Oral desensitization by oral immunotherapy using diet supplemented with allergen and development of outcome index for mild desensitization.

2018

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Introduction

Food allergy is defined as “a phenomenon in which adverse reactions are caused through antigen-specific immunological mechanisms after exposure to a given food.” [1]. The most common mechanism of food allergy is IgE-mediated reactions, which cause immediate reactions within 2 h after the exposure to food allergens [1]. Non-IgE mediated reaction is a food allergic reaction that occurs independent of IgE [1]. Food allergy symptoms occur in various organs such as skin, mucous membrane, respiratory organs, digestive organs, nerve and circulatory organs. Of the symptoms, anaphylaxis is defined as “severe hypersensitivity reaction that may cause a life-threatening risk with systemic symptoms induced at several organs.” [1] Moreover, this symptom, when blood pressure reduction or consciousness disturbance is accompanied, is called anaphylactic shock [1]. Imai et al reported that the symptom appearance frequency was most frequent with skin symptoms at 92%, with respiratory symptoms of 33.6%, mucosal symptoms of 28.0% and gastrointestinal symptoms of 18.6% [2]. Furthermore, the shock was 10.4% (307 cases) [2]. On the basis of a large-scale epidemiological survey in Japan, the prevalence of food allergy is estimated to be 5–10% in infants [3], 5% in young children, and 4.5% in school children [4]. In Japan, the treatment of food allergy is very important.

In Japan, egg allergy is the most common food hypersensitivity among pediatric population [2]. The major allergenic egg proteins are ovalbumin (OVA) and ovomucoid (OM), which are contained within egg white (EW) [5]. OVA is the major protein in EW, comprising 54% of its total protein content [5]. OM represents 11% (w/w) of EW proteins [5]. OM is known to exhibit trypsin inhibitor activity [5,6]. Moreover, OM is relatively resistant to treatment such as heat, chemistry and digestive enzymes [5,7]. The other allergenic proteins in EW are ovo transferrin, ovomucin and lysozyme [5].

The principles of dietary and nutritional instruction on the treatment of food allergy are minimum avoidance of causative foods, dietary instruction for safe ingestion,
evaluation of nutritional status, and the maintenance of QOL in the patient’s family [1]. Recently, oral immunotherapy (OIT) is attracting attention as a treatment method of food allergy. OIT is defined as “a treatment method for cases where the early acquisition of tolerance during the natural course cannot be anticipated. After a symptom induction threshold has been determined during an earlier OFC, causative foods are taken under a physician's instruction aiming to acquire the conditions of increased threshold or desensitization.” [1]. However, in Japan, OIT is not recommended as general treatment for food allergy because OIT has some problems [1]. International guidelines and systematic reviews do not propose a unified method of the OIT, and various treatment periods, methods of dose escalation, and OIT foods have been reported [1,8,9]. Yanagida reported that low-dose OIT, which continued the allergen intake of small amounts for long term, seemed to be effective for tolerating larger amounts of causative foods [10,11]. Additionally, these smaller amount therapies may lead to fewer adverse reactions than traditional OIT and be more effective than strict elimination [11]. Furthermore, egg OIT studies using raw egg, EW powder [12], omelets [13], cookies contained EW [14] and muffin contained EW [15] were reported. However, there is no OIT study on how to ingest the allergen per day. Therefore, I consider the division of allergen amount per day and propose a new OIT method that involves mixing a small amount of allergen powder in the diet. I examine whether diets supplemented with allergen ameliorate the severity of allergy in an allergic mouse model.

The important agenda is that the biomarkers are not established to predict the OIT outcome without oral food challenge. The reported biomarkers are allergen-specific IgE, IgG4 and IgA2, and CD63 and CD203c. However, these biomarkers have problems; allergen-specific IgE correlates with the presence or absence of allergy [16], but does not reflect the severity of allergy [17], the presence of IgG4 and IgA against food antigens should probably be interpreted as a result of antigen exposure, rather than of disease [18,19], and the CD63 and CD203c have a shortage of evidence. Moreover, few
studies have focused on the correlation between these biomarkers and the stage of desensitization; most previous OIT studies entailed the complete desensitization of the treatment group. Therefore, I carry out low-dose OIT against children with severe egg allergy. Then, after OIT, these children are then divided into a high effect (H-E) and a no/low effect (N/L-E) groups, and serum biomarkers are compared between the H-E and N/L-E groups.

Objects of this thesis are to propose safer OIT method and to develop the biomarker which can predict the outcome by low-dose OIT without oral food challenge. Therefore, in this thesis, I tried to establish the mouse model with ovomucoid or egg white allergy, to examine whether diets supplemented with allergen ameliorate the severity of allergy in these mice, and to analysis the serum OIT biomarker treated low-dose OIT using low egg-allergen cookies (LAC).
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Chapter 1

Intake of diet including 1% ovomucoid for 4 weeks induces oral desensitization in ovomucoid specific allergic mouse model.

Introduction

I propose a new OIT regimen that involves mixing a small amount of allergen powder in the diet. Some merits of this OIT method are: 1) the allergen dose is divided, 2) allergen doses ingested can be easily adjusted, and 3) the taste and smell of the allergen is masked. Few reports exist regarding the relationship between oral desensitization and an allergen-containing diet in allergic mouse models [1]. An egg white diet, which uses egg whites instead of total protein, for 2 weeks results in a decrease in the anti-OVA-specific IgE level and protection against systemic anaphylaxis in an OVA-allergic mouse model [1]. However, the egg white diet caused an anaphylaxis reaction, and induced a decrease in body weight and food intake during the early stages of OIT treatment in the OVA-specific allergic mouse model [1,2]. Here, I determine that diet supplemented with 1% of allergen was not enough to induce anaphylaxis reaction, and clarify that the diet can induce oral desensitization and immune tolerance.
Materials and Methods

Animals and diets

Experimental animal care and treatment conformed to the Mukogawa Women’s University guidelines for the ethical treatment of laboratory animals (Acceptance No. FSN-01-2015-01-A). Animals were housed at 22°C with 60% humidity under a 12-h light (08:00–20:00) and dark (20:00–08:00) cycle. Body weights and food intake were measured at 10:00. Plasma were stored at −40°C.

Milk casein, gelatinized cornstarch, cellulose powder, AIN-93G mineral mixture, and AIN-93 vitamin mixture were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan). L-Methionine was purchased from Wako Pure Chemical Industries (Osaka, Japan). Sucrose was purchased from Mitsui Sugar Co., Ltd (Tokyo, Japan). Corn oil was purchased from Olitalia Srl (Forlì, Italy). OM was purified from hen egg whites following a previously established method [3]. Table 1–1 shows the compositions of the control and 1% OM diet. We prepared all diets in our laboratory.

Allergy sensitization and OIT protocol

Twenty C3H/HeJ female mice were purchased from Clea Japan (Tokyo, Japan) at six weeks old. All mice were housed individually in plastic cages. Fig 1–1 shows the protocol of sensitization and OIT. Fifteen mice (allergy group) were sensitized by four intraperitoneal (i.p.) administrations of 5.0 mg OM/100 μL saline plus 100 μL of alum (Wako Pure Chemical Industries, Osaka, Japan) at 12-day intervals. Four mice in the allergy group did not survive past the fourth sensitization. The non-allergy group consisted of five mice injected with 100 μL saline plus 100 μL of alum via i.p. administration.

On day −1 after the allergy group was orally challenged with 20 mg OM/100 μL saline (oral challenge), the allergy group was further divided into the OIT group (5 mice; 1% OM diet) and the non-treated group (6 mice; 20% casein diet). The OIT treatment was performed for 4 weeks. On day 15, plasma samples of mice were
obtained under isoflurane anesthesia. On day 16, the non-treated group and OIT group underwent an oral challenge. On day 30, both the non-allergy and the allergy groups were challenged with 100 μg OM/100 μL sterilized PBS via i.p. administration (systemic challenge). The OIT group was fed a 20% casein diet for 1 week after OIT. Anatomy was performed within 1 week after the systemic challenge. The final oral challenge at the endpoint was carried out before anatomy. Mice were killed under isoflurane anesthesia to obtain plasma and dorsal skin samples.

**Allergic symptoms after food challenge**

After oral or i.p. administration, mice were monitored for allergic symptoms for 30–40 min and scored on a 6-point scale (0, no symptoms; 1, scratching around the nose and head; 2, puffiness around the eye and mouth; 3, wheezing or labored respiration or little activity after prodding; 4, no activity after prodding; 5, death).

**Vascular permeability**

We measured the vascular permeability following previously described methods [4,5]. Mice were intravenously injected with 200 μL of 1.5% FITC-albumin under isoflurane anesthesia, intradermally injected at 4 points (test (2 points), 5 μmol/L OM; control (2 points), Tyrode's solution) in the shaved dorsal skin, and orally gavaged with 20 mg OM solution. After 30-40 min, mice were sacrificed under isoflurane anesthesia to obtain their shaved dorsal skin. The vascular permeability (10 μL plasma equivalent) was calculated as follows: OM, the mean of OM fluorescence value/plasma fluorescence value; Tyrode's, the mean of Tyrode's fluorescence value/plasma fluorescence value.

**Measurement of the levels of OM-specific IgE and IgA in plasma**

We measured plasma immunoglobulin (Ig) levels by enzyme-linked immunosorbent assay (ELISA). OM-specific IgE was determined by a modified capture ELISA [6].
Goat anti-mouse IgE (2 μg/mL; Bethyl Laboratories, Montgomery, TX, USA) were used as the coating antibodies for a 96-well flat bottom ELISA microplate (Nunc-Immuno Maxisorp, Thermo Fisher Scientific Inc, Massachusetts, USA). OM was biotinylated using a biotinylation kit (Sulfo-OSu; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and the signal was detected using horseradish peroxidase (HRP)-conjugated streptavidin (Abcam, Cambridge, UK). Dilution ratio of the plasma in OM-specific IgE was 1:10 and the HRP reaction was carried out for 20 min at room temperature. The levels of OM-specific IgE was presented as the absorbance values at 492 nm.

