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Intraoral administration of a T-cell epitope peptide induces immunological tolerance in Cry j 2-sensitized mice

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Abstract: Sublingual immunotherapy using allergen-derived peptides is feasible as a novel specific immunotherapy, but its efficacy has not yet been demonstrated in either humans or animals. In addition, it remains obscure whether the oral immune system is involved in the mechanism of sublingual immunotherapy. Here, we show that the intraoral administration of the T-cell epitope peptide P2-246-259 derived from Cry j 2, a major Japanese cedar (*Cryptomeria japonica*) pollen allergen, to Cry j 2-sensitized mice induces immunological tolerance, and that *ex vivo* lymph node cell proliferation to P2-246-259 and Cry j 2 was inhibited. In addition, intraoral administration was shown to be superior to intragastric administration in terms of tolerance induction, suggesting that the oral immune system contributes to the induction of immunological tolerance. Therefore, the significant efficacy of sublingual immunotherapy using a peptide on allergen-specific T-cells was demonstrated in animals, and this may be potentiated by the oral mucosal immune system. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: sublingual immunotherapy; T-cell epitope; immunological tolerance; allergen; Cry j 2

INTRODUCTION

Specific immunotherapy is the only current treatment that has the potential to cure and reduce the symptoms and medication requirements for allergic diseases such as allergic rhinitis [1]. Sublingual-swallow immunotherapy (SLIT) is raising considerable interest as an alternative for conventional subcutaneous immunotherapy [2–4], although it remains obscure whether the oral mucosal immune system is involved in the mechanism of SLIT. Peptide immunotherapy, in which a peptide corresponding to the T-cell epitope of a protein allergen is utilized instead of the allergen extract, has also been proposed as a novel immunotherapy [5]. The combination of these two immunotherapies, i.e. SLIT using a peptide, should be feasible, but the efficacy of this treatment has not yet been demonstrated in either humans or animals.

It is well known that oral administration of an allergen or a peptide induces a state of systemic immunological unresponsiveness in animals, which is called oral tolerance, and this phenomenon is considered as a basis for SLIT [6]. In most animal studies, however, administration has been conducted by gastric intubation; an allergen or a peptide is exposed only to the intestinal mucosa but is not exposed to the oral mucosa, which is the main exposure site in SLIT. There are a few studies [7–9] in which intraoral administration was examined as a route for inducing immunological tolerance, but none of these studies used a T-cell epitope peptide as a tolerogen.

In our previous study [10,11], we investigated T-cell epitope of Cry j 2, a major Japanese cedar (*Cryptomeria japonica*) pollen allergen, using a set of overlapping peptides and we found that the peptide P2-246-259 was a major T-cell epitope in BALB/c mice. Gastric intubation of P2-246-259 to mice before and after intranasal sensitization with Cry j 2 inhibited the proliferative responses of T-cells to P2-246-259 and Cry j 2. In this study, we investigated whether intraoral administration of P2-246-259 before and after intranasal sensitization (in both prophylactic and therapeutic regimens) would induce immunological tolerance in T-cell response. In addition, we compared the effects of intraoral and intragastric administrations of P2-246-259 in order to examine whether the oral mucosal immune system is involved in the induction of tolerance. As a result, the significant efficacy of sublingual immunotherapy using a peptide was demonstrated in mice, which may be potentiated by the oral mucosal immune system.

MATERIALS AND METHODS

Animals

Five or six-week-old female BALB/c mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan)

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and housed under conventional conditions. The Institutional Animal Care and Use Committee of Sankyo approved all the experiments in this study.

Reagents

Cry j 2 was purified from Japanese cedar pollen using a monoclonal antibody specific to Cry j 2 (N26) [12]. P2-246-259 (RAEVSYVHVNGAKF), a T-cell epitope peptide derived from Cry j 2, was synthesized at Sigma-Aldrich Japan K.K. (Tokyo, Japan) and Peptide Institute, Inc. (Osaka, Japan). Cholera toxin was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Intranasal Sensitization

The mice were intranasally administered with a solution consisting of 1 μg of Cry j 2 and 1 μg of cholera toxin, which was used as a mucosal adjuvant dissolved in 10 μl of phosphate-buffered saline (PBS).

