```
GATTCAGTCT TTGCTGTATG CGATATAGAT ACTAGTTATA ATGGCAAAAG TATCAGATCT  60
                                             M  A  K  V  S  D  L
TGCGCTTCTT CTTGTGGCTG GAATGGCCAT ATCTCTTTAT ATTCAAGAGA CGGGAGCAGT 120
 A  L  L  L  V  A  G  M  A  I  S  L  Y  I  Q  E  T  G  A  V
TAAGTTTGAT ATAAAGAACC AGTGCGGGTA CACAGTCTGG GCGGCGGGAC TACCCGGAGG 180
 K  F  D  I  K  N  Q  C* G  Y  T  V  W  A  A  G  L  P  G  G
AGGGCAGCAG CTGACCCAGG GTCAGACATG GACGGTTAAT TTGGCGGCAG GGACACAGTC 240
 G  Q  Q  L  T  Q  G  Q  T  W  T  V  N  L  A  A  G  T  Q  S
GGCAAGATTC TGGGGTCGAA CCGGCTGCTC TTTCGATGCG AGCGGCAAAG GGACCTGTCA 300
A  R  F  W  G  R  T  G  C* S  F  D  A  S  G  K  G  T  C* Q
AACCGGTGAC TGCGGTGGCC AACTGAGCTG CACAGTTTCG GGAGCTGTTC CCGCCACGCT 360
 T  G  D  C* G  G  Q  L  S  C* T  V  S  G  A  V  P  A  T  L
GGCCGAGTAC ACCCAGAGCG ACCAGGACTA CTACGACGTC TCCCTGGTGG ACGGCTTCAA 420
 A  E  Y  T  Q  S  D  Q  D  Y  Y  D  V  S  L  V  D  G  F  N
CATTCCTCTT TCCATCAACC CTACCAATGC ACAGTGTACC GCCCCTGCAT GCAAAGCAGA 480
 I  P  L  S  I  N  P  T  N  A  Q  C* T  A  P  A  C* K  A  D
CGTGAACGCT GTGTGCCCTG CTGAACTGAA GGTGGATGGG GGATGCAAGA GTGCCTGCGC 540
 V  N  A  V  C* P  A  E  L  K  V  D  G  G  C* K  S  A  C* A
TGCCTTTCAA ACTGACCAGT ACTGCTGCAC TGGCACCTAT GCCAATAGCT GCCCTGCCAC 600
 A  F  Q  T  D  Q  Y  C* C* T  G  T  Y  A  N  S  C* P  A  T
AAACTACTCG ATGATATTCA AGAACCAGTG CCCCCAGGCC TACAGTTATC CCAAGGACGA 660
 N  Y  S  M  I  F  K  N  Q  C* P  Q  A  Y  S  Y  P  K  D  D
TACAGCCACA TTCGCTTGCC CCTCTGGTAC AGACTACAGT ATTGTATTCT GTCCCTAGAT 720
 T  A  T  F  A  C* P  S  G  T  D  Y  S  I  V  F  C* P  *

ATATATAGGA TTATGTTTGT GTGGAATAAT ATATTAATAA GTGCTGTCAT CTTTGTGTTG 780
ACGCTGTGGT GTAGCTGCAT AGTTGCAACT CTCACCACTT ATTATCGACC AATAAAACAT 840
GTCTGTCATC TCACCACTAA TGACAGAAGC GTAATTGACA CGCAATACAC TGTTATGAGA 900
GAATATTAAT AATAAATATA TATTTTGAAA AAAAAAAAAA AAA                   943
```

Figure 5. Nucleotide sequence of Cry j 3.8 cDNA and its deduced amino acid sequence. A consensus pattern of TLRs (residues 82–97), G-x-[GF]-x-C-x-T-[GA]-D-C-x(1,2)-G-x(2,3)-C, is shown in boldface, and conserved cysteine residues are indicated as C*. Two potential N-glycosylation sites (residues 133 and 188) are double-underlined, and the N-terminal sequence determined is single-underlined.

cross-reaction with Jun a 3 (22). In Japanese cedar pollinosis patients, the IgE-binding frequency against Jun a 3 is comparable with that against Cry j 3 (33% and 27%, respectively). Furthermore, three sequential IgE epitopes of Jun a 3 (amino acids 120–131, 132–145 and 152–165) are highly conserved in Cry j 3.8, at 92% (120–130), 100% (132–145) and 86% (152–153, 155–162 and 164–165) (23). These data strongly imply cross-reactivity between Cry j 3 and Jun a 3.

Cross-reactivity between allergens from Japanese cedar pollen and tomato have been reported in dogs and humans (24, 25), although the causative allergens remain unknown. Because two isoforms of TLP have been isolated from tomato fruit and their amounts shown to increase with ripening (26), Cry j 3 may be one of the causative allergens of the cross-reactivity. TLPs are reported to be contained in many fruits, as is chitinase. TLPs have significant amino acid sequence identity with thaumatin and are categorized in the PR-5 family (27). The PR-5 family comprises thaumatin, osmotin and zeamatin, and proteins highly homologous to them. PR-5 family proteins are well known as allergens from pollen and sweet fruits. Mal d 2 from apple (*Malus domestica*) was the first allergen described as a TLP (28). Following the identification of Mal d 2, several fruit allergens, including Pru av 2 from cherry (*Prunus avium*), Act c 2 and Act d 2 from kiwi (*Actinidia chinensis* and *A. deliciosa*, respectively), Cap a 1 from bell pepper (*Capsicum annuum*) and thaumatin from grape (*Vitis vinifera*), were reported as allergens belonging to the PR5 family (19, 29–32). Recently, PR-5 allergens from pollen have been identified as Jun a 3 from mountain cedar (*J. ashei*), Jun v 3 from eastern red cedar (*J. virginiana*) and Cup a 3 from *C. arizonica* (20, 22, 33). The PR proteins are considered important pan-allergens responsible for pollinosis and oral allergy syndrome (14). A recently

```
Cry j 3.8        : MAKVSDLALLLVAGMAISLYIQETGAVKFDIKNQCGYTVWAAGLPGGGQQ  50
Jun r 3.1        : MARVSELALLLVATLAISLHMQEAGAVKFDIKNQCGYTVWAAGLPGGGKR  50
Jun a 3          : MARVSELAFLLAATLAISLHMQEAGVVKFDIKNQCGYTVWAAGLPGGGKR  50
Cup s 3.2        : MARVSELALLLVATLAISLHMQEAGAVKFDIKNQCGYTVWAAGLPGGGKR  50
T. occidentalis  : MATVSDLALLLVAGLAISLCTQEAGAVKFNIRNQCGYTVWAAGLPGGGKQ  50

Cry j 3.8        : LTQGQTWTVNLAAGTQSARFWGRTGCSFDASGKGTCQTGDCGGQLSCTVS 100
Jun r 3.1        : LDQGQTWTLNLAAGTASARFWGRTGCTFDASGKGSCKTGDCGGQLSCTVS 100
Jun a 3          : LDQGQTWTVNLAAGTASARFWGRTGCTFDASGKGSCQTGDCGGQLSCTVS 100
Cup s 3.2        : LDQGQTWTVNLAAGTASARFWGRTGCTFDASGKGSCRSGDCGGQLSCTVS 100
T. occidentalis  : LDQGQTWSVDVAAGTKGARFWGRTGCSFDGSGRGSCQTGDCGGQLSCTLS 100

Cry j 3.8        : GAVPATLAEYT---QSDQDYYDVSLVDGFNIPLSINPTNAQCTAPACKAD 147
Jun r 3.1        : GAVPATLAEYT---QSDQDYYDVSLVDGFNIPLAINPTNAQCTAPACKAD 147
Jun a 3          : GAVPATLAEYT---QSDQDYYDVSLVDGFNIPLAINPTNAQCTAPACKAD 147
Cup s 3.2        : GAVPATLAEYT---QSDKDYYDVSLVDGFNIPLAINPTNTKCTAPACKAD 147
T. occidentalis  : GAVPATLAEYSLNGNDNKDFYDVSLVDGFNVPLSINPTNTGCTAPACKAD 150

Cry j 3.8        : VNAVCPAELKVDGGCKSACAAFQTDQYCCTGTYANSCPATNYSMIFKNQC 197
Jun r 3.1        : INAVCPSELKVEGGCNSACNVFQTDQYCCRNAYVDNCPATNYSKIFKNQC 197
Jun a 3          : INAVCPSELKVDGGCNSACNVFKTDQYCCRNAYVDNCPATNYSKIFKNQC 197
Cup s 3.2        : INAVCPSELKVDGGCNSACNVLQTDQYCCRNAYVDNCPATNYSKIFKNQC 197
T. occidentalis  : VNAACPAELKVNSGCNSACNVFQTDQYCCRGANVDNCPATHYSMIFKNQC 200

Cry j 3.8        : PQAYSYPKDDTA-TFACPSGT-DYSIVFCP 225
Jun r 3.1        : PQAYSYAKDDTA-TFACASGT-DYSIVFCP 225
Jun a 3          : PQAYSYAKDDTA-TFACASGT-DYSIVFCP 225
Cup s 3.2        : PQAYSYAKDDTA-TFACASGT-DYSIVFCP 225
T. occidentalis  : PQAYSYAKDDTSSTFTCPSGTTDYSIVFCP 230
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Figure 6. Multiple sequence alignment of Cry j 3.8 with the TLPs, Jun r 3.1 (*Juniperus rigida*, accession number AY353703), Jun a 3 (*J. ashei*, AF121776), Cup s 3.2 (*Cupressus sempervirens*, AY353706), and *T. occidentalis* (*Thuja occidentalis*, AY795850). Identical amino acid residues are indicated in boldface.