OM-specific IgA was determined by indirect ELISA following the methods [7]. The coating concentrations of OM were 500 μg/mL. The dilution ratio of the plasma in OM-specific IgA was 1:100. The signal was detected using HRP-conjugated goat anti-mouse IgA (Bethyl Laboratories) and the HRP reaction was carried out for 20 min at room temperature. The levels of OM-specific IgA was presented as the absorbance values at 492 nm.

**Statistical analysis**

Values are presented as means ± SE [8]. Allergic symptom scores were analyzed by Kruskal-Wallis tests; a post-hoc multiple comparisons test was performed with the Mann-Whitney U test and Ryan method [9]. The vascular permeability was compared by repeated two-way ANOVA followed by Bonferroni’s multiple-comparison test. Statistical significances of OM-specific IgE and IgA concentration were determined by one-way ANOVA, followed by Tukey’s multiple-comparison test. Differences with $p < 0.05$ were considered significant. Graph Pad Prism version 5.0 (Graph Pad Software, San Diego, CA) was used for all analyses.
**Results**

*Nutritional effect of 1% OM diet.*

I confirmed the nutritional effect of the 1% OM diet. Body weight change in the OIT group was significantly lower than that in the non-treated group (Fig 1–2A). However, the mice in the OIT group did not lose weight during the treatment (Fig 1–2A). Daily food intake was not significantly different between the non-treated and OIT groups (Figure 2B).

*Effect of 1% OM diet on OM-allergic mouse model.*

I determined whether the 1% OM diet improved allergic reactions. In response to the oral challenge at day 16 of OIT treatment, the OIT group displayed lower allergic symptom scores than the non-treated group (Fig 1–3A–C and 1–4A). Next, in response to the oral challenge at the endpoint, only one mouse of the OIT group exhibited scratching behavior, whereas scratching was observed in all mice of the non-treated group (Fig 1–4A). In response to the systemic challenge (day 30), the OIT group exhibited lower allergic symptom scores than did the non-treated group, although they did experience mild symptoms (scores ranged from 1–3 (Fig 1–4B). The vascular permeability of the OIT group was significantly lower than that of the non-treated group (Fig 1–5).

*Influence of the plasma levels of OM-specific IgE and IgA in OM-allergic mice fed with a 1% OM diet.*

IgE triggers type I allergic reaction, while IgA promotes allergen tolerance during human infancy [16]. At day 15 or at the endpoint, the plasma level of OM-specific IgE was comparable to that of the non-treated group to OIT group, whereas the plasma levels of OM-specific IgA was significantly higher in the OIT group than in the non-treated group (Fig 1–6A and B).
Discussion

In this experiment, the body weight change in the OIT group was significantly lower than that in the non-treated group. The inhibition percentage of body weight gain in the OIT group was 9.27% (mean body weight in the non-OIT was 30.03 g). If the 1% OM diet induces allergic reaction, body weight and food intake were predicted to decrease during the early OIT stage [1,2]. However, these results do not support this assumption. OM is a well-known trypsin inhibitor [10,11]. Oda, et al. reported that body weight gain in rats fed a 1% quail OM diet was significantly lower than in rats fed a 20% casein diet [12]. The low body weight gain in the 1% OM diet group may have caused pancreatic hypersecretion leading to energy metabolism-promoting actions. A suppression of body weight gain also has been reported for other trypsin inhibitors [13,14]. Thus, it was suggested that the low body weight gain in the OIT group caused the trypsin inhibition of OM.

I challenged this assumption with our new OIT method using a diet supplemented with small amounts of the allergen. The findings suggest that most mice in the OIT group achieved oral desensitization after 4 weeks of treatment. Moreover, allergic symptom scores at the endpoint were lower than at day 16. Therefore, long-term OIT was effective for oral desensitization. However, the dietary treatment was unable to induce immune tolerance; under i.p. challenge, the OIT group exhibited symptom scores of 1 to 3. These symptom scores in the OIT group reflected an improving trend compared with the non-treated group, indicating an increase in the threshold value of antigen amount to induce the symptoms. This is consistent with the Leonard’s report [15]. Overall, based on these results and previous reports, OIT using diet supplemented with small amount of allergen can induce desensitization but not immune tolerance in an allergy mouse model.

Recently, a relationship between the desensitization by allergen-specific immunotherapy and the serum levels of allergen-specific IgA was reported [15-18]. In the experiment, the plasma levels of OM-specific IgA was significantly higher in the
OIT group than in the non-treated group. Strait, et al. reported that IgE-mediated systemic anaphylaxis and mast cell degranulation induced by antigen ingestion are suppressed by serum antigen-specific IgA, but not by IgA from the gut lumen in an allergic mouse model [19]. In contrast, Vazquez-Otriz, et al. reported that serum allergen-specific IgA was not associated with natural or induced tolerance to egg in egg-allergic children [20]. These results and reports suggest that serum allergen-specific IgA has the potential to be used as a biomarker of OIT effectiveness.

There were several limitations of this study. Frist, the score of allergic symptoms in the experiment were subjectively observed. Second, the dose-response of allergen concentration in diet was not examined, and the minimum dietary concentration required to elicit oral desensitization was not clear. Third, the OM allergic mouse model was not similar animal model of egg allergic patients because patients with egg allergy react various egg protein such as OVA, ovotransferrin, OM and lysozyme.

**Conclusion**

Data from our mouse model study suggest that the OIT method using diet supplemented with allergen is effective for oral desensitization. However, it is now difficult to utilize the OIT method for patients with food allergy because of lacks of evidence. Next, I will carry out the experiment using an allergic mouse model to dissolve the described limitations.
References


Increased peanut-specific IgA levels in saliva correlate with food challenge outcomes after peanut sublingual immunotherapy. *J Allergy Clin Immunol* 2012;129:1159-1162.


Table 1–1. Compositions of experimental diets

<table>
<thead>
<tr>
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<th>20% casein diet</th>
<th>1% OM diet</th>
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<td>g/kg diet</td>
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<td>190</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gelatinized cornstarch</td>
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<td>453</td>
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<tr>
<td>Sucrose</td>
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<td>200</td>
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<tr>
<td>Corn oil</td>
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<td>50</td>
</tr>
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<tr>
<td>Vitamin mixture (AIN-93)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>OM</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

All dietary combinations were prepared in our laboratory. OM, ovomucoid.
Fig 1–1. Mouse experimental protocol
Fig 1–2. Body weight change (A; mean ± SE, n = 5-6) and daily food intake (B; mean ± SE, n = 5-6) in the non-allergy group (20% casein diet), the non-treated group (20% casein diet), and the OIT group (1% OM diet) during treatment.

Significant differences in body weight change are indicated by different lowercase letters (a, b) and determined by one-way ANOVA followed by Tukey’s multiple-comparison test on day 28, $p < 0.05$. N.S. means no significant difference.
**Fig 1–3.** The nasal symptoms after oral challenge at day 16.

The nasal symptoms after saline administration in the non-allergy mouse (A) are shown. The nasal symptoms after 20 mg OM oral administration in the non-treated mouse (B) and in the OIT mouse (C) are shown.
Fig 1–4. Symptom scores of oral challenge (A), symptom scores of systemic challenge (B).

Value are presented as raw data and mean. Significant differences in symptom scores (A, B) were calculated via a Mann-Whitney U test and the Ryan method (9). Significant differences are indicated by different letter (a, b), $p < 0.05$. 
Fig 1–5. Vascular permeability of dorsal skin (mean ± SE, n = 4–6).

Vascular permeability (10 μL plasma equivalent) was calculated as follows: OM, the mean of OM fluorescence value/plasma fluorescence value; Tyrode's, the mean of Tyrode's fluorescence value/plasma fluorescence value. Differences in vascular permeability were assessed using repeated two-way ANOVA followed by Bonferroni’s multiple comparison test. In the absence of significant interactions, different superscript letters (lower-case letters for points injected with Tyrode’s solution; upper-case letters for points injected with OM) indicate significant differences (p < 0.05). Superscript asterisks indicate significant differences (p < 0.05) between the Tyrode’s and OM points in the same group.
Fig 1–6. The plasma levels of OM-specific IgE (A; mean ± SE, n = 4–6) and IgA (B; mean ± SE, n = 4–6) at day 15 and at the endpoint.

Capture ELISA was used to measure OM-specific IgE. Dilution ratio of the plasma in OM-specific IgE was 1:10. OM-specific IgA was measured by indirect ELISA. Dilution ratio of the plasma in OM-specific IgA was 1:100. HRP reaction was carried out for 20 min at room temperature. Significant differences within the each points were determined by one-way ANOVA followed by Tukey’s multiple-comparison test. Significant differences are indicated by different letters (a, b), p < 0.05.
Chapter 2

Diets supplemented with 1% egg white induce oral desensitization and immune-tolerance in egg white specific allergic mouse model.

Introduction

In the previous study (Chapter 1), it was reported that diets supplemented with 1% OM induce oral desensitization and immune-tolerance in OM specific allergic mouse model. However, there were several limitations of the previous study (Chapter 1). To solve these problems; First, I measured the change in the rectal temperature and the frequency of diarrhea after oral challenge with 40 mg EW in order to quantitatively evaluate the seventy of allergy in model mouse. Second, I prepared three test diet supplemented with 0.01, 0.1 and 1% allergen due to clarify the dose-response desensitization of OIT utilizing diet. Third, mice were sensitized to EW because it need to conduct experiment similar animal model of egg allergic patients. Therefore, I examined whether 0.01–1.0% of EW-containing diets can induce immune tolerance and/or oral desensitization for 4 weeks in an EW allergic mouse model.
Materials and Methods

Animals and diets

Care and treatment of the experimental animals conformed with the Mukogawa Women’s University guidelines for the ethical treatment of laboratory animals (No. FSN-01-2016-01-A). Two to three animals were housed per cage at 22°C with 60% humidity under a 12-h light (08:00–20:00)-dark (20:00–08:00) cycle. Body weight and food intake were measured at 10:00 at 2–4 days interval. EW was diluted 3 folds in water, mixed for 1 h, filtered through gauze, and freeze-dried. The protein patterns of EW powder in SDS-PAGE were consistent with raw EW (Fig 2–1). All diets in the experiment were prepared in-house (Table 2–1).