Induction of Tolerance

The mice were intraorally administered with P2-246-259 at doses of 1, 10 or 100 μg/body dissolved in 20 μl of PBS. The intraoral administration was performed by injecting the solution slowly into the oral cavities of the mice using a micropipette. We confirmed that the Evans blue solution was distributed within the oral cavity and esophagus right after intraoral administration of the solution. In some experiments, mice were intragastrically administered with P2-246-259 at doses of 1, 10 or 100 μg/body dissolved in 200 μl of PBS. The intragastric administration was performed using a plastic animal-feeding needle. The control mice were intraorally or intragastrically administered with the same volume of PBS as the peptide solution. In the prophylactic regimen, the mice were administered with P2-246-259 on days 14 and 7, and sensitized with Cry j 2 on days 0 and 14. In the therapeutic regimen, the mice were sensitized with Cry j 2 on day 0, and administered with P2-246-259 on days 7 and 14. Then the mice were sensitized with Cry j 2 again on day 21.

Proliferation Assay

One week after the last sensitization, the cervical lymph nodes were removed from the mice and pooled in each group, and a single cell suspension of cervical lymph node cells (cLNCs) was prepared for a proliferation assay. An erythrocyte-depleted, X-ray-irradiated spleen-cell suspension was also prepared from autologous normal mice and used as antigen presenting cells (APCs). The cLNCs were cultured at 3×10^5 cells/well together with the APCs at 4×10^5 cells/well, in 0.2 ml of RPMI 1640 (Invitrogen Corporation, San Diego, CA) supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin (Invitrogen Corporation) and 1% of serum prepared from autologous normal mice, in 96-well, flat bottom plates. The cells were cultured with indicated concentrations of P2-246-259, Cry j 2 or without stimulants in triplicate wells at 37 °C with 5% CO_2 for 3 days. Then, 0.5 μCi of [^{3}H]thymidine (Amersham Biosciences UK Ltd., Little Chalfont, UK) was added to each well and the cells were incubated for another 18 h. The cells were harvested and the radioactivity of the [^{3}H]thymidine incorporated to the DNA was measured using a liquid scintillation counter. The stimulation index was calculated by dividing the counts per minute (CPM) in the presence of stimulants by the mean CPM in the absence of stimulants. The results are expressed as the mean stimulation index ± S.E.M.

RESULTS

The Induction of Immunological Tolerance by Intraoral Administration of P2-246-259 in a Prophylactic Regimen

First, we investigated whether intraoral administration of the T-cell epitope peptide P2-246-259 into mice would induce immunological tolerance in a prophylactic regimen. P2-246-259 is a major T-cell epitope peptide derived from Cry j 2. Mice were intraorally administered with 1, 10 or 100 μg/body of P2-246-259. Control mice were administered with PBS. Then, these mice were intranasally sensitized with Cry j 2. One week after the second sensitization, a cell suspension of cLNCs was prepared in each group and the cells were stimulated with P2-246-259. The cLNCs from the control mice proliferated well to P2-246-259, whereas cLNCs from the mice that had been intraorally administered with P2-246-259 showed greatly decreased proliferation to P2-246-259, indicating that intraoral administration of P2-246-259 inhibited cLNC proliferation to the P2-246-259 (Figure 1). The inhibition of proliferation was dependent on the dose of P2-246-259 administered and the proliferation was completely inhibited at doses of 10 and 100 μg/body. Therefore, intraoral administration of P2-246-259 before allergen sensitization induced immunological tolerance.

