Intake of Diet Including 1% Ovomucoid for 4 Weeks Induces Oral Desensitization in Ovomucoid-Specific Allergic Mouse Model

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Summary We propose a new oral immunotherapy (OIT) method that includes a small amount of a food allergen in the diet. However, it is not clear whether this method will induce oral desensitization and immune tolerance. Therefore, we investigated the therapeutic effectiveness using a 1% food allergen diet in an allergic mouse model. C3H/HeJ mice were sensitized to ovomucoid (OM) in alum four times at 12-d intervals. Sensitized mice were divided into two groups: the OIT group (19% casein diet with 1% OM) and the non-treated group (20% casein diet without OM). The non-sensitized mice served as the non-allergy group. The OIT treatment was performed for 4 wk. To assess desensitization and immune tolerance, we performed oral and intraperitoneal OM challenges, assessed vascular permeability of the dorsal skin, and measured allergic biomarkers. The OIT group exhibited significantly lower oral symptom scores and vascular permeability than the non-treated group, but the two groups did not differ in intraperitoneal allergy symptom scores. Furthermore, the OIT group had significantly higher OM-specific IgA levels in their plasma than the non-treated group. However, the plasma levels of OM-specific IgE, IgG1, and IgG2a were not significantly different between the OIT and the non-treated groups. These results suggest that the proposed OIT using an OM-supplemented diet may induce desensitization, but not immune tolerance, in an OM allergic mouse model.

Key Words oral immunotherapy, ovomucoid, food allergy, diet

Globally, 240–550 million people may suffer from food allergies (1). Recently, the prevalence of food allergies has increased by 18% among children under 18 y of age (2, 3). Dietary avoidance of specific allergens is recommended as the first-line approach in the management of food allergy (4). In Japan, egg allergy is the most prevalent food hypersensitivity among the pediatric population, exceeding cow’s milk allergy (4). A major cause of egg allergy is ovomucoid (OM), which constitutes approximately 11% of egg white protein (5, 6). Oral immunotherapy (OIT) is a new treatment for food allergies (4, 7). This treatment involves inducing desensitization through continuous food-allergen intake. However, according to the Japanese pediatric guidelines for food-allergy treatment, OIT is not often recommended because of the risk of inducing the allergic symptoms during the treatment (4). Moreover, current data are inadequate to establish proper OIT protocols, such as allergen dose and timing, need for escalation, therapy duration, and routes of administration.

There are some reports of the OIT protocol in an allergic mouse model (8–12). We 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 (13). An egg white diet, which uses egg whites instead of total protein for 2 wk, results in a decrease in the anti-ovalbumin (OVA) IgE level and protection against systemic anaphylaxis in an OVA-allergic mouse model (13). 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-allergic mouse model (13, 14). Here, we determine that a 1% concentration of allergen in diet was not enough to induce anaphylaxis reaction, and clarify that the allergen-containing diet can induce oral desensitization and immune tolerance.

Therefore, we sensitized C3H/HeJ mice with OM, and evaluated the immuno-therapeutic effects of the 1% OM compound diet for 4 wk in these mice.

EXPERIMENTAL

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 and splenocyte culture supernatant were stored at −40°C.

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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, Ltd. (Osaka, Japan). Sucrose was purchased from Mitsui Sugar Co., Ltd. (Tokyo, Japan). Corn oil was purchased from Olitalia S.r.l. (Forlì, Italy). OM was purified from hen egg whites following a previously established method (15). Table 1 shows the compositions of the control and 1% OM diet. We prepared all diets in our laboratory.

Twenty C3H/HeJ female mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) at 6 wk old. All mice were housed individually in plastic cages. Figure 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) at 12-d 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 wk. 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 wk after OIT. Anatomy was performed within 1 wk after the systemic challenge. One final oral challenge at the endpoint was carried out before anatomy. Mice were killed under isoflurane anesthesia to obtain plasma, spleen, and dorsal skin samples.

**Food challenge in allergy mice.** 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 (16, 17). 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: (higher test fluorescence value−higher control fluorescence value)/(plasma fluorescence value).