Sensitization with EW and OIT protocol

The experimental protocol is illustrated in Fig 2–2. Fifty female BALB/c mice were purchased from Clea Japan (Tokyo, Japan) at six-weeks old. Forty mice were sensitized by two times intraperitoneal administrations of 100 μg egg white (EW) in 100 μL saline plus 100 μL of alum (Imject™ Alum Adjuvant; Thermo Fisher Scientific Inc., Waltham, MA, USA) on week -4 and -2. The other ten mice were i.p. injected with 100 μL of saline plus 100 μL of alum (non-allergy group). On week -1, plasma was collected and assayed for OVA-specific IgE to verify sensitization to EW. Subsequently, all mice were given 20 mg EW in 200 μL saline orally three times for a week to induce allergic symptoms through oral administration of EW. A day before OIT, all mice were fasted from the previous night, and were orally challenged with 40 mg EW in 200 μL saline (oral challenge) at the morning. At before and 15 min after the oral challenge, the rectal temperature was measured using a thermometer (KN-91-AD-1687-M; Natsume Seisakusyo Co. Ltd., Tokyo, Japan), and the frequency of diarrhea in each group was recorded for 30 mins after the oral challenge.

Sensitized mice (n = 10 per group) were provided 4 weeks of OIT using diets supplemented with 0 (non-OIT group), 0.01 (0.01% EW group), 0.1 (0.1% EW group),
or 1% EW (1% EW group) in 20% casein diet; non-sensitized mice were used as non-allergy. One mouse each from non-OIT and 1% EW group died during the course of OIT. On 2 weeks (the medium of 4 weeks of OIT), plasma samples of mice were obtained under isoflurane anesthesia. On the next day, the mice were orally challenged with 40 mg EW in 200 μL saline (oral challenge), and the rectal temperature was measured at before and 15 min after the oral challenge, and the frequency of diarrhea was recorded for 30 mins after the oral challenge. On the final day of OIT, the mice were i.p. challenged with 200 μg EW in 100 μL sterilized PBS (systemic challenge), and the rectal temperature was measured at before and 30 min after the systemic challenge. The mice were also orally challenged the following day, and assessed as described. Within 1 week of the final oral challenge, five mice from each group were sacrificed under isoflurane anesthesia to obtain the plasma and spleen. Plasma were stored at -40°C before analysis. At a later date, the remaining animals (4–5 mice/group) were tested for vascular permeability according to published methods [1,2].

**Vascular permeability**

Mice were injected intravenously with 200 μL of 1.5% FITC-albumin (Albumin–fluorescein isothiocyanate conjugate, Sigma-Aldrich, St. Louis, MO, USA) under isoflurane anesthesia and intradermally at 4 positions with EW (2 positions; 20 μg EW in 50 μL) and Tyrode's (2 positions; 50 μL of Tyrode's solution) on the shaved dorsal skin. After 30 min, mice were scarified under isoflurane anesthesia to obtain the shaved dorsal skin and plasma. The fluorescent intensity of the dorsal skin and plasma extracts was measured automatically using a fluorescent plate reader (Infinite M200; TECAN Ltd., Männedorf, Switzerland) with 485 nm excitation and 530 nm emission. The vascular permeability (50 μL plasma equivalent) was calculated as follows: EW, the mean of EW fluorescence value/plasma fluorescence value; Tyrode's, the mean of Tyrode's fluorescence value/plasma fluorescence value.
**Measurement of Plasma OVA- and OM-Specific Antibodies (IgE, IgA, IgG1 and IgG2a)**

The levels of OVA- and OM-specific IgE and IgA in the plasma were determined by capture ELISA [1,3]. Goat anti-mouse IgE and IgA (2 μg/mL; Bethyl Laboratories, Montgomery, TX, USA) were used as the coating antibodies for a 96-well flat bottom ELISA microplate (Nunc-Immuno Maxisorp, Thermo Fisher Scientific Inc, Massachusetts, USA). The following antigens were used for the ELISA: OVA (OVA grade VI; Sigma-Aldrich, St. Louis, MO, USA) and OM, which was purified from egg white by ethanol precipitation [7]. Both antigens were biotinylated using a biotinylation kit (Sulfo-OSu; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and the signal was detected using HRP-conjugated streptavidin (Abcam, Cambridge, UK). Mouse anti-OVA IgE monoclonal antibody E-G5 and mouse anti-OVA IgA monoclonal antibody 2G12E12 (Chondrex Inc., Redmond, WA, USA) were used as standards to determine the concentration of OVA-specific IgE and IgA. The levels of OM-specific IgE and IgA were presented as the absorbance values. The dilution of OM-specific IgE and IgA were 1:50. The HRP reaction time for OM-specific IgE at 2 weeks, OM-specific IgE at endpoint, OM-specific IgA at 2 weeks, and OM-specific IgA at endpoint were 15 min, 10 min, 15 min, and 7 min, respectively.

Additionally, the levels of OVA- and OM-specific IgG1 and IgG2a were determined by indirect ELISA [4]. In the assay of OVA- and OM-specific IgG1 and IgG2a, the coating concentrations of antigens were 1 (IgG1) and 10 (IgG2a) μg/mL in the ELISA plates, respectively. The following secondary antibodies were used: HRP-conjugated goat anti-mouse IgG1 and IgG2a (Bethyl Laboratories). The absorbance values represent the levels of OVA- and OM-specific IgG1 and IgG2a. The dilution ratios of OVA-specific IgG1 at 2 weeks and endpoint, OM-specific IgG1 at 2 weeks and endpoint, OVA-specific IgG2a at 2 weeks and endpoint, OM-specific IgG2a at 2 weeks, and OM-specific IgG2a at endpoint were 1:50000, 1:10000, 1:1000, 1:1000, and 1:250, respectively. The HRP reaction time for OVA-specific IgG1 at 2 weeks, OVA-specific IgG1 at endpoint, OM-specific IgG1 at 2 weeks, OM-specific IgG1 at endpoint, OVA-
specific IgG2a at 2 weeks and endpoint, OM-specific IgG2a at 2 weeks, and OM-IgG2a at endpoint were 10 min, 7 min, 7 min, 5 min, 10 min, 15 min, and 7 min, respectively.

*Spleen Lymphocyte Culture and Assessment of Cytokine Secretions*

Mice spleens were removed and homogenized in Hank’s balanced salt solution [HBSS: 0.98% Hank’s solution (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 0.46% HEPES (Dojindo Molecular Technologies, Inc.), 0.2% NaHCO₃, 1.0% penicillin-streptomycin (Nacalai Tesque, Inc., Kyoto, Japan), 10% fetal calf serum (Corning, Inc., New York, NY, USA)]. Spleen lymphocytes were isolated from the spleen homogenate using Lympholyte-M (Lympholyte(M) Cell Separation Media, Cedarlane Laboratories, Ontario, Canada). Spleen lymphocytes were plated at a density of 1.5×10⁷/well in RPMI-1640 complete medium [1% RPMI-1640 (Nissui Pharmaceutical Co., Ltd.) supplemented with 0.23% NaHCO₃, 1.0% penicillin-streptomycin, 10% fetal calf serum, 1% glutamine (Nacalai Tesque, Inc.), and 1× 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Tokyo, Japan)] in a 24-well plate. Cells were stimulated with or without 2 mg EW per well, and incubated at 37°C and 5% CO₂. Culture supernatants were collected after 48 h, and then stored at -80°C before analysis. Concentrations of interleukin-4 (IL-4), IL-10, IL-17, and interferon-γ (IFN-γ) were assayed by ELISA according to the manufacturer instructions (BioLegend, San Diego, CA, USA). Data are presented as the difference in cytokine production between stimulated and non-stimulated spleen lymphocytes.

*CD4⁺ Foxp3⁺ Cells in Spleen Lymphocytes*

To quantify the cell population, 3.0×10⁶ of spleen lymphocytes isolated using Lympholyte-M were transferred into another tube. Extracellular staining was performed using FITC-conjugated anti-mouse CD4 and intracellular staining was performed Alexa 647-conjugated anti-mouse Foxp3 (BioLegend) as described by the manufacturer. Cells were acquired using BD FACS Calibur and analyzed using Cell Quest Pro (BD
Biosciences, Franklin Lakes, NJ, USA).

**Statistical Analysis**

Values are presented as means ± SE [5]. Statistical differences except for vascular permeability were analyzed by one-way ANOVA followed by Tukey’s multiple-comparison test. The vascular permeability was compared by repeated two-way ANOVA followed by Bonferroni’s multiple-comparison test. Differences with $p < 0.05$ were considered significant. The GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all analyses.
Results

Effect of EW-Containing Diet in EW Allergic Mouse Model

I measured the change in rectal temperature and frequency of diarrhea after EW challenge to assess the improvement of allergic severity in the EW allergic mouse model. After an oral challenge before OIT, there were no differences in the change in rectal temperature and the percentages of diarrhea occurrence among allergy groups (Fig 2–3A and 2–4A). After an oral challenge at 2 and 4 weeks, the change in rectal temperature in the 1% EW group was not significantly different than that of the non-allergy and non-OIT groups (Fig 2–3B and 2–3C). In addition, the percentages of diarrhea occurrence in the 1% EW group at 2 and 4 weeks were 40% and 67%, and were lower than of the non-OIT group (Fig 2–4B and 2–4C). However, I noted that in the 0.1% EW group, the decrease in rectal temperature was greater at 4 weeks than before OIT.

After systemic (intraperitoneal) challenge, the change in rectal temperature in the 0.1% EW and 1% EW groups were significantly lower than that of the non-OIT group (Fig 2–5A). Moreover, the change in rectal temperature in the 1% EW group was not significantly different than that of the non-allergy group (Fig 2–5A). Of note, the effect of OIT on the change in rectal temperature after the systemic challenge was dependent on antigen-dose. However, there was no significant difference in vascular permeability of the skin injected with EW among all allergy groups regardless of OIT (Fig 2–5B). Therefore, a dose of at least 1% EW appears to be required to adequately mitigate allergic symptoms in the EW allergic mouse model.