The Induction of Immunological Tolerance by Intraoral *vs* Intragastric Administration of P2-246-259 in a Therapeutic Regimen

Next, we investigated whether intraoral administration of P2-246-259 would induce immunological tolerance in a therapeutic regimen. In addition, in order to examine whether the oral immune system is involved in the induction of immunological tolerance, we compared intraoral and intragastric administrations. Mice were first intranasally sensitized with Cry j 2. Then, they were intraorally or intragastrically administered with 1, 10 or 100 μg/body of P2-246-259. After the mice were re-sensitized with Cry j 2, a cell suspension of cLNCs was prepared and the cells were stimulated with P2-246-259 or Cry j 2. While the cLNCs from the control mice proliferated well to P2-246-259, the cells from the mice that were intraorally administered with P2-246-259 at all doses showed greatly diminished proliferation to P2-246-259 (Figure 2(A)). On the other hand, the cLNC from mice that were intragastrically administered with P2-246-259 showed decreased

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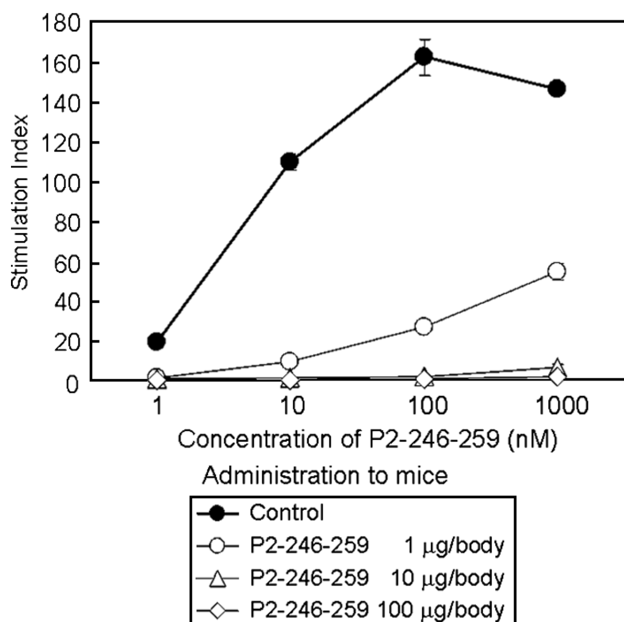

Figure 1 Intraoral administrations of P2-246-259 to mice before allergen sensitization showing inhibited cLNC proliferation to P2-246-259. The background responses (CPM) were 138.4 ± 20.1, 126.1 ± 10.7, 139.9 ± 30.9 and 121.2 ± 25.1 for the doses of 0 (control), 1, 10 and 100 μg/body groups, respectively.

