Food allergy is currently a major health and lifestyle hazard for many Japanese. Most allergens are proteins, and those that are ingested in food are absorbed from the intestine into the body to a degree, retaining their intact form (1–3). In most cases, these induce oral tolerance, which is a state of local and systemic unresponsiveness induced by oral administration of innocuous antigens such as food proteins and commensal bacteria (4). On the other hand, oral tolerance is sometimes broken, and allergic reactions are induced as a result (5). The mechanism of oral tolerance has not yet been fully defined.

Recently, a lot of attention has been focused on oral immunotherapy for pediatric patients, where a small amount of antigen is administered initially by mouth, and the amount of allergen is then gradually increased to induce immune tolerance (6). It is therefore important to clarify the mechanisms that regulate tolerance and the development of allergy, and to find an effective approach for induction of oral tolerance.

There have been many reports of studies designed to investigate oral tolerance using various allergens and administration schedules in experimental animals (7, 8), but few of them have attempted development of an oral immunotherapy model. The B10.A mouse strain has shown that sensitization can be induced by intragastric administration of lysozyme instead of oral tolerance; however, anaphylactic shock induced by subsequent intragastric administration of lysozyme is suppressed. This mouse model would be useful for assessing the method of oral immunotherapy.

Key Words food allergy, oral immunotherapy, egg white lysozyme, anaphylactic reaction

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin class E; PBST, phosphate buffered saline containing 0.05% Tween 20.
either allergy or tolerance, and the development of suitable therapy for food allergy.

**Experimental Conditions**

**Animals and diets.** Female B10.A mice (5 wk old, weighing 20–23 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were housed in a room with a 12-h light-dark cycle, and the temperature was maintained at 24˚C. All mice were fed a commercial diet (CE-2; Oriental Yeast Co., Ltd., Tokyo, Japan). The experimental design was in accordance with the guidelines for animal experimentation, and was approved by the Animal Experiment Committee of Nagoya University of Arts and Sciences.

**Animal treatment.** Mice were administered 0, 0.2, 2, 20 or 100 mg LY intragastrically (i.g.) once a day for 6 d. At day 26 and day 33, mice were administered 50 µg LY with 1 mg alum in 200 µL of phosphate-buffered saline (PBS) via the intraperitoneal (i.p.) route. During the experimental period, samples of blood were collected and their antibody content was measured. Finally, the mice were administered 100 mg LY orally on day 43 to induce anaphylactic shock, and their rectal temperature was measured.

**Preparation of blood samples and measurement of LY-specific IgG1 and IgE levels.** Blood was collected from the mice via the tail vein, and the plasma was recovered by centrifugation at 3,000 × g for 10 min. Each sample was then treated with sodium azide to a final concentration of 0.05%. All samples were stored at −20˚C until further assays.

Lysozyme-specific IgG1 and IgE were measured by ELISA. ELISA plates (Nunc) were incubated with 10 µg/mL LY at 4˚C overnight, and then blocked with 1% BSA/PBS at 37˚C for 1 h. Each plasma sample was diluted 1 : 100 for the analyses of effects during intragastrical (i.g.) administration (day 0–19), and 1 : 1,000 for the analyses of effects by intraperitoneal (i.p.) administration (day 19 and day 43) in 1% BSA/PBST. Then, a 100 µL plasma sample diluted was applied to each well of LY-coated plates and incubated at 37˚C for 1 h. After the wells had been washed, they were incubated with 100 µL 1 : 5,000-diluted horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 (Bethyl) or IgE (Lifespan) in 1% BSA/PBST at 37˚C for 1 h, respectively. Each well was washed and incubated with 100 µL of substrate buffer (0.05 M citrate/0.1 M disodium hydrogen phosphate, pH 5.0) containing 0.04% o-phenylenediamine and 0.003% H2O2 for about 20 min, and then 25 µL of 2 N sulfuric acid was added. The absorption at 492 nm was measured using a Microplate Reader MTP-650FA (Corona Electric Hitachinaka, Japan).

**Assessment of hypersensitivity reactions.** To obtain a more objective measure of anaphylaxis severity, rectal temperatures were measured 0–120 min after challenge using a digital thermometer (TD-320, Shibaura Electronics Corp., Tokyo, Japan).

**Statistical analysis.** The results are expressed as the mean±SD. All statistical analyses were performed using SPSS ver. 20.0 statistical software (SPSS Inc., Chicago, IL). Statistical analyses was tested by using Student’s t-test or one-way ANOVA, followed by Tukey’s multiple comparison tests or Dunnett’s test. Statistical differences were considered significant at p<0.05.