OVA- and OM-Specific Antibodies in the EW Allergic Mouse Model Fed with Diet Containing EW

IgE triggers type I allergic reaction, while IgA promotes allergen tolerance during human infancy [6]. The plasma concentration of OVA-specific IgE at 2 weeks and the study endpoint was not significantly different between the non-OIT and EW diet groups
The plasma level of OM-specific IgE in the 1% EW group at 2 weeks was significantly higher than that of the non-OIT group (Fig 2–6C). However, at the study endpoint, the plasma level of OM-specific IgE in the 0.1% and 1% EW groups was significantly lower than that of the non-OIT group (Fig 2–6D). The plasma concentration of OVA-specific IgA at 2 weeks in the 1% EW group was significantly higher than that of other groups (Fig 2–7A), but at the study endpoint, the concentration of OVA-specific IgA was comparable to that of the non-OIT group to 1% EW group (Fig 2–7B). Further, the plasma level of OM-specific IgA in the 0.1% and 1% EW groups at 2 weeks was significantly higher than that of the non-OIT group (Fig 2–7C). In contrast, at the study endpoint, the plasma level of OM-specific IgA in the 1% EW group was significantly lower than that of the non-OIT group (Fig 2–7D).

Imbalanced in type 1 and type 2 helper T cells (T\textsubscript{h1}/T\textsubscript{h2}) triggers allergy [6]. T\textsubscript{h2} induces a B-cell class-switch of IgG1 and IgE, while T\textsubscript{h1} induces a B-cell class-switch of IgG2a [6]. The plasma levels of OVA-specific IgG1 and IgG2a at 2 weeks and the study endpoint were comparable among the allergy groups (Table 2–2). The plasma level of OM-specific IgG2a in the 1% EW group at 2 weeks was higher than that of the non-OIT group (Table 2–2). However, at the study endpoint, the plasma level of OM-specific IgG2a in the 1% EW group was significantly lower than that of the non-OIT group (Table 2–2). Collectively, the data suggest that the intake of diet supplemented with 1% EW for 2 weeks promotes production of antibodies, and 1% EW-supplemented diet for 4 weeks induces anergy and/or active suppression in the EW allergic mouse model.

\textbf{Effect of Diets Containing EW on T\textsubscript{h} cell Differentiation}

T\textsubscript{h1} response drives cell-mediated immunity and releases substance such as IFN-\gamma, while T\textsubscript{h2} drives humoral immunity and releases substance such as IL-4 [6]. The concentration of IFN-\gamma was 7 to 8 fold higher in the 0.1% EW and 1% EW groups than in the non-OIT group (Table 2–3). However, IL-4 concentration was not significantly
different between the non-OIT group and EW diet groups (Table 2–3). The concentration of IL-17, which indicates the differentiation of Th17 cells, was comparable in non-OIT group to EW diets groups (Table 2–3). The concentration of IL-10, an immune suppressor cytokine, were significantly 2 folds higher in the 1% EW group than in the non-OIT group (Table 2–3). Moreover, the percentage of CD4+ Foxp3+ cell in the 1% EW group was higher than that of the non-OIT group (Table 2–3). Therefore, supplementation of diet with 1% EW appears to affect Th cell the differentiation in the EW allergic mouse model.
Discussion

In previous reports, the amount of allergen needed to induce oral desensitization in mouse approximately 1–5 mg [7–10]. In this study, because the daily food intake was approximately 2.7 g for each mouse (Table 2–4), supplementation with 0.1% EW should have been sufficient to elicit desensitization. It is possible that the different oral administration methods may differently affect oral desensitization. Thus, we orally administrated 3 mg EW to each EW allergic model mouse for 4 weeks; however, the oral gavages failed to induce oral desensitization (Fig 2–8). Previous mouse studies have used cholera toxin as the oral adjuvant [7–10]; in contrast, I used alum as an intraperitoneal adjuvant. Different allergic mouse models are thought to require different minimum allergen amount to induce oral desensitization. In this study, the feeding amount of allergen in the 1% EW diet was 5% of the protein intake per day. Recommended dietary allowance of protein in 3–7 year old children in Japan is 25–35 g/day [11], and the allergic protein for OIT should be between 1.25 and 1.75 g per day. Moreover, the amount of EW powder are approximate equivalent of one third of an egg per day (100 g of raw EW contain 10.5 g protein, and the percentage of raw EW in an egg approximately is 70%) [12]. In the human OIT studies, the maximum administration of EW powder for OIT was 2 g per day [13] and 4 g per day [14]. The main advantage point in OIT based on allergen-supplemented diet is that the daily amount of allergen protein can be divided into 3 meals. Thus, I considered that this OIT may be applicable for allergic children.

Notably, the decrease in rectal temperature after the systemic challenge was significantly less in the 0.1% EW group than in the non-OIT group; however, the decrease in temperature was comparable among these groups after an oral challenge. Because immune tolerance is thought to follow oral desensitization, the basis for these seemingly contradictory observations are unknown. Nevertheless, these results demonstrated that diets containing more than 0.1% of allergen modulate the immune response to a certain extent in the allergic mouse model.
Allergic reaction promotes Th2 activation, which induces IgE class-switching of B cells, causing the production of allergen-specific IgE by the augmented B cells [6]. In the non-OIT group, the concentration of OVA-specific IgE at the study endpoint was higher than at 2 weeks. However, in the 1% EW group, the concentration of OVA-specific IgE at 2 weeks was comparable to that at the study endpoint. Moreover, the plasma level of OM-specific IgE in the 1% EW group was lower than that of the non-OIT group at the study endpoint. In vitro and in vivo, IL-10 and regulatory T cells (Treg) induce IgG4 production and decrease IgE switching in human [15–17]. In this study, IL-10 secretion of spleen lymphocytes and was significantly higher in the 1% EW group than in the non-OIT group. Therefore, the data suggest that there is a relationship between the producing suppression of allergen-specific IgE and IL-10 production in vivo.

IgA’s role in mucosal immunity is well known and it is particularly critical for antigen exclusion in the gastrointestinal tract [6]. Recently, a correlation between desensitization and serum levels of allergen-specific IgA has been reported [17–21]. Among others, T cells derived from gut-associated lymphoid tissue [22] and transforming growth factor-β (TGF-β), which inhibits inflammatory responses [23], are known to regulate IgA production. Pilette et al. reported that IgA response to immunotherapy is selective for IgA2, is correlated with increased local TGF-β expression, and induces monocyte IL-10 expression [17]. In this study, the plasma levels of OVA- and OM-specific IgA were remarkably higher (approximately 7 folds) in the 1% EW group than in the non-OIT groups at 2 weeks. However, increased level of these antibodies in the 1% EW group was no longer observed at the study endpoint. I hypothesized that a continuous allergen intake induces anergy and active immune suppression, and inhibits the promotion of antibody production after allergic reactions. Therefore, the level of allergen-specific IgA in the plasma may predict the therapeutic course of OIT.

In human OIT studies, allergen-specific IgG4 are frequently used serum
biomarkers. Allergen-specific IgG4 could inhibit allergen-specific IgE binding to B cells [17, 24], and increased when oral desensitization was induced [14, 25]. However, the IgG subclass in mouse are IgG1, IgG2a, IgG2b and IgG3, and IgG1 in mouse is equivalent with IgG4 in human [6]. In this study, it appeared that the behavior of the plasma levels of OM-specific IgG1 from at 2 weeks to at the study endpoint was different with the other OM-specific antibodies such as IgE, IgA and IgG2a. Therefore, it was suggested that there might be an association between oral desensitization and allergen specific IgG1 in an allergic mouse model.

Th cell differentiation is regulated by various cytokines such as IFN-γ, IL-4, and IL-10. IL-10 suppresses antigen-induced proliferation more effectively in Th2 than in Th1 cells [26]. Thus, the increase in IL-10 production may block excessive Th2 cell differentiation. IL-10 is produced by various T cell, of which Treg are the most prolific cells and abundantly produce IL-10 but not IL-4 [27]. In addition, IFN-γ selectively inhibits the proliferation of Th2 cells [26]. I found that IL-10 and IFN-γ secretions and the percentage of CD4⁺ Foxp3⁺ cell in spleen lymphocytes were higher in the 1% EW group than in the non-OIT group. Moreover, IL-4 secretion was lower in the 1%EW group than in the non-OIT group although the difference was not statistically significant. Therefore, results of this study suggested that diet containing 1% allergen may elicit the production of IL-10 and IFN-γ, thereby preventing excessive Th2 cell differentiation in the allergy mouse model.

From this study and previous studies, it was suggested allergen specific immunotherapy induced the differentiation to Treg cells, and that the induced Treg related desensitization against allergen. However, there is no report that the downregulation of Treg inhibits desensitization by allergen specific immunotherapy. The anti-CD25 monoclonal antibody (clone PC61) depleted CD4⁺Foxp3⁺ Treg cells in vivo [28, 29]. Therefore, I examined whether the intraperitoneal administration of anti-CD25 monoclonal antibody (clone PC61) canceled the oral desensitization by OIT using diet supplemented with 1% EW in EW allergic mouse model. The experimental schedule
was shown in Fig 2–9A. The intraperitoneal administrations of antibody used 150 μg of anti-mouse CD25 (clone PC 61, BioLegend) per week (Fig 2–9B). Spleen lymphocytes were isolated using Lympholyte-M. In the analysis of flow cytometry, extracellular staining was performed using FITC-conjugated anti-mouse CD4 (BioLegend), and intracellular staining was performed Alexa 647-conjugated anti-mouse Foxp3 and CD25 clone PC61 (BioLegend) as described by the manufacturer. The results was that the change in rectal temperature in the sensitized mice fed 1% EW and not administrated anti CD25 after oral challenge was significantly suppressed than that of the sensitized mice not fed 1% EW diet and not administrated anti CD25 (Fig 2–10A). Moreover, the change in rectal temperature in the sensitized mice fed 1% EW diet and administrated anti CD25 was not significantly different than that of the sensitized mice not fed 1% EW diet and not administrated anti CD25 (Fig 2–10A). In the analysis of flow cytometry, the percentage of CD4⁺ CD25⁺ spleen lymphocytes was significantly lower in the sensitized mice fed 1% EW diet and administrated anti CD25 than other groups (Fig 2–10B). Additionally, the percentage of CD4⁺ Foxp3⁺ spleen lymphocytes in the sensitized mice fed 1% EW diet and administrated anti CD25 was significantly lower than that of the sensitized mice fed 1% EW diet and not administrated anti CD25 (Fig 2–10C). Therefore, it was suggested that the differentiation to T_{reg} was one of important factors to oral desensitization for OIT.