**Measurement of plasma IgE, IgA, IgG1, and IgG2a levels.** We measured plasma immunoglobulin (Ig) levels by enzyme-linked immunosorbent assay (ELISA). Capture

| Table 1. Compositions of experimental diets. |
|---------------------------------------------|
|                | 20% casein diet | 1% OM diet |
| Milk casein    | 200             | 190        |
| L-Methionine   | 2               | 2          |
| Gelatinized cornstarch | 453          | 453        |
| Sucrose        | 200             | 200        |
| Corn oil       | 50              | 50         |
| Cellulose      | 50              | 50         |
| Mineral mixture (AIN-93G) | 35          | 35         |
| Vitamin mixture (AIN-93) | 10          | 10         |
| OM             | 0               | 10         |

All dietary combinations were prepared in our laboratory. OM, ovomucoid.
antibodies (goat anti mouse IgE) and horseradish peroxidase (HRP) conjugated detection antibodies (goat anti mouse IgG-HRP, IgG1-HRP, IgG2a-HRP, and IgA-HRP) were purchased from Bethyl Lab, Inc. (Montgomery, TX). OM-specific IgE was determined by a modified capture ELISA (18). The coating antibody used 2.0 μg/mL of goat anti-mouse IgE. 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. OM-specific IgA, IgG1, and IgG2a were determined by indirect ELISA following the method of Maeta et al. (8) and Birmingham et al. (19). The coating concentrations of OM were 500 μg/mL (IgA) and 10 μg/mL (IgG1 and IgG2a). The dilution ratio of the plasma in OM-specific IgA was 1:100 and the HRP reaction was carried out for 20 min at room temperature. The reference curves of OM-specific IgG1 and IgG2a were drawn by mouse OM-specific monoclonal IgG antibody and goat anti-mouse IgG-HRP.

**Mouse spleen cell cultures and cytokine secretions.** We measured cytokine secretion in spleen cell cultures following methods modified from Maeta et al. (8). Cell cultures were stimulated in 5.0 μg/mL concanavalin A incubated at 37°C under 5% CO2. Supernatants of cultures were collected after 72 h. The concentrations of interleukin-4 (IL-4) and interferon-γ (IFN-γ) were determined using ELISA, according to the manufacturer’s instructions (BioLegend, San Diego, CA).

**Statistical analysis.** Values are presented as means±SE. 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 (20). Statistical significances of vascular permeability and IgE and IgA concentration were deter-

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**Fig. 2.** Body weight change (A) and daily food intake (B) in the non-allergy group (20% casein diet), the non-treated group (20% casein diet), and the OIT group (1% OM diet) during treatment. Data are presented as values and SE (n=5–6). 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. 3.** Symptom scores of oral challenge (A), vascular permeability of dorsal skin (B), and symptom scores of systemic challenge (C). Significant differences in symptom scores (A, C) were calculated via a Mann-Whitney U test and the Ryan method (20). Significant difference in vascular permeability of dorsal skin (B) was determined by one-way ANOVA followed by Tukey’s multiple-comparison test. Significant differences are indicated by different letter (a, b), p<0.05.
1% Ovomucoid Diet Induces Desensitization in Ovomucoid Allergic Mouse Model

RESULTS
Nutritional effect of 1% OM diet
We 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. 2A). However, the mice in the OIT group did not lose weight during the treatment (Fig. 2A). Daily food intake was not significantly different between the non-treated and OIT groups (Fig. 2B).

Effect of 1% OM diet on OM-allergic mouse model
We 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. 3A and supplemental Fig. 1). 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. 3A). Moreover, the vascular permeability of the OIT group was significantly lower than that of the non-treated group (Fig. 3B). In response to the systemic challenge (day 30), the OIT group exhibited lower allergic symptom scores than did the non-treated group (Fig. 3C), although they did experience mild symptoms (scores ranged from 1–3 (Fig. 3C). These results suggest that the OIT method using the 1% OM diet induced desensitization but not immune tolerance in the OM-allergic mouse model.