**Results and Discussion**

To clarify the mechanisms underlying tolerance and induction of allergy and to find an effective way of inducing oral tolerance, we used B10.A mice. B10.A mice were administered 0.2, 2, 20 and 100 mg of LY intragastrically once a day for 6 d (i.g. administration). Sera were collected from the mice on days 0, 6, 9, 12, 16 and 19. LY-specific IgG1 and IgE in each serum sample were measured by ELISA. Values are means±SD of 5 animals. Differences were determined by one-way ANOVA followed by Dunnett’s test. **p<0.05 and *p<0.01 versus day 0.
ELISA (Fig. 1). LY-specific IgG1 and IgE were increased only after i.g. administration without adjuvant in the experimental groups, and the effects were highest in the 20 mg group. On the other hand, the group that received 100 mg showed lower antibody levels than the 20 mg group.

In addition to the i.g. route, mice were also administered LY intraperitoneally (i.p. administration) with alum. Their sera were then collected, and serum IgG1 and IgE to LY were measured by ELISA. White and black bars represent before (day 19) and after (day 43) i.p. administration, respectively. Values are means±SD of 5 animals. Differences were determined by paired t-test between before i.p. and after i.p. in each group and one-way ANOVA followed by Tukey’s multiple comparison tests among the groups: **p<0.05, *p<0.01.

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In addition to the i.g. route, mice were also administered LY intraperitoneally (i.p. administration) with alum. Their sera were then collected, and serum IgG1 and IgE to LY were measured by ELISA (Fig. 2). In all groups, serum LY-specific antibody levels were increased to various degrees in comparison with those before i.p. administration. We found that LY-specific IgG1 and IgE in serum of B10.A mice were increased only after i.g. administration without adjuvant (Fig. 1). Moreover, regardless of the amount administered orally, all groups showed an increase of LY-specific antibody following administration of LY by the i.p. route (Fig. 2). Oral tolerance can be defined as inhibition of specific immune responsiveness to subsequent parenteral injection of protein to which an individual or animal has been exposed previously via the oral route. Thus, priming is induced in B10.A mice instead of oral tolerance, as this is a high-responder strain.

After oral and intraperitoneal treatment, LY was administered orally to mice to induce anaphylactic shock. The rectal temperature of mice was decreased at 30 min and 60 min after the oral challenge with
LY, and then showed a mild recovery (Fig. 3A). Therefore, the rectal temperature at 30 min after induction of shock was compared among the groups (Fig. 3B). In comparison with before the induction of shock, almost all mice showed a reduction of rectal temperature after the shock induction. When the difference in rectal temperature measured before and after shock induction was compared among the groups (Fig. 3C), the experimental groups showed a smaller decrease in rectal temperature than the control group, and the decrease in the 20 mg group was smallest. We found that anaphylactic shock induced by the oral route after induction of allergy was suppressed in all experimental groups, and that the effect increased dose-dependently up to administration of 20 mg (Fig. 3). On the other hand, it was interesting that anaphylactic shock was induced in the group that received 100 mg of LY, which was the highest dose used in this study (Fig. 3). It appears to be important for a certain amount of allergen to be ingested and for allergen-specific antibody responses to be induced, in order to inhibit the anaphylactic shock caused by allergen reuptake via the oral route.

As has been reported clinically, oral immunotherapy was provided the patients based on the tests of specific IgE and oral food challenge (10). It started from a small amount of allergen which evoked no symptom and the dose of allergen then gradually increased to induce immune tolerance. The amount of allergen at the start and end of therapy varies greatly between individual patients. In the present study, therefore, this mouse model assumes this situation, which means that the mice were administrated allergen orally and intraperitoneally to induce allergy, then examined by measuring the subsequent specific-antibody responses and antigen-induced anaphylactic reactions to estimate the effects of oral and multiple pre-administration of allergen.

Furthermore, this mouse model might make it possible to improve the method of oral immunotherapy by examining the dose and frequency of oral administration of allergen after induction of allergy. Many patients take the allergenic food raw or just heating as if it were medicine in oral immunotherapy. However, in most cases, foods are cooked and processed with other foods and undergo significant changes in such qualities as digestibility, absorbability and allergenicity. Some studies of the intestinal absorption of food protein have shown that the concentration of protein in the blood increases in a dose-dependent manner and that co-administration with other food components such as protein or corn oil markedly affects the intestinal absorption of allergen (11, 12). Furthermore, allergenicity is altered by denaturation and modification (13–15), such as that occurring through processing or cooking. Thus, additional evaluation of the form of allergen and co-administered components that could alter immune responses will be required. This mouse model would be practical and useful for assessing the allergenic potential of proteins in processed food relative to that in unprocessed food, as a pilot study preceding clinical trials for patients.

It is anticipated that the present findings will provide useful information for understanding the mechanisms underlying the induction of allergy or tolerance, and for devising suitable therapies for allergy.

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REFERENCES