identified allergen from Japanese cedar pollen, CJP-4, also belongs to the PR family. CJP-4 has been identified as a 34 kDa protein with endochitinase activity that cross-reacts with latex allergens (34). Therefore, both Cry j 3 and CJP-4 may act as causative allergens of the cross-reactivity in Japanese cedar pollinosis and oral allergy syndrome.

We purified native Cry j 3 from Japanese cedar pollen. The N-terminal sequence of purified Cry j 3 did not completely coincide with any previously reported sequences of Cry j 3, but did coincide with that of Jun a 3. Cry j 3.8 has the highest amino acid identity with Jun a 3 (85.8%) among the Cry j 3 isoforms (Cry j 3.1–3.6, 42.5–54.8%; Cry j 3.7, 73.6%) (12, 13). However, it remains to be elucidated whether the other Cry j 3 isoforms exist in mature Japanese cedar pollen.

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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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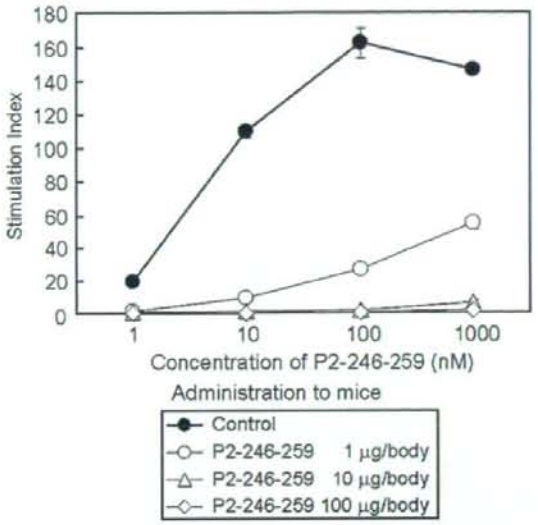

Figure 1 Intraoral administrations of P2-246-259 to mice before allergen sensitization showing inhibited cLNC proliferation to P2-246-259. The background responses (CPM) were 138.4 ± 20.1, 126.1 ± 10.7, 139.9 ± 30.9 and 121.2 ± 25.1 for the doses of 0 (control), 1, 10 and 100 μg/body groups, respectively.

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

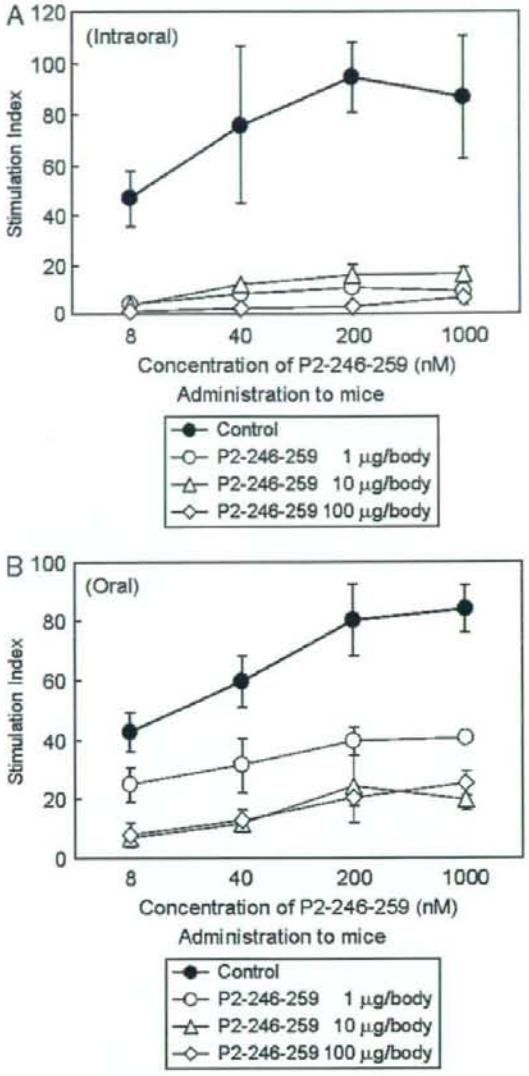

Figure 2 Intraoral (A) and intragastric (B) administrations of P2-246-259 to mice after allergen sensitization showing inhibition of cLNC proliferation to P2-246-259. The background responses (CPM) in (A) were 63.6 ± 6.1, 229.9 ± 34.2, 85.5 ± 12.3, and 109.5 ± 7.6 for the doses of 0 (control), 1, 10 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.

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

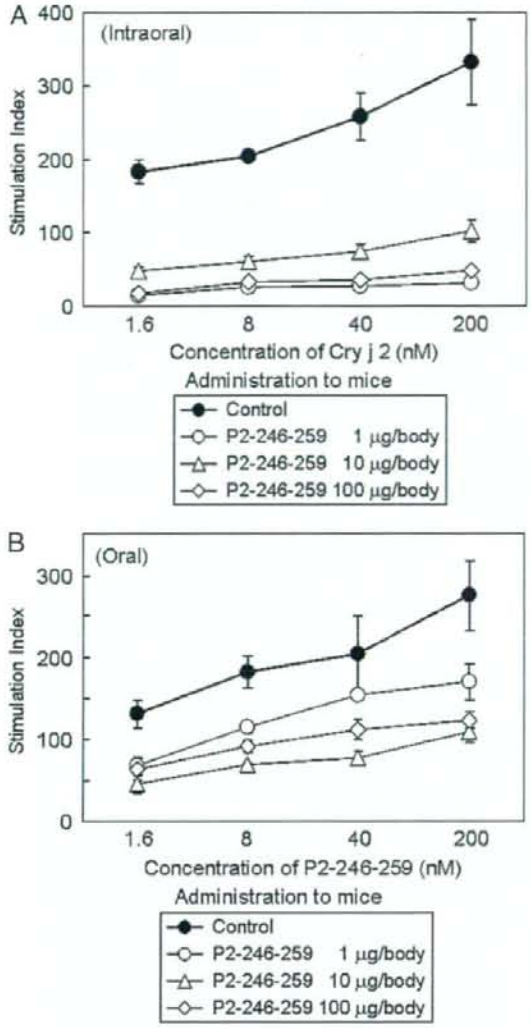

Figure 3 Intraoral (A) and intragastric (B) administrations of P2-246-259 to mice after allergen sensitization showing inhibition of cLNC proliferation to Cry j 2. The background responses (CPM) in (A) and (B) were the same as in Figure 2(A) and (B), respectively.