There were several limitations of this study. First, the pathogenic mechanism of food allergy in this model mouse was different to that of food allergic patients. Second, human’s diets have variations than mouse. Therefore, when supplementing allergen to the diet of allergy patients, I must consider the cooking characteristics of each allergens. Third, I cannot conclude the correlation between oral desensitization by OIT and plasma levels of allergen-specific antibodies because IgG and IgA may probably be interpreted as a result of antigen exposure.
Conclusion

Collectively, the results showed that intake of 1% EW diet for 4 weeks induced oral desensitization and immune tolerance in the EW allergic mouse model. Future studies will focus on examining the effects of diet supplemented with 1% low-reactive allergen, which is safer than raw allergen for OIT. Furthermore, I expect that the proposed OIT method can be adopted as a standard protocol to treat food allergies. Next, I examined the correlation between oral desensitization by OIT and plasma levels of allergen-specific antibodies.
References


Table 2–1. Composition of the experimental diets†

<table>
<thead>
<tr>
<th>Component (g/kg of diet)</th>
<th>20 % casein diet</th>
<th>0.01 % EW diet</th>
<th>0.1 % EW diet</th>
<th>1 % EW diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk casein</td>
<td>200</td>
<td>199.9</td>
<td>199</td>
<td>190</td>
</tr>
<tr>
<td>Egg white (EW)</td>
<td>0</td>
<td>0.1</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gelatinized cornstarch</td>
<td>453</td>
<td>453</td>
<td>453</td>
<td>453</td>
</tr>
<tr>
<td>Sucrose</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture (AIN-93G)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture (AIN-93)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

†Milk casein, gelatinized cornstarch, cellulose powder, AIN-93G mineral mixture, and AIN-93 vitamin mixture were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). DL-Methionine and corn oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sucrose was purchased from Mitsui Sugar Co., Ltd (Tokyo, Japan). EW was diluted 3 folds in water, mixed for 1 h, filtered through gauze, and freeze-dried. All diets were prepared in-house.
<table>
<thead>
<tr>
<th></th>
<th>Non-allergy</th>
<th>Allergy</th>
<th>P value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-OIT</td>
<td>0.01% EW</td>
<td>0.1% EW</td>
</tr>
<tr>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA-IgG1 (OD 415 nm)</td>
<td>0.000 ± 0.000 b</td>
<td>0.322 ± 0.017 a</td>
<td>0.277 ± 0.027 a</td>
</tr>
<tr>
<td>OVA-IgG2a (OD 492 nm)</td>
<td>0.001 ± 0.001 b</td>
<td>0.388 ± 0.058 a</td>
<td>0.459 ± 0.106 a</td>
</tr>
<tr>
<td>OM-IgG1 (OD 415 nm)</td>
<td>0.001 ± 0.001 c</td>
<td>0.295 ± 0.071 b</td>
<td>0.284 ± 0.026 b</td>
</tr>
<tr>
<td>OM-IgG2a (OD 492 nm)</td>
<td>0.013 ± 0.001 b</td>
<td>0.193 ± 0.073 ab</td>
<td>0.188 ± 0.041 ab</td>
</tr>
<tr>
<td>Endpoint</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVA-IgG1 (OD 415 nm)</td>
<td>0.000 ± 0.000 b</td>
<td>0.563 ± 0.031 a</td>
<td>0.578 ± 0.041 a</td>
</tr>
<tr>
<td>OVA-IgG2a (OD 492 nm)</td>
<td>0.005 ± 0.004 b</td>
<td>0.779 ± 0.054 a</td>
<td>1.152 ± 0.203 a</td>
</tr>
<tr>
<td>OM-IgG1 (OD 415 nm)</td>
<td>0.008 ± 0.004 b</td>
<td>0.676 ± 0.053 a</td>
<td>0.658 ± 0.051 a</td>
</tr>
<tr>
<td>OM-IgG2a (OD 492 nm)</td>
<td>0.007 ± 0.004 c</td>
<td>0.345 ± 0.086 a</td>
<td>0.302 ± 0.062 ab</td>
</tr>
</tbody>
</table>

† Values are presented as the means ± SE (n = 4–5). We only used the data with 2 points: 2 weeks and the study endpoint.

‡ Significant differences were determined by one-way ANOVA followed by Tukey’s multiple-comparison test and were indicated by different letters (a, b, and c). p < 0.05.
Table 2–3. The concentrations of interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-10 and IL-17 following in vitro stimulation of spleen lymphocytes with egg white (EW) and the percentage of CD4+ Foxp3+ cells in spleen lymphocytes †

<table>
<thead>
<tr>
<th>Allergy</th>
<th>Non-OIT</th>
<th>0.01% EW</th>
<th>0.1% EW</th>
<th>1% EW</th>
<th>$P$ value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ (pg/mL)</td>
<td>119 ± 53 b</td>
<td>184 ± 49 b</td>
<td>708 ± 97 a</td>
<td>860 ± 145 a</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>260 ± 62</td>
<td>245 ± 62</td>
<td>154 ± 10</td>
<td>154 ± 11</td>
<td>N.S.</td>
</tr>
<tr>
<td>IL-10 (ng/mL)</td>
<td>1.03 ± 0.12 bc</td>
<td>0.64 ± 0.12 c</td>
<td>1.61 ± 0.27 ab</td>
<td>2.05 ± 0.35 a</td>
<td>0.003</td>
</tr>
<tr>
<td>IL-17 (pg/mL)</td>
<td>31.8 ± 6.5</td>
<td>35.1 ± 6.7</td>
<td>17.9 ± 1.7</td>
<td>36.1 ± 4.8</td>
<td>N.S.</td>
</tr>
<tr>
<td>CD4+ Foxp3+ (%)</td>
<td>3.61 ± 0.15 b</td>
<td>3.20 ± 0.28 b</td>
<td>3.61 ± 0.12 b</td>
<td>4.61 ± 0.32 a</td>
<td>0.004</td>
</tr>
</tbody>
</table>

† Values are presented as means ± SE (n = 5).
‡ Significant differences were determined by one-way ANOVA followed by Tukey’s multiple-comparison test and were indicated by different letters (a, b, and c), $p < 0.05$. N.S., not significant.
Table 2–4. Nutritional effects in egg white (EW) allergic mouse model of oral immunotherapy (OIT) using diets supplemented with 0.01–1% EW. †

<table>
<thead>
<tr>
<th></th>
<th>Non-allergy</th>
<th>Non-OIT</th>
<th>0.01% EW</th>
<th>0.1% EW</th>
<th>1% EW</th>
<th>P value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight at day 1 (g)</td>
<td>23.6 ± 0.4</td>
<td>23.4 ± 0.4</td>
<td>23.5 ± 0.3</td>
<td>23.8 ± 0.3</td>
<td>23.7 ± 0.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Body weight at day 28 (g)</td>
<td>24.4 ± 0.4</td>
<td>23.9 ± 0.4</td>
<td>25.0 ± 0.4</td>
<td>25.1 ± 0.5</td>
<td>24.4 ± 0.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Body weight gain during OIT (g)</td>
<td>0.7 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>1.3 ± 0.5</td>
<td>0.7 ± 0.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Daily food intake (g)</td>
<td>2.7 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.8 ± 0.0</td>
<td>2.7 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

† Values displays means ± SE (N = 9–10).

‡ Significant differences were determined by one-way ANOVA followed by Tukey’s multiple-comparison test and were indicated by different letters, $p < 0.05$. N.S., not significant.
Fig 2–1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of raw EW and EW powder.

SDS-PAGE used the laemmli method (Laemmli UK. 1980). Protein separations used 5–20% gradient Poly-Acrylamide Gel (ATTO Corporation, Tokyo, Japan). Lane 1, 2 and 3 were applied on pre-stained molecular marker (SMOBIO), standard OVA (1.5 µg/lane), and standard OM (1.5 µg/lane), respectively. Raw EW put on lane 4 and 5 (1.5 and 3.75 µg/lane, respectively). The EW powder in the experiment put on lane 6 and 7 (1.5 and 3.75 µg/lane, respectively). EW samples were reduced by heating (95 ºC 2 min) with 4 x SDS-PAGE sample buffer containing 8% SDS, 20% 2-mercaptoethanol and 40% glycerol. The detection of protein in the gel used silver stain kit (Wako Pure Chemical Industries, Ltd. Osaka, Japan).
Fig 2-2. Mouse experimental protocol.

The detailed method were described in the section of Animals, Diets, and OIT Protocol.

Alum; aluminum hydroxide gel, EW; egg white, i.p.; intraperitoneal, OIT; oral immunotherapy.
Fig 2–3. Change in rectal temperature after oral challenge with single oral dose of 40 mg egg white (EW) at 3 time points {before OIT (A), 2 weeks (B), and 4 weeks (C)}. Values displays means ± SE (N = 9–10). Differences in the change in rectal temperature were determined by one-way ANOVA followed by Tukey’s multiple-comparison test, and were indicated by different lower-case letters, $p < 0.05$. 
Fig 2-4. Occurrence of diarrhea after oral challenge with single oral dose of 40 mg egg white (EW) at 3 time points {before OIT (A), 2 weeks (B), and 4 weeks (C)}.

Values displays the percentage of mice with diarrhea (N = 9-10).
Fig 2–5. Change in rectal temperature after systemic challenge (A; mean ± SE, n = 9–10) with single intraperitoneal dose of 200 μg EW, and vascular permeability (B; mean ± SE, n = 4–5).

Differences in the change in rectal temperature were determined by one-way ANOVA followed by Tukey’s multiple-comparison test, and were indicated by different lower-case letters, \( p < 0.05 \). Vascular permeability (50 μL plasma equivalent) was calculated as follows: EW, the mean of EW fluorescence value/plasma fluorescence value; Tyrode’s, the mean of Tyrode’s fluorescence value/plasma fluorescence value. Differences in vascular permeability were assessed using repeated two-way ANOVA followed by Bonferroni’s multiple comparison test. In the absence of significant interactions, different superscript letters (lower-case letters for points injected with Tyrode’s solution; upper-case letters for points injected with EW) indicate significant differences (\( p < 0.05 \)). Superscript asterisks indicate significant differences (\( p < 0.05 \)) between the Tyrode’s and EW points in the same group.
Fig 2-6. Plasma levels of ovalbumin (OVA) and ovomucoid (OM) specific IgE at 2 weeks and the study endpoint.