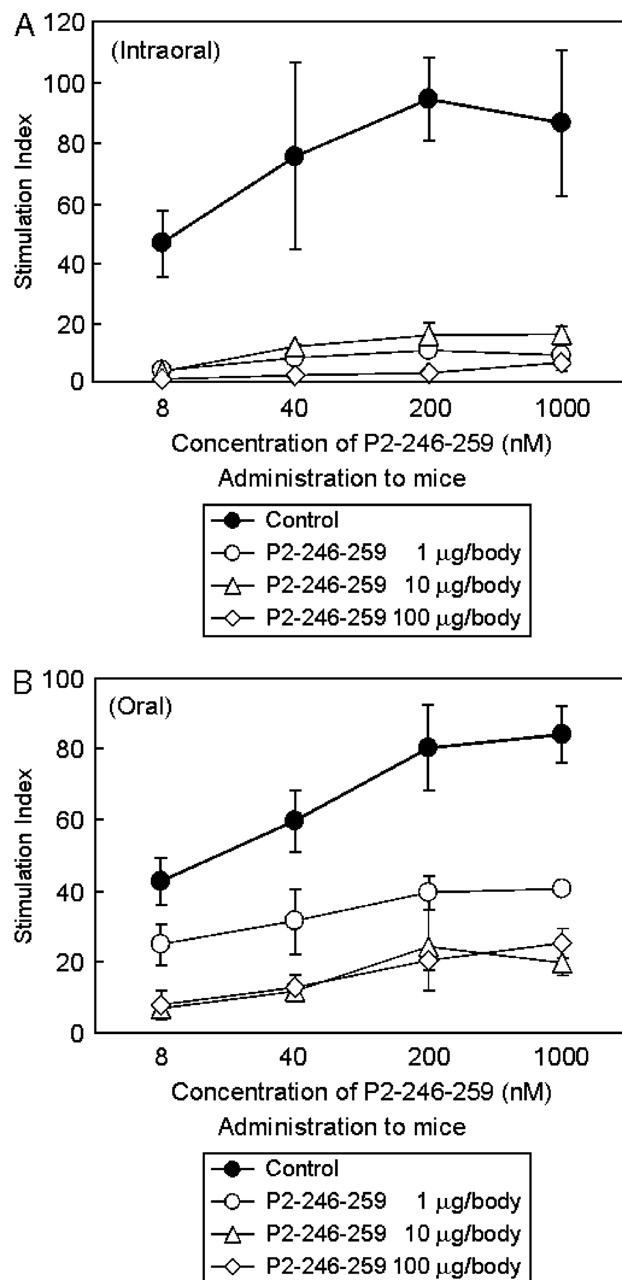

Figure 2 Intraoral (A) and intragastric (B) administrations of P2-246-259 to mice after allergen sensitization showing inhibition of cLNC proliferation to P2-246-259. The background responses (CPM) in (A) were 63.6 ± 6.1, 229.9 ± 34.2, 85.5 ± 12.3, and 109.5 ± 7.6 for the doses of 0 (control), 1, 10 and 100 μg/body groups, respectively. The background responses (CPM) in (B) were 45.2 ± 1.0, 99.5 ± 9.8, 63.8 ± 12.2, and 70.3 ± 9.0 for the doses of 0 (control), 1, 10 and 100 μg/body groups, respectively.

proliferation to P2-246-259 except the 1 μg/body dose group in which inhibition was partial (Figure 2(B)). Similar inhibition and differences between intraoral and intragastric administration were also observed in the cLNC proliferation to Cry j 2, the native protein allergen which P2-246-259 is derived from (Figure 3). Taken together, intraoral administration of P2-246-259 after allergen sensitization induced profound immunological tolerance, and the therapeutic efficacy of intraoral administration of P2-246-259 on allergen-specific T-cells was greater than that of intragastric administration, which suggests that the oral mucosal immune system contributes to the induction of immunological tolerance.

DISCUSSION

In the present study, we showed that intraoral administration of T-cell epitope peptide to mice before and even after allergen sensitization induced immunological tolerance in the T-cell response. In addition, the superiority of intraoral administration of the peptide to intragastric administration was shown, suggesting that the mucosal immune system around the oral cavity has an intensive role in inducing T-cell tolerance. From these results, the efficacy of SLIT using a T-cell epitope peptide was clearly demonstrated in mice.

We have shown for the first time that intraoral administration of a T-cell epitope peptide induces immunological tolerance in experimental animals. There have been a few studies in which intraoral administration of a protein allergen was examined. Sun *et al.* and Holt *et al.* showed that intraoral administration of an allergen to animals inhibited delayed-type hypersensitivity and specific IgE antibody

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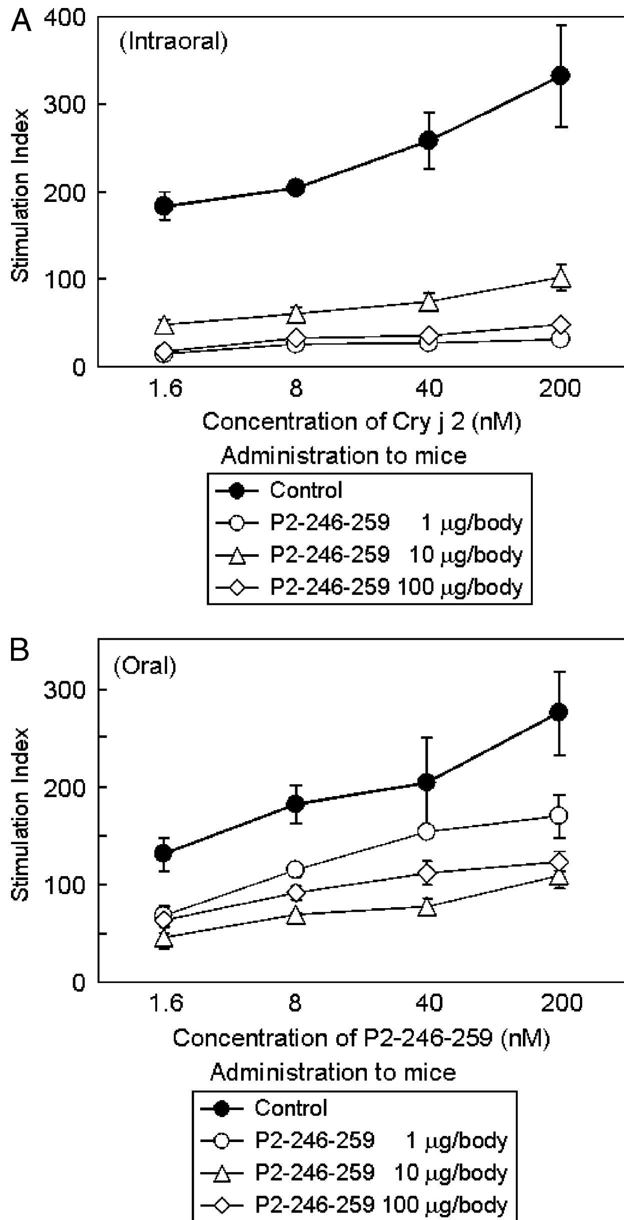