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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Dietary Pulverized Konjac Glucomannan Prevents the Development of Allergic Rhinitis-Like Symptoms and IgE Response in Mice

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Konjac is a traditional Japanese food with a peculiar texture, and it has been suggested that its main ingredient, konjac glucomannan (KGM), ameliorates metabolic disorders such as diabetes and hypercholesteremia. We have found that feeding with pulverized KGM (PKGM) prevents skin inflammation in a murine model of atopic dermatitis. Here, we show that dietary PKGM suppresses allergic rhinitis-like symptoms in mice upon immunization and nasal sensitization with ovalbumin (OVA). The PKGM-fed mice showed a much lower frequency of sneezing than in control animals. We also found that PKGM supplementation exclusively suppressed OVA-specific IgE response without affecting IgG1/IgG2a responses as well as systemic Th1/Th2 cytokine production. These results suggest that PKGM can be a beneficial foodstuff in preventing nasal allergy such as seasonal pollinosis.

Key words: allergic rhinitis; IgE; pulverized konjac glucomannan

Konjac, long consumed as a Japanese food, is manufactured using a konjac powder, which is obtained from the tubers of *Amorphophallus konjac*. The main ingredient of konjac powder is konjac glucomannan (KGM), a highly viscous polysaccharide composed of glucose and mannose residues at a molar ratio of 2:3 with β1-4 linkages. Several lines of evidence suggest that dietary KGM has beneficial effects on health: oral intake of KGM prevents hyperglycemia and hyperlipidemia, and its potency is correlated with high viscosity.[1)] Lim *et al.*[2)] reported that dietary KGM enhances IgA and IgG secretion by lymphocytes from the rat mesenteric lymph node, suggesting that KGM can also modulate the immune system. However, the mode of action of dietary KGM in regulating the metabolic and/or immune pathway is largely unknown.

We have found a novel, beneficial immunomodulatory function of processed KGM: feeding with pulverized low-viscous KGM (PKGM) prevented the development of eczematous skin inflammation and hyper-IgE production in NC/Nga mice,[3,4)] a well-known animal model of atopic dermatitis (AD).[5)] More recently, we found that AD-like skin lesions (dermal thickening, eosinophilia, and mastocytosis), as well as local overproduction of substance P and proinflammatory cytokines, were all impaired in PKGM-fed NC/Nga mice,[6)] suggesting that dietary PKGM suppresses a wide array of skin inflammatory immune responses. We have also reported that oral intake of PKGM inhibited the increase in serum IgE and IgG levels induced by continuous injection of syngeneic keratinocyte extracts in BALB/c mice.[7)] Taken together, these results suggest that dietary PKGM can be beneficial in preventing allergy, although the precise mechanism underlying the anti-inflammatory action of PKGM and its effect on allergen-induced inflammatory disorders remain to be investigated.

In the present study, we found that dietary PKGM prevents the development of allergic rhinitis-like inflammation in mice upon nasal challenge with OVA. We also found that PKGM exclusively suppresses OVA-specific IgE antibody response without affecting systemic Th1/Th2 cytokine profile.

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Abbreviations: AD, atopic dermatitis; ELISA, enzyme-linked immunosorbent assay; i.p., intraperitoneal; KGM, konjac glucomannan; OVA, ovalbumin; PBS, phosphate-buffered saline; PKGM, pulverized konjac glucomannan

Materials and Methods

Konjac glucomannan. Two kinds of konjac glucamannan (KGM) powder, high viscous KGM (PROPOL® A, PA) and pulverized KGM (S-P), were prepared by Shimizu Chemical Corporation (Hiroshima, Japan), and their physicochemical properties were analyzed as previously described.[3,4] The GM contents of PA and S-P powder were 98.1% and 97.0% respectively, and the viscosity-average molecular weight of both samples were estimated to be over 1,000 kDa (as assessed using an Ubbelohde viscometer). The mean particle size of these KGM powders was estimated to be 300 μm for PA and 75–100 μm for S-P. The peak viscosity of 1% solution was more than 100,000 mPa·s for PA and approximately 35,000 mPa·s for S-P.

Animals and diets. Four-week-old female BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained under specific pathogen-free conditions for 8 weeks on a control diet (MF diet, Oriental Yeast, Tokyo) or a KGM diet (an MF diet containing 5% w/w KGM powder) *ad libitum*. All animals were housed in an animal facility kept at 22 ± 2 °C under a 12-h light/12-h dark cycle. All animal studies were carried out using protocols reviewed and approved by the Committee on Animal Experimentation of Hiroshima University.

Immunization, intranasal sensitization, and evaluation of nasal symptoms. Immunization and nasal challenge with ovalbumin (OVA) were performed by previously described protocols,[8] with some modifications. Briefly, at 7 and 9 weeks of age (*i.e.*, 3 and 5 weeks after the start of feeding with KGM), the mice were immunized intraperitoneally (i.p.) with either phosphate-buffered saline (PBS) or 20 μg of OVA (Sigma, St. Louis, MO) emulsified in 2.25 mg of alum adjuvant (LSL, Tokyo) in 100 μl of total volume. Two weeks after the second immunization, the mice were intranasally challenged with OVA solution (25 mg/ml solution in PBS, 20 μl/mouse) by daily instillation without anesthesia (Fig. 1A). To evaluate allergic rhinitis-like symptoms, the mice were placed into an observation cage (one animal/cage) just after intranasal instillation with OVA, and the number of sneezes was counted for 5 min under blinded conditions.

Analysis of plasma immunoglobulins. Twelve hours after the last (eleventh) nasal challenge with OVA, blood samples were collected from all the groups of mice. Total plasma IgE, IgG1, and IgG2a levels were determined by sandwich enzyme-linked immunosorbent assay (ELISA), as previously described.[3] Plasma OVA-specific IgE, IgG1, and IgG2a titers were also analyzed by ELISA, as described previously.[7] Briefly, 96-well microtiter plates were coated with OVA solution (100 μg/ml) at 4 °C overnight, and then incubated with diluted plasma samples (× 10 for IgE, and × 500 for IgG1 and IgG2a). After reaction with secondary biotinylated rat anti-mouse IgE, IgG1, or IgG2a antibody (BD Bioscience, San Jose, CA, 1:250 dilution) and subsequent incubation with streptavidin-conjugated alkaline phosphatase (Zymed Laboratories, San Francisco, CA, 1:1,000 dilution), enzyme reaction was performed using the AttoPhos® substrate system (Promega Corporation, Madison, MI). Fluorescence intensity of each well was then analyzed with a Wallac 1420 ARVO Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA).

Cell culture and cytokine analysis. Spleens were removed from the mice, and total mononuclear cell suspension was prepared by treatment with lysis buffer (150 mM NH_4Cl, 15 mM $NaHCO_3$, 0.1 mM EDTA–2Na, pH 7.3) to lyse red blood cells. After washing 3 times with PBS, splenocytes (4×10^6 cells/ml) were stimulated with OVA (100 μg/ml) for 96 h in RPMI-1640 medium (Sigma) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 μM of 2-mercaptoethanol, and 10% fetal bovine serum (BioWest, Rue de la Caille, France) at 37 °C in 5% CO_2/95% air. The IL-4, IL-5, and IFN-γ levels in the culture supernatant were determined by sandwich ELISA using reagents and instructions from BD Biosciences. IL-13 assay was carried out using DuoSet mouse IL-13 (R&D Systems, Minneapolis, MN).

Statistical analysis. Statistical analysis was performed by Student's *t*-test. $P < 0.05$ was accepted as the level of significance.