Data are presented as the mean ± SE (n = 5). The levels of OVA-specific IgE (A and B) were expressed in μg/mL, and those of OM-specific IgE (C and D) were expressed as the absorbance at 492 nm. Differences were determined by one-way ANOVA followed by Tukey’s multiple-comparison test, and were indicated by different lower-case letters, p < 0.05.
Fig 2–7. Plasma levels of ovalbumin (OVA) and ovomucoid (OM) specific IgA at 2 weeks and the study endpoint.

Data are presented as the mean ± SE (n = 5). The levels of OVA-specific IgA (A and B) were expressed in μg/mL, and those of OM-specific IgA (C and D) were expressed as the absorbance at 492 nm. Differences were determined by one-way ANOVA followed by Tukey’s multiple-comparison test, and were indicated by different lower-case letters, p < 0.05.
Fig 2–8. Effects of daily oral gavage of egg white (EW; 3 mg) for 4 weeks in an EW allergic mouse model.

The change in rectal temperature was measured 15 min after an oral challenge with a single dose of 40 mg EW at before (A) and after oral immunotherapy (OIT) for 4 weeks (B). Data are presented as the mean ± SE (n = 7 per group). Twenty-one female BALB/c mice were purchased from Clea Japan (Tokyo, Japan) at six-weeks old. Fourteen mice with EW allergy were prepared by the described method. Sensitized mice were divided into non-OIT and daily oral gavage groups (n = 7 per group), and non-sensitized mice were used as the non-allergy group. The mice in the daily oral gavage group were orally administrated 3 mg EW in 100 μL saline. The mice in the non-allergy and non-OIT group were orally administrated saline instead of EW solution. The oral gavage was carried for 4 weeks every day. Statistically significant difference was assessed using a one-way ANOVA followed by Tukey’s multiple-comparison test, and were indicated by different lower-case letters, p < 0.05.
Fig 2-9. Mouse experimental protocol (A) and grouping (B) in the suppression of oral desensitization for anti-mouse CD25 (PC 61).

The detailed method were described in the part of Discussion.

EW; egg white, i.p.; intraperitoneal, OIT; oral immunotherapy.
Fig 2–10. Change in rectal temperature (A) after oral challenge with single oral dose of 40 mg egg white (EW) after OIT for 2 weeks, and the percentage of CD4+ CD25+ cells (B) and CD4+ Foxp3+ cells (C) in spleen lymphocytes.

Data are presented as the mean ± SE (n = 7). Differences were determined by one-way ANOVA followed by Tukey’s multiple-comparison test, and were indicated by different lower-case letters, p < 0.05.
Chapter 3

Low-dose oral immunotherapy using low-egg allergen cookies for severe egg allergy children reduces allergic severity and affects allergen-specific antibodies in serum in a single-center study.

Introduction

In the previous study (Chapter 2), it was reported the dose-response desensitization of OIT utilizing diet supplemented with 0.01, 0.1 and 1% allergen in an EW allergic mouse model. Moreover, it was suggested that the level of allergen-specific IgA in the plasma may predict the therapeutic course of OIT. However, I cannot conclude the correlation between oral desensitization by OIT and plasma levels of allergen-specific antibodies because IgG and IgA may probably be interpreted as a result of antigen exposure.

Yanagida et al indicated that smaller amount allergen-specific immunotherapy such as epicutaneous immunotherapy, sublingual immunotherapy, low-dose OIT may be less effective than traditional OIT using larger dose although these smaller amount therapies may lead to fewer adverse reactions than traditional OIT and be more effective than strict elimination [1]. Patients with severe egg allergy cannot safely eat even 0.5 g of hard-boiled EW. Thus, egg OIT using larger dose carries a high risk for such patients. Few studies have focused specifically on patients with severe egg allergy, for whom safe OIT methods have not yet been developed. Takahashi reported on heated meringue prepared in a domestic pressure cooker, and developed the recipe of low-egg allergen cookies (LAC) using this meringue [2]. Following up on this work, I used the cookies for OIT prior to beginning the use of hard-boiled EW.

Therefore, I evaluated whether low-dose OIT using LAC against patients with severe egg allergy was safe and effective. Moreover, I examined the relationship between mild desensitization by low-dose OIT and serum biomarkers of allergy.
Materials and Methods

Ethics statement

The study protocol was approved by the Ethics Committees of Mukogawa Women’s University (Permitted Number: 11-52) and Osaka Habikino Medical Center (Permitted Number: 577, UMIN000008012). Written informed consent was obtained from the parents of all study participants.

Study participants

Participants included children with egg allergy who, by their treating doctor’s recommendation, could not receive OIT using hard-boiled EW (Fig 3–1). Participants were recruited at Osaka Habikino Medical Center. Detailed clinical history of all participants (age, sex, family and personal history of allergies, symptoms) were obtained from clinical records.

Preparation of LAC

Ingredients for LAC were a hen egg, water, sugar, potato starch, and baking powder, all of which can be purchased from a general supermarket. The heated meringue were prepared by Takahashi’s method [2]. Ingredients were mixed using a ratio of 1.0 g heated meringue, 0.2 g egg yolk, 1.64 g sugar, 29 mg baking powder, 0.3 mL soft water, and 4.0 g potato starch. The mixture, which then weighted 0.5–0.7 g, was rolled, and then baked at 160°C for 4.5 minutes in an oven (GMO-5910: Osaka Gas Co., Ltd, Osaka, Japan). After baking, the cookies kept in the oven for 1.5 minutes, and then left at room temperature for about 10 minutes. All LAC were made in our lab, and one LAC contained approximately 7.9–11 mg of egg white protein.

Open food challenge and OIT schedule

The detailed study design is illustrated in Fig 3–1. Prior to conducting OIT,
participants were screened with open hard-boiled EW challenge tests and blood samples were taken. In the challenge tests, participants were administered 0.1 g, 0.2 g, 0.5 g, 1.0 g, and 2.0 g of hard-boiled EW at 20-minute intervals, the results of which were determined by the doctor. Symptom scores of allergic reaction were assessed according to the modified Sampson’s anaphylaxis grades in the Japanese Pediatric Guideline for Food Allergy [3] (Table 3–1). The initial numbers of LAC were determined by the open food challenges with LAC. Participants were administered 1, 2, 4, and 10 pieces at LAC in 20-minute intervals, the results of which were determined by the doctor (Table 3–1). The low-dose OIT was performed for 4 months (Fig 3–1 and Table 3–2). The maximum numbers of LAC once a day were 10 pieces (containing 79–110 mg of egg white protein). After OIT, open hard-boiled EW challenge tests were carried out and blood samples retaken. Participants were then divided into a high effect (H-E) group and a no/low effect (N/L-E) group (Table 3–3). Those in the H-E group had either no allergic reaction to ingesting 2 g of hard-boiled EW, or had an increase to the final hard-boiled EW dose (compared to before the intervention) without worsening of allergic symptoms. The other participants were assigned to the N/L-E group.

**Measurement of allergic biomarkers in serum**

Levels of serum total IgE and egg yolk (EY)-, EW-, and OM-specific IgE were measured using the ImmunoCAP 100 instrument (Thermo-Fisher Scientific, Massachusetts, USA). The percentage of CD203c+ basophils in whole blood was measured using the Allergenicity kit (Beckman Coulter, Inc., California, USA). These biomarkers were analyzed at Osaka Habikino Medical Center. Moreover, serum OVA- and OM-specific, IgG4, IgA1, and IgA2 were measured by indirect enzyme-linked immunosorbent assay at Mukogawa Women’s University [4]. Coating antigens (500 μg/mL) were used: OVA (OVA grade VI, Sigma-Aldrich Co.LLC, Missouri, USA) and OM, which was purified from an egg white by the method of ethanol precipitation [2]. The following secondary antibodies were used: biotin-conjugated mouse monoclonal
anti-human IgG4 (Sigma-Aldrich Co., LLC, Missouri, USA) and horseradish peroxidase-conjugated mouse monoclonal B3506B4 anti-human IgA1 (Abcam, Cambridge, UK), mouse monoclonal A9604D2 anti-human IgA2 (Abcam, Cambridge, UK) and streptavidin (Abcam, Cambridge, UK). We used reference human pool serum as a standard (OVA-specific IgG4; 15.3 µgA/mL, OM-specific IgG4; 15.6 µgA/mL). The levels of OVA-and OM-specific IgA1 and IgA2 in serum were calculated against the reference human pool serum as 100%.

Statistical analysis

Statistically significant differences were assessed using a repeated two-way ANOVA followed by Bonferroni’s multiple comparison test. When two-way ANOVA indicated the presence of a group-time interaction, one-way ANOVA was conducted followed by Tukey’s multiple-comparison test. P values less than 0.05 were considered statistically significant. All analyses were conducted using GraphPad Prism version 5.0 (GraphPad Software, San Diego, USA).
Results

Reduction of allergic severity for low-dose OIT using LAC

In the center, 45 out of 116 patients cannot participate in OIT using hard-boiled EW. 13 out of 45 patients, which assessed for eligibility, were enrolled in the study. 2 patients were excluded because they showed an allergic reaction after eating 1–2 LAC (Table 3–3). 5 participants were able to eat 2 g of hard-boiled EW after the OIT without an allergic reaction (no symptoms) (Table 3–3). 2 participants increased to the final hard-boiled EW dose without worsening of symptom score after OIT (Table 3–3). These 7 participants in the H-E group improved to a level at which they could start OIT using hard-boiled EW. 4 participants did not show improvement in response to OIT (Table 3–3).