Figure 3 Intraoral (A) and intragastric (B) administrations of P2-246-259 to mice after allergen sensitization showing inhibition of cLNC proliferation to Cry j 2. The background responses (CPM) in (A) and (B) were the same as in Figure 2(A) and (B), respectively.

production, respectively [7,8]. However, the dose dependency of the inhibition was not apparent in these studies. We further demonstrated that intraoral administration of a T-cell epitope peptide that targets only specific T-cells inhibited cLNC proliferation to the peptide and a native protein allergen in a dose-dependent manner. Thus, the direct inhibitory effect on the T-cell response, which is a primary and crucial step for immune regulation, was clearly indicated by using a T-cell targeting peptide. Our finding in mice strongly implies that the inhibition of T-cell response is operative as the mechanism of SLIT in humans.

In SLIT, it is recommended that the solution be retained and sufficiently exposed to the oral mucosa [13], but the physiological role of secondary lymphoid tissues around the oral mucosal area in SLIT remains obscure. In clinical studies, it has been reported that sublingually administered radiolabeled-allergen was detected in the mouth for more than 2 h, even after rinsing. It is plausible to think that the remaining allergen is continuously exposed to the oral lymphoid tissues. Nasal-associated lymphoid tissues (NALT) in mice are thought to correspond to oral lymphoid tissues in humans. To date, there have been no reports indicating the direct involvement of NALT in inducing immunological tolerance, which would require mice that lack NALT. Instead, we showed that intraoral administration was superior to intragastric administration by carefully evaluating their dose-dependent inhibitions. Our result is consistent with other investigators' observations that intraoral administration of an allergen was more effective for tolerance induction than intragatstric administration [7,8]. It is strongly suggested that the mucosal immune system around the oral cavity has an intensive role in inducing T-cell tolerance.

The precise cellular mechanism of the oral immune system is not clear, but growing evidence illustrates the participation of dendritic cells on the mucosal surface. Recently, it was reported that particular dendritic cells exist in the oral mucosa and that they have important roles in inducing immunological tolerance [14,15]. P2-246-259 injected into the oral cavity might be captured by these dendritic cells, and the cells might migrate into NALT and work as tolerance-inducing APCs that are specialized for inhibiting P2-246-259-specific T-cell responses. Further studies are necessary to clarify the involvement of oral mucosal immunity in immune regulation, but the mouse model of SLIT we have shown here will be useful to address many important questions regarding the mucosal immune system in the oral cavity.

CONCLUSIONS

In conclusion, we showed the efficacy of intraoral administration of a T-cell epitope peptide for the first time. In addition, we showed the importance of intraoral administration as a route to induce immunological tolerance to sensitized mice by comparing intraoral administration with intragastric administration. It is anticipated that the mechanism of SLIT will be elucidated and that the efficacy of SLIT using a T-cell epitope peptide will be demonstrated in clinical studies.

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