Results

Dietary PKGM prevents the development of allergic rhinitis-like sneezing symptoms in OVA-sensitized mice

To investigate the effect of low-viscous pulverized KGM (PKGM) on allergic nasal inflammation, BALB/c mice were fed a PKGM-containing diet or diets containing non-pulverized KGM powder for 3 weeks, and then immunized twice with OVA/alum at a 2-week interval. Two weeks after the second immunization, they were intranasally challenged with OVA solution to induce nasal hyperreactivity (Fig. 1A). All groups of mice were continuously fed KGM-containing diets or a control MF diet during the entire sensitization period.

First we tested the effect of dietary PKGM on rhinitis-like symptoms of the OVA-sensitized mice by counting their sneezing behavior. Control mice immunized with OVA/alum and intranasally challenged with OVA (OVA/OVA) started marked sneezing at the eighth nasal sensitization, and this symptom was further exaggerated towards the eleventh challenge (shown closed circles in Fig. 1B). In contrast, the development of sneezing was significantly prevented in PKGM-fed OVA/OVA mice (indicated by closed triangles in

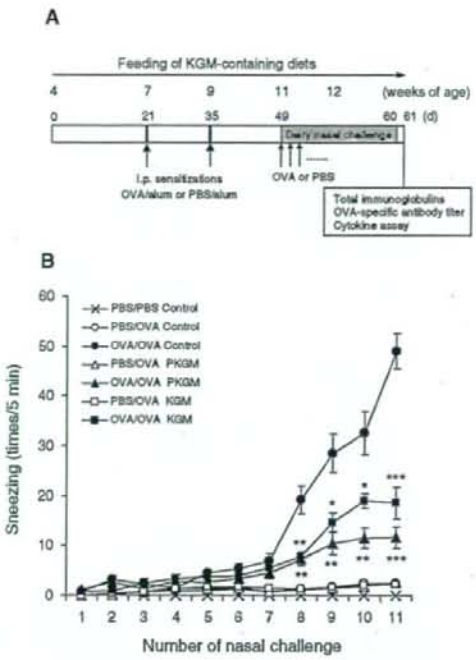

Fig. 1. Dietary KGM Prevents the Development of Nasal Sneezing in BALB/c Mice upon Sensitization with OVA.

A, Experimental scheme. Four-week-old BALB/c mice were continuously fed a PKGM- or non-pulverized KGM-containing diet. Three weeks later, those mice were immunized and intranasally challenged with OVA, as described in "Materials and Methods." B, The numbers of sneezes per 5 min were counted just after nasal challenge with OVA. Values are expressed as means ± SD with six mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (as compared to control OVA/OVA mice).

Fig. 1B). Sham control groups fed a control diet (PBS/PBS or PBS/OVA, shown as x-marks and open squares respectively) or the PKGM diet (PBS/OVA, shown open triangles) did not show sneezing. These data indicate that oral intake of PKGM prevents rhinitis-like symptoms in mice sensitized with OVA. We observed that feeding the non-pulverized KGM diet also significantly suppressed the development of sneezing (shown as closed squares in Fig. 1B), although the potency was less prominent than that observed in PKGM-fed mice.

Dietary PKGM exclusively suppresses OVA-specific IgE response and total IgE level

To evaluate the effect of dietary PKGM on the immune response, we next examined OVA-specific antibody responses 24 h after the eleventh nasal challenge with OVA. The specific IgE response was evident in control mice immunized and challenged with OVA (OVA/OVA), but the increase in IgE titer was significantly suppressed in PKGM-fed OVA/OVA mice (Fig. 2A). In contrast to the inhibitory effect on IgE response, the OVA-specific IgG1 and IgG2a responses were indistinguishable as between the PKGM-fed mice and the control mice. We also found that PKGM-fed mice showed a significant decrease in plasma total IgE concentration, while IgG1 and IgG2a levels were unaffected (Fig. 2B). Mice fed non-pulverized KGM also showed antibody responses similar to those seen in PKGM-fed mice; *i.e.*, a significant down-regulation of OVA-specific IgE titer and a trend toward decrease ($P = 0.07$) in total IgE levels. These results indicate that oral supplementation with KGM-containing diets preferentially down-regulates *in vivo* IgE production.

Oral intake of KGM had no effect on the secretion of Th1/Th2 cytokines from splenocytes stimulated with OVA

Antigen-driven isotype switching of immunoglobulins is tightly regulated by cytokines, which drive germline transcription at the immunoglobulin constant region loci: Th2-dominated cytokine milieu favors the IgE or IgG1 class switch recombination,[9] whereas isotype switching to the IgG2a subclass is directly modulated by a Th1-associated transcription factor, T-bet.[10] Above exclusive suppression of the OVA-specific IgE response (with no effect on IgG1 or IgG2a responses) in KGM-fed mice implies that down-regulation of IgE production by dietary PKGM or non-pulverized KGM may not be attributable to its effect on the Th1/Th2 cytokine milieu. To test this possibility, we analyzed cytokine production by splenocytes from these mice upon *in vitro* stimulation with OVA. We found that secretion of Th2 cytokines (IL-4, IL-5, and IL-13) by splenocytes from PKGM- or non-pulverized KGM-fed OVA/OVA mice was comparable to those seen in control OVA/OVA mice (Fig. 3). Similarly, splenic IFN-γ production was not affected by feeding of KGM samples. These results indicate that oral intake of KGM has no effect on systemic Th1/Th2 cytokine production by splenocytes.

Discussion

We have reported that dietary PKGM of small particle size suppressed skin inflammation and hyper-IgE production in AD-prone NC/Nga mice.[3,4] In the present study, we found that PKGM prevented allergic rhinitis-like symptoms and IgE response in mice upon nasal challenge with OVA, further providing evidence that PKGM is a beneficial foodstuff in preventing allergy and allergen-specific IgE response.

Our previous studies with NC/Nga mice indicated that neither non-pulverized KGM nor re-granulated PKGM suppressed AD-like skin lesions, suggesting that a small particle size of PKGM powder is critical for its eczema-preventive effect.[3,4] By contrast, here we found a significant anti-allergic effect of non-pulverized KGM powder, although its potency was less prominent than that of PKGM (Fig. 1B). The differential efficacy of

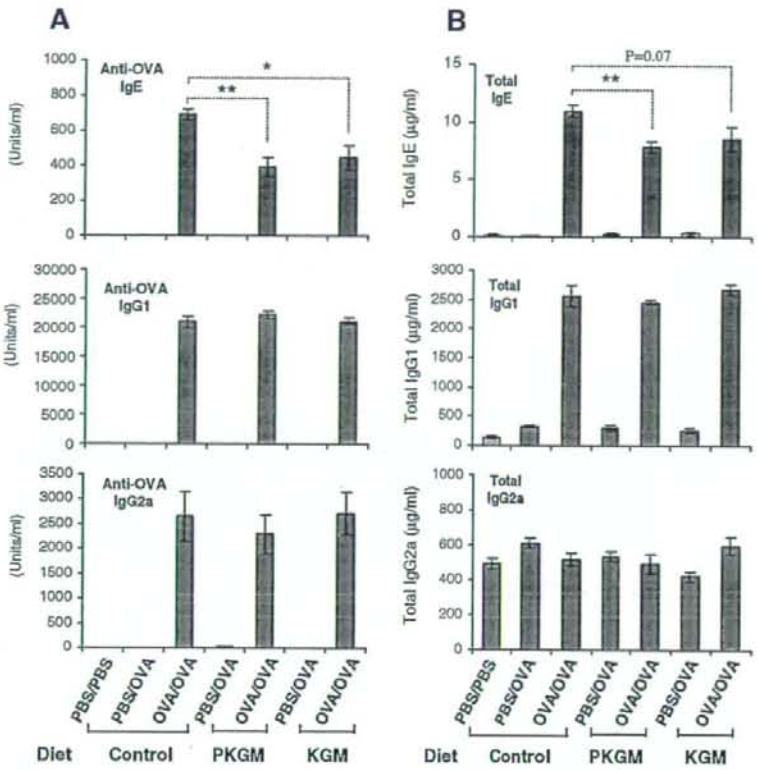

Fig. 2. Dietary PKGM Preferentially Suppresses Allergen-Specific IgE Response and Total IgE Levels upon Sensitization with OVA.