Influence of the allergic biomarkers for low-dose OIT using LAC

The serum levels of allergic biomarkers before OIT were not significantly different between the N/L-E and the H-E groups (Table 3–4). The EW- and OM-specific IgE levels in the H-E group did not differ before vs. after OIT (Table 3–4). In addition, the OVA- and OM-specific IgG4 concentrations in the H-E group were not significantly different before vs. after OIT (Table 3–4). However, the ratio of OM-specific IgG4/OM-specific IgE after OIT was significantly higher in the H-E group than in the N/L-E group after OIT, although the ratio of H-E group did not differ before or after OIT (Fig 3–2D). Serum levels of OVA-specific IgA2 in the H-E group were significantly higher after OIT compared to before (Table 3–4). Moreover, the ratio of OM-specific IgA2/OM-specific IgE in the H-E group was significantly higher after OIT compared to before (Fig 3–2F). The medians fold increase in the ratio of OVA-specific IgA2/EW-specific IgE in the H-E and N/L-E groups was 4.1-and 1.7-fold, respectively (Fig 3–2C).
**Discussion**

Yanagida et al reported that low-dose \(\frac{1}{100}–\frac{1}{32}\) of a whole egg (62–192 mg of egg protein) in scrambled form once a day OIT induced sustained unresponsiveness to \(\frac{1}{32}\) and \(\frac{1}{2}\) of a whole egg in scrambled form, with no severe symptoms [5]. Moreover, Brian et al reported that sustained unresponsiveness was achieved at high rates with both low-dose (300 mg of peanut flour once a day) and high-dose (3000 mg of peanut flour once a day) OIT in young peanut-allergic children [6]. However, in an allergic mouse model, the intake of a small amount of allergen did not induced oral desensitization [7]. In the present study, 7 out of 11 participants improved to a level at which they could begin OIT using hard-boiled EW. They could safely eat 0.5 g of hard-boiled EW after the OIT without an allergic reaction (no symptoms). Therefore, it is suggested that low-dose OIT can reduce the risk of allergic symptoms by accidental ingestion of food including egg, and improve the quality of life in allergic children and these parents. Moreover, no patients discontinued intake of LAC because of the LAC rejection, and there were no severe adverse reactions reported during OIT (data not shown). These results thus confirm low-dose OIT using LAC as an effective and safe treatment against severe egg allergy.

Several authors have reported on serum biomarkers to predict of clinical outcome [8, 9]. Allergen-specific IgG4 are the most frequently used serum biomarkers in studies of OIT because IgG4 could inhibit allergen IgE binding to B cells [10], and increased when oral desensitization was induced [5, 9]. Wright et al. reported that the ratio of serum OM-specific IgG4/OM-specific IgE was significantly higher in the group that achieved sustained unresponsiveness, whereas this was not the case for OVA [9]. These results are consistent with those in this report. IgA is well known for its role in mucosal immunity and is particularly important for antigen elimination in the gastrointestinal tract [11]. Kulis et al. reported that the concentration of peanut-IgA measured in saliva or serum was found to be higher in patients who achieved desensitization after 12 months of peanut sublingual immunotherapy [12]. Moreover, serum EW-specific IgA2
has been associated with the development of tolerance in egg allergy [13]. Pilette et al. reported that the IgA response to immunotherapy is selective for IgA2, is correlated with increased local TGF-β expression, and induces monocyte IL-10 expression [14]. In an allergic mouse model, IgE-mediated systemic anaphylaxis and mast cell degranulation induced by antigen ingestion are suppressed by antigen-specific IgA in serum, but not by IgA in the gut lumen [15]. In this study, the level of OVA-specific IgA2 and the ratios of OVA-specific IgA2/EW-specific IgE and OM-specific IgA2/OM-specific IgE were higher in the H-E group after OIT vs. before. However, Vazquez-Otriz et al. reported that serum allergen-specific IgA was not associated with natural or induced tolerance to egg in children with egg allergy [16]. Therefore, these results indicated the relationship between mild desensitization by low-dose OIT and production of allergen-specific IgG4 and IgA2 in serum.

There were several limitations of this study. The most important limitation is the absence of a placebo group, owing which the improvement of egg allergy in the study could not distinguish between the effect from OIT and natural tolerance. However, it is difficult to explain the improvement only due to natural tolerance for 3–4 months. In addition, the number of patients in H-E and N/L-E groups was not comparable, and the total number of subject was small. Moreover, the concentration of IgA1 and IgA2 did not display percentage against control pool serum of egg allergy patients.

**Conclusion**

The findings suggest that low-dose OIT using low-allergen food is an effective and safe treatment, and an adequate prior step to OIT using hard-boiled EW in patients with severe egg allergy. Moreover, it was suggested that small amount of antigen administration influences the immunologic function. In the future, I recommend that a randomized, double-blind, placebo-controlled trial of LAC be carried out in order to eliminate the effect of natural tolerance.
References


Table 3-1. The age and sex of all subjects, and the final dose and symptom scores in open hard-boiled egg white and low-egg allergen cookies (LAC) challenge tests before treatment.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age at the start (year, month)</th>
<th>Sex</th>
<th>Before treatment</th>
<th>Final dose that evoked symptoms (g)</th>
<th>Symptom scores † (according Ref 3)</th>
<th>Final pieces of LAC that evoked symptoms</th>
<th>Symptom scores † (according Ref 3)</th>
<th>The initial numbers of LAC once a day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5, 7/12</td>
<td>M</td>
<td></td>
<td>1.0</td>
<td>GS (2)</td>
<td>10</td>
<td>NS (2)</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>5, 11/12</td>
<td>M</td>
<td></td>
<td>1.0</td>
<td>GS (2)</td>
<td>10</td>
<td>No symptoms</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>6, 0/12</td>
<td>M</td>
<td></td>
<td>0.2</td>
<td>GS (2)</td>
<td>10</td>
<td>GS (3)</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>5, 10/12</td>
<td>M</td>
<td></td>
<td>0.2</td>
<td>RS (2)</td>
<td>5</td>
<td>RS (2)</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>3, 10/12</td>
<td>F</td>
<td></td>
<td>0.5</td>
<td>GS (3)</td>
<td>10</td>
<td>RS (2)</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>4, 8/12</td>
<td>F</td>
<td></td>
<td>1.0</td>
<td>GS (2), NS (2)</td>
<td>10</td>
<td>No symptoms</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>6, 11/12</td>
<td>M</td>
<td></td>
<td>1.0</td>
<td>GS (2)</td>
<td>10</td>
<td>No symptoms</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>8, 7/12</td>
<td>M</td>
<td></td>
<td>1.0</td>
<td>SS (1), RS (2), GS (2)</td>
<td>10</td>
<td>No symptoms</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>4, 1/12</td>
<td>M</td>
<td></td>
<td>0.1</td>
<td>GS (2)</td>
<td>5</td>
<td>GS (2)</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>8, 6/12</td>
<td>M</td>
<td></td>
<td>2.0</td>
<td>SS (2), GS (3)</td>
<td>5</td>
<td>RS (2)</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>5, 11/12</td>
<td>F</td>
<td></td>
<td>1.0</td>
<td>SS (2)</td>
<td>10</td>
<td>SS (1) RS (3) GS (2)</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>6, 0/12</td>
<td>M</td>
<td></td>
<td>0.5</td>
<td>GS (2)</td>
<td>1</td>
<td>SS (1) GS (2)</td>
<td>No treatment</td>
</tr>
<tr>
<td>13</td>
<td>4, 8/12</td>
<td>F</td>
<td></td>
<td>0.1</td>
<td>GS (2)</td>
<td>2</td>
<td>GS (2)</td>
<td>No treatment</td>
</tr>
</tbody>
</table>

† Symptom scores indicated reacted region (GS; Gastrointestinal tract Symptoms SS; Skin Symptoms, RS; Respiratory tract Symptoms, CS; Cardiovascular Symptoms, NS; Neurological Symptoms) and severity grades (1–5) according to the modified Sampson’s anaphylaxis grades in the Japanese Pediatric Guideline for Food Allergy [3] and these challenge results were determined by a doctor.
Table 3–2. The provided amounts of low-egg allergen cookies (LAC) during oral immunotherapy (OIT).

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Provided amounts / week for OIT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
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</tr>
<tr>
<td>2</td>
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<tr>
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<tr>
<td>10</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 3–3. The final dose and symptom scores in open hard-boiled egg white challenge tests before and after treatment.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>Grouping ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final dose that evoked symptoms (g)</td>
<td>Symptom scores † (according Ref 3)</td>
<td>Final dose that evoked symptom (g)</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>GS (2)</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>GS (2)</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>GS (2)</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>RS (2)</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>GS (3)</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>GS (2), NS (2)</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>GS (2)</td>
<td>2.0</td>
</tr>
<tr>
<td>8</td>
<td>1.0</td>
<td>SS (1), RS (2), GS (2)</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>0.1</td>
<td>GS (2)</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>2.0</td>
<td>SS (2), GS (3)</td>
<td>2.0</td>
</tr>
<tr>
<td>11</td>
<td>1.0</td>
<td>SS (2)</td>
<td>1.0</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>GS (2)</td>
<td>No treatment</td>
</tr>
<tr>
<td>13</td>
<td>0.1</td>
<td>GS (2)</td>
<td>No treatment</td>
</tr>
</tbody>
</table>

† Symptom scores indicated reacted region (GS; Gastrointestinal tract Symptoms SS; Skin Symptoms, RS; Respiratory tract Symptoms, CS; Cardiovascular Symptoms, NS; Neurological Symptoms) and severity grades (1–5) according to the modified Sampson’s anaphylaxis grades in the Japanese Pediatric Guideline for Food Allergy [10] and these challenge results were determined by a doctor.

