p < 0.05$ ) groups compared with in the control group. However, no significant difference was observed between the esophagus and control groups. These results suggest that increased splenocyte proliferation is associated with sensitization itself, but not only with application. The lack of a significant difference in splenocyte proliferation between the skin and oral mucosa groups may be related to the similarity in ear swelling in these groups. An earlier study using the LTT with  $\text{HgCl}_2$  as a reagent noted non-specific blast formation of human lymphocytes, making accurate judgment problematic<sup>6,9</sup>. Another study showed no difference in peripheral lymphocyte proliferation between patients with oral mucosal diseases treated using dental amalgam and healthy subjects<sup>11</sup>. These results suggest that it is difficult to identify  $\text{HgCl}_2$ -sensitized T lymphocytes by routine LTT, although the results of our preliminary study were different from those of human studies. This indicates the need to establish new methods of examination such as cytokine profiling.

In the cytokine profile of splenocytes, Th1 and Th2 reactions were observed in the skin group. In patients positive for nickel patch tests, an increase in Th1/Th2 cytokines has

been reported<sup>13</sup>). Herein, we obtained similar results. However, in our experiments, only the Th2 reaction increased in the oral mucosa group. We speculate that the evaluation of blood cytokines may be useful in determining the presence or absence of sensitization and in indentifying exposure sites. Further studies on the regional lymph nodes in sensitized mice will help our understanding on the relationship between Th1/Th2 balance and sensitization sites. We are now engaged in these studies.

In summary, HgCl<sub>2</sub> applied at a single dose resulted in similar degrees of sensitization in the skin and oral mucosa. Splenocyte proliferation increased in the skin and oral mucosa-sensitized mice compared with in the control mice. The cytokine profile of splenocytes in skin-sensitized mice showed increases in Th1 and Th2 reactions, but oral mucosa-sensitized mice revealed only an increase in Th2 reaction. These results suggest that there are differences in the Th1/Th2 balance between sensitization through different sites in DTH.

### References

- Ahlfors E, Czerkinsky C (1991) Contact sensitivity in the murine oral mucosa. An experimental model of delayed-type hypersensitivity reactions at mucosal surfaces. *Clin Exp Immunol* 86:449–456.
- Ahlfors E, Jonsson R, Czerkinsky C (1996) Experimental T cell-mediated inflammatory reactions in the murine oral mucosa. II. Immunohistochemical characterization of resident and infiltrating cells. *Clin Exp Immunol* 104:297–305.
- Ahlfors E, Lyberg T (2001) Contact sensitivity reactions in the oral mucosa. *Acta Odontol Scand* 59:248–254.
- Bains VK, Loomba K, Loomba A, Bains R (2008) Mercury sensitization: review, relevance and a clinical report. *Br Dent J* 205:373–378.
- Böhm I, Brody M, Bauer R (1997) Comparison of personal history with patch test results in metal allergy. *J Dermatol* 24:510–513.
- Cederbrant K, Gunnarsson LG, Hultman P, Norda R, Tibbling-Grahn L (1999) *In vitro* lymphoproliferative assays with HgCl<sub>2</sub> cannot identify patients with systemic symptoms attributed to dental amalgam. *J Dent Res* 78:1450–1458.
- Dunsche A, Kästel I, Terheyden H, Springer IN, Christophers E, Brasch J (2003) Oral lichenoid reactions associated with amalgam: improvement after amalgam removal. *Br J Dermatol* 148:70–76.
- Garner L (2004) Contact dermatitis to metals. *Dermatol Ther* 17:321–327.
- Henderson DC, Clifford R, Young DM (2001) Mercury-reactive lymphocytes in peripheral blood are not a marker for dental amalgam associated disease. *J Dent* 29:469–474.
- Laine J, Happonen RP, Vainio O, Kalimo K (1997) *In vitro* lymphocyte proliferation test in the diagnosis of oral mucosal hypersensitivity reactions to dental amalgam. *J Oral Pathol Med* 26:362–366.
- Loftenius A, Skoglund A, Ekstrand J, Hovmark A, Möller E (1999) No evidence for specific *in vitro* lymphocyte reactivity to HgCl<sub>2</sub> in patients with dental amalgam-related contact lesions. *J Oral Pathol Med* 8:364–370.
- Martins LE, Duarte AJ, Aoki V, Nunes RS, Ogusuku S, Reis VM (2008) Lymphocyte proliferation testing in chromium allergic contact dermatitis. *Clin Exp Dermatol* 33:472–477.
- Minang JT, Arestrom I, Troye-Blomberg M, Lundeberg L, Ahlberg N (2006) Nickel, cobalt, chromium, palladium and gold induce a mixed Th1- and Th2-type cytokine response *in vitro* in subjects with contact allergy to the respective metals. *Clin Exp Immunol* 146:417–426.
- Natsuaki M, Yano N, Yamaya K, Kitano Y (2000) Immediate contact hypersensitivity induced by repeated hapten challenge in mice. *Contact Dermatitis* 43:267–272.
- Nordlind K, Liden S (1993) *In vitro* lymphocyte reactivity to heavy metal salts in the diagnosis of oral mucosal hypersensitivity to amalgam restorations. *Br J Dermatol* 128:38–41.
- Okamura T, Morimoto M, Yamane G, Takahashi S (2003) Langerhans' cells in the murine oral mucosa in the inductive phase of delayed type hypersensitivity with 1-chloro-2, 4-dinitrobenzene. *Clin Exp Immunol* 134:188–194.
- Takahashi S, Kawashima J, Morimoto M, Yamane G (2003) Lymphocyte transformation test in comparison with patch test using nickel, cobalt, palladium, gold, chromium and mercury. *Environ Dermatol* 10:64–69.
- Thornhill MH, Pemberton MN, Simmons RK, Theaker EO (2003) Amalgam-contact hypersensitivity lesions and oral lichen planus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 95:291–299.

- 19) Vreeburg K, Groot K, Hoogstraten I, Blomberg B, Scheper R (1991) Successful induction of allergic contact dermatitis to mercury and chromium in mice. *Int Arch Allergy Appl Immunol* 96:179–183.
- 20) Yiannias JA, Winkelmann RK, Connolly SM (1998) Contact sensitivities in palmoplantar pustulosis (acropustulosis). *Contact Dermatitis* 39:108–111.

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