After the eleventh nasal challenge, plasma samples were collected from each group of mice, and antibody responses were analyzed by ELISA. A, OVA-specific IgE, IgG1, and IgG2a responses. B, Total immunoglobulin levels. Values are expressed as means ± SD with six mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ (as compared to control OVA/OVA mice).

non-pulverized KGM on these two atopy models might be due to distinct mechanisms underlying the pathogenesis of OVA-induced rhinitis in BALB/c mice and those of eczema development in NC/Nga mice. Indeed, OVA-induced nasal hyperreactivity requires FcεRI as well as IL-13,[8,11] whereas AD-like skin lesions are seen in STAT6-deficient NC/Nga mice that have no Th2 cells or IgE antibodies.[12] Thus the OVA-induced rhinitis model appears to be more dependent on the classical IgE-mast cell axis of the allergic response than the NC/Nga model. Non-pulverized KGM might preferentially down-regulate these type-I allergic pathways, although which are largely dispensable for eczema development in NC/Nga mice.

We found that dietary PKGM exclusively suppressed the OVA-specific IgE response without affecting the IgG1/IgG2a responses and systemic Th1/Th2 cytokine production (Figs. 2A and 3). These data imply that suppression of the IgE response is not attributable to a skewed Th1 response, but rather driven by hitherto unknown mechanisms other than canonical Th1/Th2 dogma. We also found that PKGM feeding specifically suppressed the increase in total IgE levels, but not that of IgG1 (Fig. 2B). This result differs from our previous data, which indicated that dietary PKGM suppressed both total IgE and IgG1 levels in NC/Nga mice[4,6] and in BALB/c mice upon injection with syngeneic keratinocyte extract.[7] This discrepancy in the data might be due to differences in experimental settings (*e.g.*, mouse strain, immunization protocols).

How does dietary PKGM preferentially down-regulate the IgE response in our rhinitis model? We have several assumptions about the underlying mechanisms. The first possibility is that dietary PKGM suppresses only the IgE class switch recombination (CSR), but not IgG1 CSR. One such IgE-inhibitory candidate is IL-21, which specifically down-regulates IL-4-driven Cε germline transcription without abrogating STAT6 activation.[13] *In vivo*, IL-21-deficient mice show exaggerated IgE production.[14] More recently, natural killer T cell-derived IL-21 has been found to trigger Bε cell apoptosis to inhibit IgE production.[15] All these data

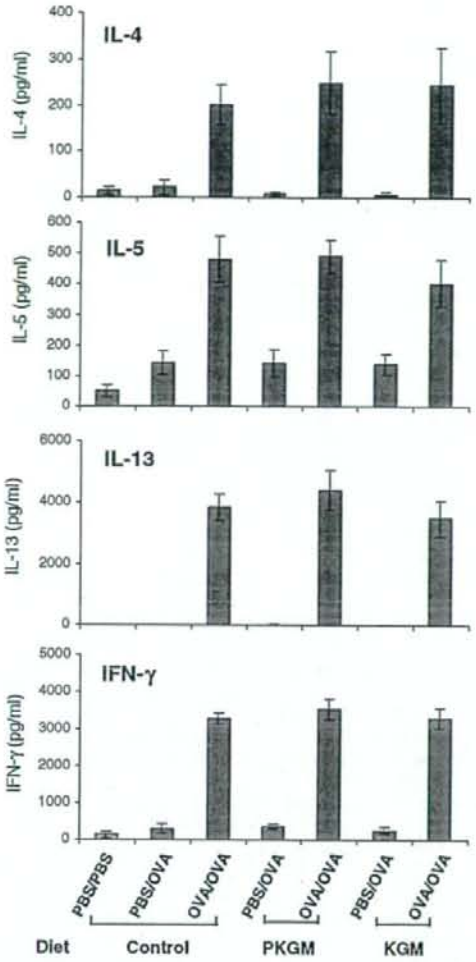

Fig. 3. Dietary KGM Did Not Affect Th1/Th2 Cytokine Production by Splenocytes from OVA-Sensitized Mice.

Total splenocytes from KGM-fed mice after the eleventh nasal challenge were stimulated with OVA for 96 h, and IL-4, IL-5, IL-13, and IFN-γ levels in the culture supernatant were quantified by sandwich ELISA, as described in "Materials and Methods." Values are expressed as means ± SD with 6 mice per group.

establish IL-21 as a critical negative regulator of IgE synthesis, although further investigation is needed to assess the actual involvement of this cytokine in our system. Second, IgE CSR might be down-regulated by a cell-mediated pathway. Obayashi *et al.*[16] recently demonstrated that dendritic cells (DCs) selectively inhibited IgE CSR, and thus a similar IgE inhibitory loop might also be activated in the mucosal DC-B cell interface of PKGM-fed mice. Finally, dietary PKGM might specifically suppress IgE^+ plasma cell differentiation *in vivo*. This possibility is based on a recent report indicating that IgE^+ plasma cells are generated *via* an exceptional *in vivo* maturation program, in which somatic hypermutation and affinity maturation take place in $IgG1^+$ cells, and a post-IgE-switching phase in which IgE^+ B cells differentiate swiftly into plasma cells.[17] Hence it is also possible that dietary PKGM negatively regulates the latter unique plasma cell differentiation pathway to inhibit IgE production. Whatever the mechanism is, analysis of *in vivo* IgE^+ and $IgG1^+$ B cell/plasma cell subsets in PKGM-fed mice should be the first experiment to address these possibilities.

In addition to the IgE-suppressive mechanisms, the precise mode of action by which dietary PKGM prevents allergic rhinitis is still unknown. Since FcεRI signaling is essential to OVA-induced nasal hyperreactivity,[8] suppression of allergen-specific IgE would be the primary target of dietary PKGM in inhibiting rhinitis. However, other anti-inflammatory roles of PKGM might also be involved, because we have found that PKGM feeding abrogated a wide array of skin inflammatory immune responses in NC/Nga mice, including defective skin thickening, decreased local mastocytosis/eosinophilia, and overall impairment in cutaneous overproduction of substance P, proinflammatory cytokines, and a Th2 cell-attractive chemokine, CCL17/TARC.[5] Our preliminary histopathological analysis indicates that nasal mast cell infiltration is severely impaired in PKGM-fed mice (unpublished data), suggesting that nasal proinflammatory immune responses might be down-modulated upon feeding with PKGM.

Another important issue is how dietary KGM interacts with gut mucosa to fulfill an anti-allergic function. One plausible possibility is that KGM modulates gut microflora, which in turn prevent IgE production and nasal inflammation. Indeed, it has been reported that dietary KGM increases fecal bifidobacteria,[18] an intestinal microflora whose reduction is highly correlated with atopy predisposition.[19] Another noteworthy evidence is that acid-hydrolyzed KGM has a greater prebiotic effect than does KGM in increasing cecal and fecal bifidobacteria.[20] This result might provide a mechanistic insight into our observation that PKGM showed a more potent anti-rhinitis effect than did non-pulverized KGM (Fig. 1B). That is, the superior anti-allergic effect of PKGM might be attributable to its better bioavailability to gut microbiota. Practically, PKGM, with its finer particle size, appears to be degraded more easily into oligosaccharides than non-pulverized KGM, because the increase in specific surface area promotes bacterial adherence and/or enzymatic access to the surface area of KGM molecule. In addition to the possible prebiotic action, the direct effect of dietary PKGM on the gut immune system is another intriguing possibility to be addressed.