‡ The high-effect (H-E) group could ingest 2 g of hard-boiled EW despite allergic reactions (no symptoms) or the increase final hard-boiled EW dose without the allergic symptoms worsening before OIT, and the no/low effect (N/L-E) group included the other subjects.
Table 3–4. The final dose of hard-boiled egg white in open food challenge and levels of allergic biomarker in serum among no/low-effect (N/L-E) and high-effect (H-E) group.†

<table>
<thead>
<tr>
<th></th>
<th>N/L-E group (N=4)</th>
<th>H-E group (N=7)</th>
<th>P value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Final dose that evoked</td>
<td>1.0 (0.3–1.8) b</td>
<td>1.0 (1.0–1.8) a</td>
<td>1.0 (2.0–1.0) b</td>
</tr>
<tr>
<td>symptom</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total IgE (IU/mL)</td>
<td>705 (401–1150)</td>
<td>1067 (605–2081)</td>
<td>846 (254–1609)</td>
</tr>
<tr>
<td>EY-IgE (U/mL)</td>
<td>13.3 (2.1–79.5)</td>
<td>16.4 (2.3–81.8)</td>
<td>10.2 (8.6–65.0)</td>
</tr>
<tr>
<td>EW-IgE (U/mL)</td>
<td>47.0 (15.7–91.4)</td>
<td>64.2 (14.9–96.9)</td>
<td>43.6 (26.9–100)</td>
</tr>
<tr>
<td>OVA-IgG4 (μgA/mL)</td>
<td>0.225 (0.160–1.92)</td>
<td>0.345 (0.180–2.23)</td>
<td>1.31 (0.21–1.57)</td>
</tr>
<tr>
<td>OVA-IgA1 (Ref %)</td>
<td>22.3 (8.3–51.0)</td>
<td>22.7 (6.7–83.1)</td>
<td>15.1 (5.2–59.1)</td>
</tr>
<tr>
<td>OVA-IgA2 (Ref %)</td>
<td>19.5 (6.0–31.1)</td>
<td>24.0 (9.3–38.1)</td>
<td>12.8 (7.8–22.8) a</td>
</tr>
<tr>
<td>OM-IgE (U/mL)</td>
<td>39.1 (14.0–69.7)</td>
<td>47.3 (12.5–69.1)</td>
<td>18.9 (12.1–87.1)</td>
</tr>
<tr>
<td>OM-IgG4 (μgA/mL)</td>
<td>0.265 (0.138–2.32)</td>
<td>0.300 (0.128–3.04)</td>
<td>1.04 (0.18–1.78)</td>
</tr>
<tr>
<td>OM-IgA1 (Ref %)</td>
<td>55.9 (7.4–134)</td>
<td>62.2 (10.6–293)</td>
<td>59.1 (23.0–315)</td>
</tr>
<tr>
<td>OM-IgA2 (Ref %)</td>
<td>14.4 (4.8–27.2)</td>
<td>17.9 (7.0–48.3)</td>
<td>13.5 (9.5–86.5)</td>
</tr>
<tr>
<td>CD203c+ (%)</td>
<td>67.9 (62.1–75.0)</td>
<td>33.6 (5.3–67.8)</td>
<td>34.9 (5.31–45.0)</td>
</tr>
</tbody>
</table>

† Values are displayed as the median and 25–75% percentile.
‡ Statistically significant differences were assessed using a repeated two-way ANOVA followed by Bonferroni’s multiple comparison test. When the interaction was significant, different superscript letters indicate significant differences (p < 0.05). When the interaction was not significant, different superscript letters (a b (before vs. after in N/L-E group) and A B (before vs. after in H-E group)) indicate significant differences (p < 0.05) and superscript asterisks (* (N/L-E group vs. H-E group at one time point) in the H-E group indicate significant differences (p < 0.05).
Fig 3-1. A flow chart of oral immunotherapy (OIT) using low-egg allergen cookies (LAC) with severe egg allergy.
Fig 3-2. Ratios of OVA-specific IgG4 (A), IgA1 (B), and IgA2 (C) to EW-specific IgE, and OM-specific IgG4 (D), IgA1 (E), and IgA2 (F) to OM-specific IgE in serum before and after treatment. Values indicate the raw data points and median. Statistically significant differences were assessed using a repeated two-way ANOVA followed by Bonferroni’s multiple comparison test. When two-way ANOVA indicated the presence of a group-time interaction, one-way ANOVA was conducted followed by Tukey’s multiple-comparison test among all columns, and superscript letters without a common letter indicate significant differences (p < 0.05). When the interaction was not significant, different superscript letters (a b [before vs. after in N/L-E group] and A B [before vs. after in H-E group]) indicate significant differences (p < 0.05) and superscript asterisks (* [N/L-E group vs. H-E group at one time point]) in the H-E group indicate significant differences (p < 0.05).
Discussion and conclusion

The therapeutic effect by allergen-specific immunotherapy are reported in a lot of studies [1]. However, there are not a unified method of OIT. Then, I propose a new OIT method that involves mixing a small amount of allergen powder in the diet. In the chapter 1 and 2, I examined whether a continuous intake of diet supplemented with allergen induced oral desensitization in an allergic mouse model. In the results of these experiments, feeding a diet containing 1% allergen for 4 weeks ameliorated the severity of allergy in an allergic mouse model. Moreover, I found if the amount of allergen ingested per a day is small and equivalent, the method of intake does not influence the amelioration of the severity of allergy by OIT. These results are useful to recommend not only mixing a small amount of allergen powder in the diet but also the division intake of OIT food. Furthermore, it was suggested that oral desensitization can be induced even if allergen and other nutrients are taken at the same time. Therefore, I expect that that it will be possible to instruct the division intake of allergen such as mixing the OIT food into meal or ingesting the OIT food after every meal.

In the chapter 2, it was showed the dose-response desensitization of OIT utilizing diet supplemented with 0.01, 0.1 and 1% allergen in an EW allergic mouse model. In previous mouse studies, there are problems that the therapeutic effects do not differ between low-dose and high-dose administrated groups [2] and that the OIT using peptides of allergen strongly induces oral desensitization [3,4]. However, I think it is difficult to replace these results with OIT for patients with food allergy because it was indicated that, in the OIT studies for patients with allergy, smaller amount allergen-specific immunotherapy may be less effective than traditional OIT using larger dose [5]. In the chapter 2, the mice fed diet supplemented with 1% of EW clearly ameliorated the severity of allergy than the mice fed diet supplemented with 0.1% of EW. Replacing the results with hen egg allergic children, the intake amounts of allergen in 0.1% EW diet (25–35 g protein×0.5/100 = 0.125–0.175 g protein/day) is consistent with the intake
amount in low-dose OIT (0.14 g protein/day) [6]. Moreover, the intake amount of allergen in 1% EW diet (25–35 g protein×5/100 = 1.25–1.75 g protein/day) are approximate equivalent to with the intake amount in common OIT [7]. Furthermore, the mice fed diet supplemented with 1% of commercial enzymatic hydrolyzed EW powder or spray-dried EW powder poorly ameliorated the severity of allergy than the mice fed diet supplemented with 1% of freeze-dried EW (not publish). Therefore, I consider that the result of OIT experiment using this allergic mouse model can extrapolate the therapeutic effect of human OIT.

In this thesis, I focused on T\textsubscript{reg} as a factor affecting desensitization. In the chapter 2, the concentration of IL-10 and the percentage of CD4\textsuperscript{+} Foxp3\textsuperscript{+} cell were significantly higher in the 1% EW group than in the non-OIT group. These results are consistent with previous OIT experiments using an allergic mouse model [2–4]. Furthermore, I clarified that the downregulation of Treg suppressed the amelioration of the severity of allergy by OIT. Therefore, it was suggested that the differentiation to T\textsubscript{reg} was one of important factors to oral desensitization by OIT.

The improvement of the severity of food allergy is diagnosed by oral food challenge. It is considered that the therapeutic effect by OIT can be estimated from the increase amount fed the OIT food during treatment. However, in the initialing period of OIT and the low-dose OIT, it is difficult to estimate the amelioration of the severity of allergy because the intake amount of OIT food is low than the threshold evoked symptom. Moreover, oral food challenge when oral desensitization is not shown has a high risk and is not meaningful. Therefore, it needs to develop the outcome index which can predict mild desensitization without oral food challenge. In the chapter 3, the serum allergen-specific IgG4/IgE and IgA2/IgE ratios in the H-E group increased after OIT than before despite not in the N/L-E group. In the double blind placebo studies of low-dose OIT using LAC, the increase of serum allergen-specific IgG4/ IgE ratios from before to after OIT showed moderate accuracy for the prediction of OIT outcomes (not publish). Moreover, there are a positive correlation between the change of serum
allergen-specific IgG4/ IgE and IgA2/IgE ratios although the increase of serum allergen-specific IgA2/IgE ratios from before to after OIT showed low accuracy for the prediction of OIT outcomes (not publish). From these result and a previous study [8], it is suggested that the serum allergen specific-IgG4/IgE and IgA2/IgE ratios have the potential to predict low-dose OIT outcomes without an oral food challenge even if with mild desensitization.

Ippoliti et al showed correlation between stress and poor response to antimicrobial vaccines and suggested that chronic psychological stress might influence the response to sublingual immunotherapy, which may account for some response failures [9]. Furthermore, Epstein et al reported that quality of life in children with food allergy when initiating OIT is significantly affected, not only by the severity of previous reactions, but also by their tolerated starting dose [10]. Some children with severe egg allergy feel nauseous even if intake of LAC [11]. Future, it is very important not only to establish a uniform OIT protocol and to improve the safety for overall treatment but also to consider psychological burden of children and their parents during OIT.

From the results of chapter 1 and 2, it is suggested that oral desensitization can be induced even if allergen and other nutrients are continually taken at the same time. Furthermore, I expect that the OIT method such as mixing the OIT food into meal or ingesting the OIT food after every meal can be adopted as a standard intake protocol to treat patients with food allergies. Moreover, in the result of chapter 3, I clarified that low-dose OIT using LAC is an effective and safe treatment in patients with severe egg allergy, and indicated that the change of biomarkers such as the serum allergen-specific IgG4/IgE and IgA2/IgE ratios might estimate low-dose OIT outcomes without an oral food challenge even if with mild desensitization. Finally, in the conclusion of this thesis, I consider that results of the experiments are useful to instruct a safer intake of OIT food and to reduce the frequency of oral food challenge, and can contribute the safer treatment of patients with food allergy by OIT.
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Acknowledgments

This study was part of the project “The development of new oral immunotherapy using allergen-compound diet” (principal investigator: Akihiro Maeta), which was supported by Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science. Moreover, this work was supported by JSPS KAKENHI Grant Number JP26350137.

I extend our sincere gratitude to the children who participated in the study and their guardians, as well as the members of the division of pediatrics in Osaka Habikino Medical Center. Finally, I am especially grateful to Professor Takahashi Kyoko and the students of Takahashi’s laboratory for supporting this work.
Achievements

Main-works


4. 松島麻鈴, 前田晃宏, 高橋享子. 0.1%卵白添加食餌による経口免疫療法は、強制経口投与による治療と同等の症状緩和を誘導した。日本栄養・食料学会誌 (2019年 第72巻 1号 掲載予定).


Sub-works