Influence of the allergic biomarker in OM-allergic mice fed with a 1% OM diet
Type 1 and type 2 helper T cell (T<sub>H</sub>1/T<sub>H</sub>2) imbalance causes allergic response. T<sub>H</sub>2 induces a B-cell class-
switch of IgE and IgG1. We did not observe significant differences in OM-specific IgE (Fig. 4A). IgG1 (Table 2), or IgG2a (Table 2) plasma concentrations between the non-treated group and the OIT group at day 15 or at the endpoint. The OM-specific IgG2a/IgG1 ratio was not significantly different between the OIT and the non-treated groups (Table 2). In addition, the IFN-γ/IL-4 ratio, which reflects the Th1/Th2 balance, did not differ between the OIT and the non-treated groups (data not shown).

The presence of IgA plays a major role in allergen tolerance during human infancy (21). The plasma concentrations of OM-specific IgA in the OIT group (at day 15 and at the endpoint) were higher than those in the non-treated group (Fig. 4B). Therefore, we suggest that the OM compound diet induced the production of OM-specific IgA in the OM-allergic mouse model.

## DISCUSSION

We proposed a new OIT methodology mixing a small amount of allergen powder in the entire diet. The merits of this method are the division of allergen dose, masking the taste and smell of the allergen. However, it is not clear that diets mixed with small amounts of allergens can actually induce oral desensitization and immune tolerance. In the present study, we evaluated the effects of a 1% OM diet on an OM-allergic mouse model.

In our 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 (13, 14). However, our results do not support this assumption. OM is a well-known trypsin inhibitor (5, 6). Oga 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 (22). 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 (23, 24). Thus, it was suggested that the low body weight gain in the OIT group caused the trypsin inhibition of OM.

We challenged this assumption with our new OIT method using a diet that included small amounts of the allergen as a powder. Our findings suggest that most mice in the OIT group achieved oral desensitization after 4 wk 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 reports from Leonard et al. (9). Overall, based on our results and previous reports, the use of an allergen compound in the diet as OIT was able to induce desensitization but not immune tolerance in an allergy mouse model.

Recently, a relationship between the oral desensitization and the serum levels of allergen-specific IgA was reported (9, 25–27). In these experiments, OM-specific IgA was 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 (28). Previous studies on humans and mice have also shown that the specific-IgA serum concentration in the OIT-treated group was higher than in the placebo group (9, 25–28). In contrast, Vazquez-Oritz et al. reported that serum allergen-specific IgA was not associated with natural or induced tolerance to egg in egg-allergic children (29). These results and reports suggest that serum allergen-specific IgA has the potential to be used as a biomarker of OIT effectiveness.

Currently, no standard has been set for the target amount of allergen that should be used in OIT. The feeding amount of OM was 1.25 mg/h/mouse (30 mg/24 h/mouse). Previous OIT research used one orally administered dose of 25 mg/mouse of egg white (9), 5.0 mg/mouse of egg white protein hydrolyte (11), and 5.0 mg/mouse of heated ovomucoid-depleted egg white (12). The feeding amount of allergen in the experimental diet was 5% of the protein intake per day. Recommended dietary allowance of protein in 3- to 7-y-old Japanese children is 25–35 g/d. Therefore, the allergen intake should be 0.4–0.6 g/diet (25–35 g×0.05/3 meal times). Thus, it was suggested that the OIT method using an allergen-containing diet could apply to human allergy patients. However, the minimum dose to induce oral desensitization was not clear in this experiment. In the future, the minimum amount of allergen needed in the diet to induce oral desensitization will be examined.

In conclusion, data from our mouse model study suggest that the OIT method using an allergen-containing diet is effective for oral desensitization. We expect that this OIT method can address many of the disadvantages of conventional OIT protocols, such as the risk of anaphylaxis and the stress in patients due to allergen intake. Furthermore, the OIT method of using an allergen-containing diet has the potential to be the standard method for treating food allergies.

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Supplemental Fig. 1. The nasal symptoms after oral OM 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.