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Transcutaneous immunization by merely prolonging the duration of antigen presence on the skin of mice induces a potent antigen-specific antibody response even in the absence of an adjuvant

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Abstract

Transcutaneous immunization (TCI) is a promising needle-free technique for vaccination. In this method, strong adjuvants, such as the cholera toxin, are generally crucial to elicit a robust immune response. Here, we showed that prolonged antigen presence on the skin of mice during TCI could effectively enhance the immune response. Substantial antigen-specific antibodies were produced in the sera of mice even after non-adjuvanted TCI when the antigen presence was for longer than 16 h. This non-adjuvanted TCI method was applied using the tetanus toxoid, and potent tetanus toxoid-specific antibodies were successfully induced in the sera of mice; they survived a lethal tetanus toxin challenge with no clinical signs. Thus, non-adjuvanted approach might be a possible option for TCI, and this method might improve the safety and practicality of transcutaneous vaccination.

Keywords: Transcutaneous immunization; Adjuvant; Tetanus toxoid

1. Introduction

The skin is one of the first lines of defense of the body. During the course of evolution, the skin has developed a dense immune system comprising draining lymph nodes and various immunocompetent cells such as Langerhans cells, keratinocytes, dermal dendritic cells, and mast cells [1,2]. Together with its high accessibility, the skin's immunocompetence makes it an ideal target for vaccination.

Recently, there have been reports of needle-free vaccinations that target the intact skin surface and use peptides, proteins, or virus particles as antigens [3–7]. These novel methods, referred to as transcutaneous immunization (TCI), are performed by the topical application of antigens along with adjuvants. It is generally considered that some amount of a potent adjuvant is crucial in order to elicit a robust immune response against antigens co-administered via skin delivery [8,9]. The most common adjuvants used in the TCI method are the cholera toxin (CT) or the heat-labile enterotoxin (LT) from *Escherichia coli* [10].

Needle-free vaccination methods are desirable because they are convenient, painless, and relatively safe. In particular, vaccinations in developing countries and mass vaccinations against expected pandemics would benefit greatly from needle-free approaches [11]. Although TCI is a promising needle-free approach, the indispensable use of potent bacterial toxins as adjuvants might raise some concerns regarding the safety of this method; however, CT and LT may be less toxic when applied on the skin surface [12].

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In this study, we merely prolonged the duration of antigen presence on the skin of mice during the TCI procedure and observed that the serum antibody titre increased in a duration-dependent manner. We induced substantial serum antibody responses by a TCI of 16-h duration even in the absence of an adjuvant. In this report, we applied a modified TCI method termed "prolonged TCI" that involved no adjuvant. In this method, the tetanus toxoid (Ttd) was used as a model vaccine antigen, and this method successfully induced potent tetanus toxoid-specific antibody responses in the sera of mice; these mice survived a lethal tetanus toxin challenge without any clinical signs. These results indicate that the non-adjuvanted approach might be a possible option for TCI. This might improve the safety and practicality of transcutaneous vaccination.

2. Materials and Methods

2.1. Mice

We used female C57BL/6, BALB/c, and C3H/He mice (Japan SLC Inc., Hamamatsu, Japan) aged 7–8 weeks at the primary immunizations.

The animals were housed in a specific pathogen-free facility and provided with free access to water and food. The use of the animals and the study protocols were approved by the institutional animal care and use committee.

2.2. Antigens and adjuvant

Ovalbumin (OVA) and CT were purchased from Sigma (St. Louis, MO, USA). The Ttd was kindly provided by Kaketsuken (Kumamoto, Japan).

2.3. Conventional TCI

Conventional TCI was performed as previously described [10]. In brief, the abdomen of the mice was shaved using a No. 40 clipper, and the mice were rested for 48 h. They were anaesthetized intraperitoneally with a ketamine–xylazine mixture to prevent self-grooming. The bare abdominal skin was gently swabbed with 70% ethanol and allowed to dry. Next, 50 μl of antigen solution in PBS was placed on the bare abdominal skin over an approximate area of 1 cm^2 for 2 h. The mice were then washed extensively with lukewarm tap water and patted dry with paper towels.

2.4. Prolonged TCI

Mice were shaved and anaesthetized in the same manner as that used for the conventional TCI. The bare abdominal skin was gently swabbed with 70% ethanol and allowed to dry. Next, a 0.64-cm^2 square gauze patch with an adhesive lining (Shirojuhji, Tokyo, Japan) was soaked with 50 μl of antigen solution, and was fixed to the bare abdominal skin using medical tape (Fig. 1A and C). The mice were placed back in the cage and left for 16 h or more. Then, the medical tape and gauze patch were removed, and the abdominal skin was extensively washed with lukewarm tap water and patted dry with paper towels.

Prolonged TCI of the dorsal side of the ear was performed as described above without shaving (Fig. 1B and D).

2.5. Fecal extract

Fecal samples were collected and weighed. A hundred milligram of feces were suspended in 400 μl of PBS containing 100 μg/ml of soybean trypsin inhibitor (Wako, Ohsaka, Japan), 50 mM of EDTA, 1 mM of phenylmethylsulfonyl fluoride (Sigma), 1% of bovine serum albumin (Sigma), 5% of fetal bovine serum (Sigma), and 0.05% of sodium azide, were vigorously vortexed to homogeneity, centrifuged at 6000 × *g* for 5 min and the supernatants were collected and stored at −20 °C until assayed.

2.6. ELISA for antigen-specific IgG and IgA antibodies

Antigen-specific IgG and IgA antibody titres of the sera and fecal extracts were determined by ELISA. In brief, 96-well plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with antigen in 0.1 M carbonate/bicarbonate buffers, pH 9.0, and blocked with PBS containing 1% bovine serum albumin (Sigma). After blocking, serial dilutions of the serum samples or fecal extracts were added to the plates, which were incubated at room temperature for 1.5 h. The plates were washed 3 times with wash buffer (PBS containing 0.05% Tween 20), and peroxidase-labeled rabbit anti-mouse IgG antibodies (Zymed, San Francisco, CA, USA) or peroxidase-labeled goat anti-mouse IgA antibodies (Zymed) were added. After 1.5-h incubation at room temperature, the plates were washed 3 times with the wash buffer, and *o*-phenylenediamine (Sigma) in phosphate/citrate buffer containing 0.03% H_2O_2 was added. The reactions were arrested 10 min later by adding 1 N H_2SO_4, and optical densities were measured at 492 nm. Endpoint titres were expressed as reciprocal $\log_2$ of the limit dilutions that recorded an optical density greater than 1.0.

2.7. Tetanus toxin challenge

The left hind thigh of the mice was injected subcutaneously with 10 median lethal dose (LD_{50}) of tetanus toxin in 0.5 ml PBS, and the mice were observed daily for up to 7 days. The mice that developed severe paralysis were euthanized.

2.8. Statistical analysis

The data are represented as the geometric means of the values obtained from individual animals. The groups were compared using unpaired two-tailed Student's *t*-tests, and *p*-values ≤ 0.05 were regarded as significant.

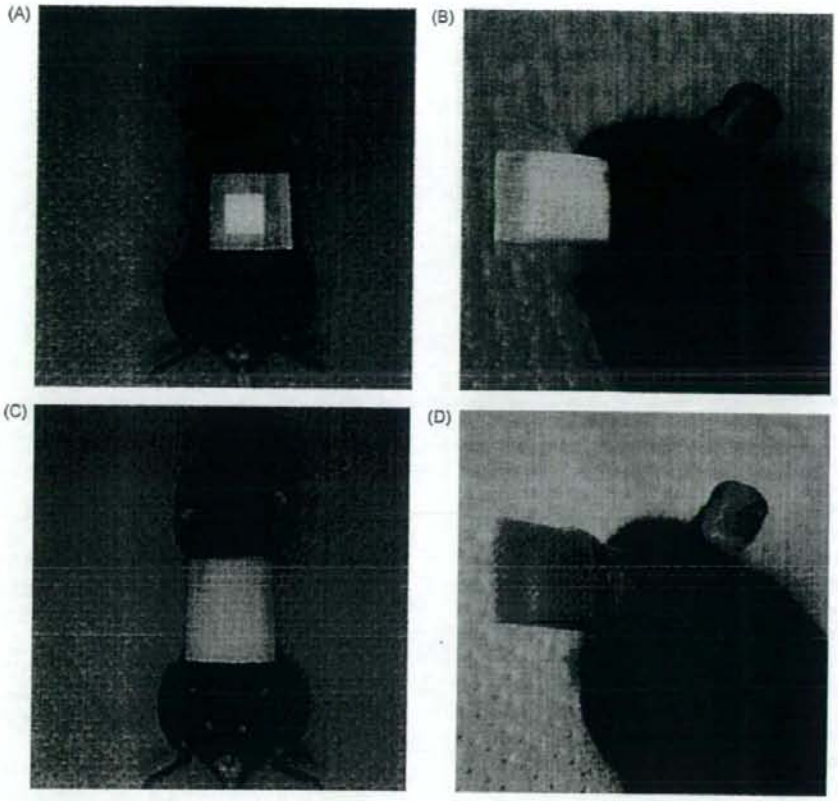

Fig. 1. Prolonged TCI using gauze patch. The gauze patch soaked with 50 μl of antigen solution was applied to the bare abdominal skin (A) or the dorsal side of the ear skin (B), and fixed with medical tape (C, D).

3. Results

3.1. Conventional TCI induced a substantial antigen-specific serum antibody response; however, the use of an adjuvant was crucial

We confirmed the crucial roll of CT as an adjuvant in the conventional TCI method.

The C57BL/6 mice were immunized by the conventional TCI procedure with 100 μg of OVA with or without 10 μg of CT as an adjuvant. Another group of mice was injected intradermally with an identical dose of OVA solution without CT. They were immunized in an identical manner 3 times with 2-week intervals. At 2 weeks after the last immunization, we collected serum samples and determined the OVA-specific and CT-specific IgG antibody titres by ELISA. When an adjuvant was used, as in the conventional TCI, we observed that the level of OVA-specific IgG antibodies induced was comparable to that induced by the intradermally injected OVA solution (Fig. 2A). CT-specific IgG antibody responses were also induced by the adjuvanted TCI (Fig. 2B). In contrast, the non-adjuvanted conventional TCI induced no significant serum antibody response.

3.2. Prolonged TCI induced a substantial antigen-specific serum antibody response even in the absence of an adjuvant

We examined the effect of prolongation of the duration of antigen presence on the skin to the immune response induced by TCI.

The bare abdominal skin or the dorsal side of the ears of the C57BL/6 mice were immunized with 100 μg of OVA with or without 10 μg of CT as an adjuvant according to the prolonged TCI procedure; the duration of the patch immunizations varied from 2 to 32 h. Booster immunizations were performed in an identical manner 2 times with 2-week intervals. At 2 weeks after the last immunization, we collected serum samples from the mice and determined their OVA-

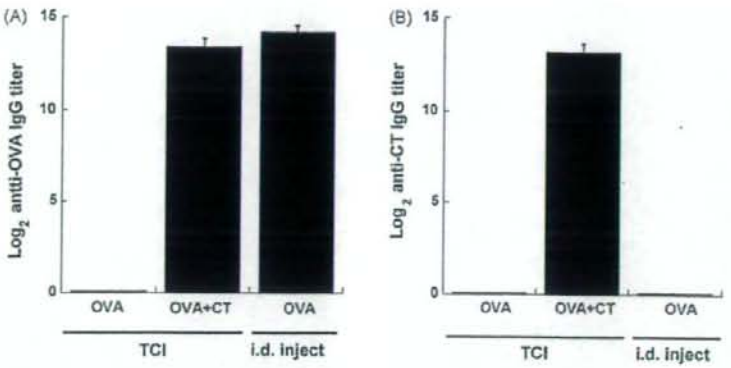

Fig. 2. Antigen- or adjuvant-specific serum IgG antibody response after conventional TCI. The bare abdominal skin of C57BL/6 mice ($n=5$) was applied directly with 50 μl of OVA solution (2 mg/ml) with or without CT (200 μg/ml) as an adjuvant for 2 h. Another group of mice ($n=5$) was injected intradermally with an identical dose of OVA solution without CT. Immunizations were performed in an identical manner for each group of mice at 0, 2, and 4 weeks. OVA-specific (A) or CT-specific (B) IgG titres in the serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.

specific IgG antibody titres. The 2-h patch immunization with an adjuvant to the abdomen induced substantial OVA-specific serum IgG antibody production, while the 2-h immunization without an adjuvant did not induce a significant antibody response. However, the 16-h patch immunization without an adjuvant also induced a significant antibody response (Fig. 3A). On the other hand, the 2-h patch immunization of the ear induced a significant antibody response even without an adjuvant, and the titres of serum OVA-specific IgG antibodies increased with the duration of the patch immunization. The antibody titres produced by the non-adjuvanted 16-h and 32-h patch immunizations of the ear were comparable to that produced by the adjuvanted 2-h patch immunization (Fig. 3B). Thus, the prolonged TCI with patch immunization for greater than 16 h induced a substantial antigen-specific serum antibody response even when it was non-adjuvanted.

Next, we compared the immunization to the bare abdominal skin with that to the dorsal side of the ear skin for determining the most suitable target site for the prolonged TCI.

C57BL/6 mice were immunized using the prolonged TCI procedure with 10 or 100 μg of OVA without an adjuvant. We boosted the mice 2 times at 2-week intervals using the same manner of immunization as that used for the primary TCI and determined the OVA-specific serum IgG antibody titres

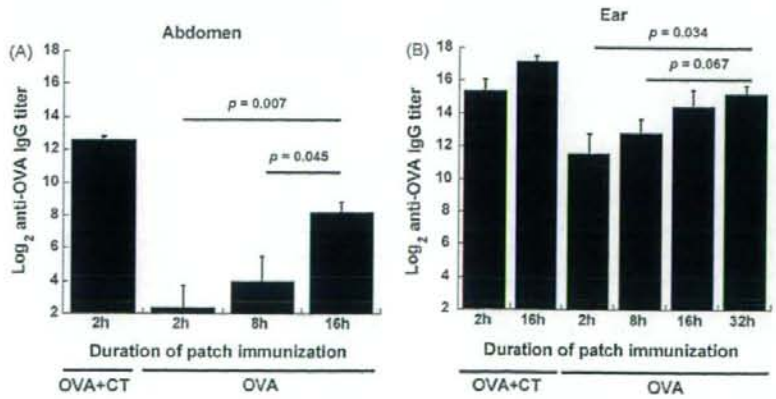

Fig. 3. Prolonged antigen presence on the skin. Gauze patches soaked with 50 μl of OVA solutions (2 mg/ml) with or without CT (200 μg/ml) as an adjuvant were taped to the bare abdominal skins (A) or the dorsal side of the left ears (B) of C57BL/6 mice ($n=5$) for 2, 8, 16, or 32 h. Immunizations for each group of mice were performed in an identical manner at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.

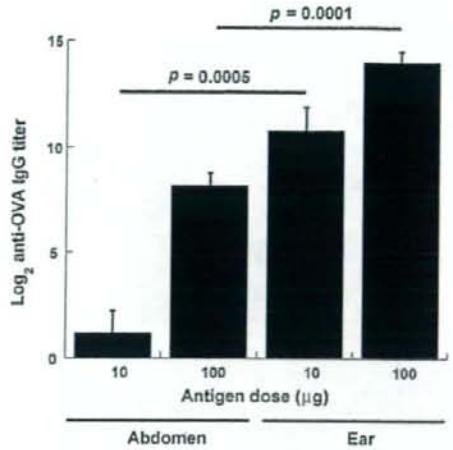

Fig. 4. Comparison between the abdominal skin and the ear skin for determining the most suitable target site for prolonged TCI. The bare abdominal skins or the dorsal side of the left ears of C57BL/6 mice ($n=5$) were immunized by non-adjuvanted prolonged TCI procedure with 10 or 100 μg of OVA for 16 h. Immunization of each group of mice was performed in an identical manner at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.

2 weeks after the last immunization. We found that, with both doses of OVA, the immunization of the dorsal sides of ears induced higher titres of antibodies than that of the bare abdominal skin (Fig. 4). Thus, the prolonged TCI procedure immunized the ear skin more efficiently than the abdominal skin.

3.3. Non-adjuvanted prolonged TCI of the ear skin induced a serum antibody response comparable induced by the adjuvanted TCI

We compared the serum antibody responses induced by the non-adjuvanted prolonged TCI and adjuvanted TCI by using dose–response and time-course studies.

Patches containing 1.0, 12.5, 25, 50, or 100 μg of OVA and with or without 10 μg of CT were taped to the dorsal side of the ears of the C57BL/6 mice for 16 h according to the prolonged TCI procedure. We boosted the mice twice with 2-week intervals in a manner identical to that used for the primary immunization and determined the serum OVA-specific IgG antibody titres 2 weeks after the last immunization. The titres of the serum IgG antibodies increased in a dose-dependent manner, and there was no significant difference between the magnitudes of the serum antibody response induced by the non-adjuvanted prolonged TCI and that induced by the adjuvanted TCI (Fig. 5A).

In the time-course experiment, we immunized the ears of the C57BL/6 mice with100 μg of OVA with or without 10 μg of CT according to the prolonged TCI procedure and boosted the mice twice at 2-week intervals in a manner identical to that used for the primary immunization. We collected serum samples prior to the immunization and every week for 1–6 weeks after the primary immunization. Further, we determined the time-course of the OVA-specific IgG antibody productions. Significant serum anti-OVA antibody production was observed after the first booster immunization, and this production was enhanced by the second booster immunization. There were no significant differences between the serum antibody responses induced by the non-adjuvanted prolonged TCI and the adjuvanted one, except with regard to

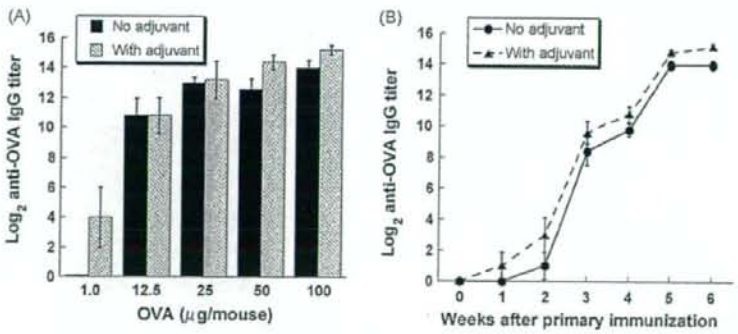

Fig. 5. Dose–response (A) and time-course (B) of serum antibody production by the non-adjuvanted or adjuvanted prolonged TCI procedure. (A) The ears of C57BL/6 mice ($n=5$) were immunized by prolonged TCI procedure for 16 h with the indicated doses of OVA from 1.0 to 100 μg with (hatched bars) or without (solid bars) 10 μg of CT as an adjuvant. Each group of mice was immunized in an identical manner at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization using ELISA. The geometric mean and the standard error of the mean are shown for each group. (B) The ears of C57BL/6 mice ($n=5$) were immunized by the prolonged TCI procedure for 16 h with 100 μg of OVA with (closed triangles and a broken line) or without (closed circles and an unbroken line) 10 μg of CT as an adjuvant at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined every week up to 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group and time.

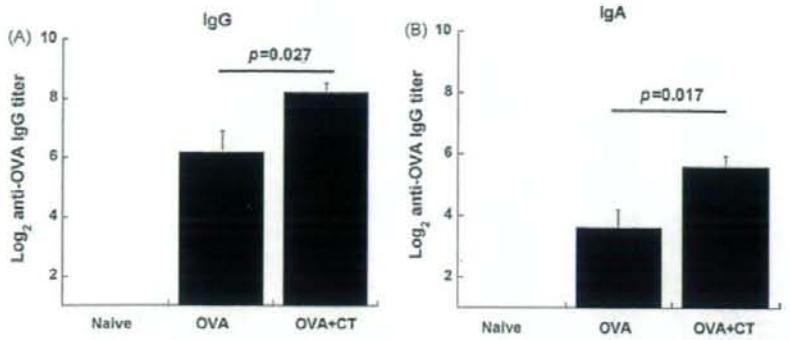

Fig. 6. Fecal antibody responses induced by the non-adjuvanted or adjuvanted prolonged TCI procedure. The ears of C57BL/6 mice ($n=5$) were immunized by prolonged TCI procedure for 16 h with 100 μg of OVA with or without 10 μg of CT as an adjuvant at 0, 2, and 4 weeks. Another group of mice ($n=5$) was inoculated nothing as a negative control. The fecal samples were collected at 6 weeks after the primary immunization and OVA-specific IgG (A) and IgA (B) titers in the fecal extracts were determined. The geometric means and the standard error of the means are shown for each group.

the antibody titres at 6 weeks after the primary immunization (Fig. 5B).

Thus, the non-adjuvanted prolonged TCI comprising the 16-h antigen presence induced serum antigen-specific antibody responses of a level comparable to that observed with the adjuvanted TCI.

3.4. Non-adjuvanted prolonged TCI induced significant fecal antibody responses

Previous studies demonstrated that CT-adjuvanted TCI elicited not only systemic but also mucosal antibody responses. We examined the fecal IgG and IgA antibody responses after adjuvanted and non-adjuvanted TCI.

Patches containing 100 μg of OVA and with or without 10 μg of CT were applied to the dorsal side of the ears of the C57BL/6 mice for 16 h. We boosted the mice twice with 2-week intervals in a manner identical to that used for the primary immunization. Other group of mice were inoculated nothing as a negative control. We collected the fecal samples at 2 weeks after the last immunization.

Both groups of the immunized mice produced significant fecal IgG and IgA antibodies (Fig. 6). The CT-adjuvanted TCI group indicated approximately 4-fold higher titers of antibody production than the non-adjuvanted group.

3.5. Non-adjuvanted prolonged TCI of the ear skin induced a significant serum antibody response, regardless of mice strains

We examined strain differences with regard to the serum antibody responses induced by the prolonged TCI procedure. The ears of the C57BL/6, BALB/c, or C3H/He mice were immunized with 100 μg of OVA with or without 10 μg of CT according to the prolonged TCI procedure. The mice were boosted twice at 2-week intervals, and the OVA-specific IgG antibody production in their sera was determined 2 weeks after the last immunization. We observed significant differences in the magnitude of the serum OVA-specific IgG antibody responses among the different strains of mice. Among the 3 strains, the C57BL/6 mice produced the highest amounts of serum antigen-specific IgG antibodies, while the C3H/He mice produced the least amounts. Besides, both the non-adjuvanted prolonged TCI and the adjuvanted one induced substantial serum antibody responses in all the strains of mice that were examined (Fig. 7).

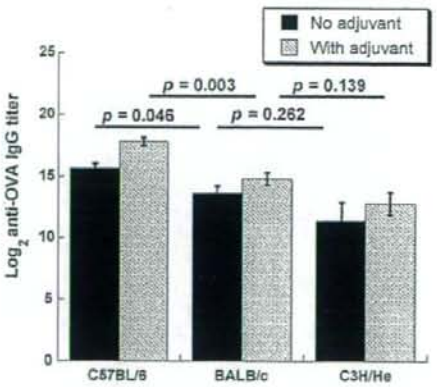

Fig. 7. Differences between the mice strains with regard to the serum antigen-specific IgG responses induced by the non-adjuvanted or adjuvanted prolonged TCI procedure. The ears of C57BL/6 ($n=5$), BALB/c ($n=5$), or C3H/He ($n=5$) mice were immunized with 100 μg of OVA with (hatched bars) or without (solid bars) 10 μg of CT as an adjuvant according to the prolonged TCI procedure for 16 h at 0, 2, and 4 weeks. OVA-specific IgG titres in serum samples were determined at 6 weeks after the primary immunization. The geometric means and the standard error of the means are shown for each group.