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Effects of oral hyposensitization with recombinant Der f2 on immediate airway constriction in a murine allergic model

M. Yasue*, T. Yokota*, M. Yuasa*, Y. Kajiwara*, M. Suko**, H. Okudaira**

Effects of oral hyposensitization with recombinant Der f2 on immediate airway constriction in a murine allergic model. M. Yasue, T. Yokota, M. Yuasa, Y. Kajiwara, M. Suko, H. Okudaira. ©ERS Journals Ltd 1998.

ABSTRACT: Recombinant Der f2 (rDer f2) has recently been developed as a promising allergen for the diagnosis and immunotherapy of house-dust mite allergy, and studies in immunology. The aim of the present study was to evaluate whether oral administration of rDer f2 could suppress an immediate allergic reaction in mice sensitized with mite allergen. We developed a murine allergic model that showed bronchoconstriction after inhalation of rDer f2, and studied the effect of oral administration of rDer f2 on the reaction.

Seven week old male A/J mice were intranasally immunized with rDer f2 12 times. Sensitized mice showed anti-rDer f2 immunoglobulin (Ig)E production and immediate airway constriction after inhalation of 10 mg·mL^{-1} of rDer f2, as determined by the Konzett-Rössler method. Immunized animals were divided into three groups, and fed phosphate-buffered saline (PBS), 0.1 mg·day^{-1}, or 1 mg·day^{-1} of rDer f2 for 4 weeks, respectively. Seven days after the last feeding, the mice were examined for their immediate response.

Animals fed with 1 mg·day^{-1} rDer f2 showed significantly reduced bronchoconstriction after inhalation of both 2 mg·mL^{-1} and 10 mg·mL^{-1} of rDer f2 compared with PBS-fed mice. Similar results were obtained when we examined mice 10 weeks after the last feeding. Reactions in the 0.1 mg·day^{-1} rDer f2-fed group also tended to decrease in comparison with PBS-fed animals. Plasma anti-rDer f2 IgE, IgG1, IgG2a, and IgG2b levels were not changed by feeding with rDer f2.

We conclude that recombinant Der f2 exhibits both sensitizing and hyposensitizing activities in mice. rDer f2 may be useful in immunotherapy and diagnosis of house-dust mite allergy.

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*Bioscience Research and Development Laboratory, Asahi Breweries Ltd., Ibaraki, Japan. **Dept of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo, Japan.

Corresponding: M. Yasue
Bioscience Research and Development Laboratory
Asahi Breweries Ltd
1-21, Midori 1-Chome
Moriya-machi
Kitasoma-gun
Ibaraki 302-0106
Japan
Fax: 00 81 297 461505

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To date, several clinically important allergens such as Der 1 and Der 2 have been identified in crude mite extract [1–4]. Among others, the structural gene of Der f2, one of the major mite allergens found in *Dermatophagoides farinae* was cloned, and the product (recombinant Der f2 (rDer f2)) shown to exhibit allergic activity in humans comparable with native Der f2 prepared from crude mite extract [5, 6]. Since Der f2 has been shown to sensitize over 80% of patients with house-dust mite allergy, rDer f2 should prove useful in the diagnosis and immunotherapy of allergic asthma and rhinitis caused by house-dust mites. It should also provide a basis for research into allergies and immunology.

In the present study, with special reference to the therapeutic application of rDer f2, we established a murine allergic model by intranasal or intraperitoneal injection of rDer f2 to A/J mice, which are highly responsive to Der f2 [7]. We examined the effect of oral-hyposensitization therapy with rDer f2 against the immediate bronchoconstriction in the intranasally sensitized animals because their reaction after rDer f2 inhalation was more intense than that of intraperitoneally immunized mice.

Materials and methods

Antigen

rDer f2, clone 1 [5], was kindly supplied by the Nikka Whisky Distilling Co., Ltd. (Chiba, Japan), and dissolved in phosphate-buffered saline (PBS).

Animals

Male A/J mice and Sprague-Dawley (SD) rats were purchased from Japan SLC, Inc (Shizuoka, Japan). They were housed under controlled conditions with 12 h alternating lights/dark cycles. Water and commercial food were allowed *ad libitum*. They were kept undergoing no treatment for at least 1 week after arrival at our laboratory.

Immunization and blood collection

Mice were immunized with rDer f2 by either intranasal (*i.n.*) or intraperitoneal (*i.p.*) injection of the antigen. In the *i.n.* immunization, mice were sensitized once a week

for 12 weeks from the age of 7 weeks. The animals were lightly anaesthetized with ethyl ether and a PE-10 polyethylene tube was inserted into their left nasal cavity. Twenty five microlitres of a 4 mg·mL^{-1} rDer f2 solution was slowly injected into the cavity from a 50 μL microsyringe through the tube. No adjuvant was used. For the *i.p.* immunization, 14 week old animals were sensitized by the injection of 10 μg of rDer f2 adsorbed in 4 mg of aluminum hydroxide three times at intervals of 2 weeks. Both *i.n.* and *i.p.* sensitized mice were 18 weeks old when they were last immunized.

Determination of airway constriction

Antigen provocation tests were carried out by the Konzett-Rössler method. This is one of the most widely used techniques for determining airway constriction provoked by sensitizing antigen or chemical mediators in immunological and pharmacological studies using small experimental animals [8–12]. This method measures "ventilation-overflow (VO)", which is the volume of air which cannot enter into an animal's lung when the respirator inflates the lung (fig. 1). It has been known that VO accurately reflects the severity of airway constriction [8, 12]. Mice were anaesthetized with pentobarbital sodium (50 mg·kg^{-1}, *i.p.*) and immobilized with pancuronium bromide (0.5 mg·kg^{-1}, *i.v.*). Tracheal cannulation was performed with an 18 gauge needle connected to an animal respirator. The lung was inflated by a fixed volume of air under 5 cmH_2O pressure at a rate of 60 breaths·min^{-1}, and VO was continuously measured by a pneumotachograph. The ventilation volume (7–10 mL·kg^{-1}) was adjusted so that VO was about 3 mL·min^{-1}.

When VO was stabilized, the antigen challenge was started. An aerosol mist of either 2 or 10 mg·mL^{-1} rDer f2 solution was generated by an ultrasonic nebulizer and inhaled by mice for 8 min. VO was recorded for at least 15 min from the initiation of antigen inhalation. Since reduction of airway reactivity had occurred when the measuring time exceeded 15–20 min, mice were only challenged with either 2 or 10 mg·mL^{-1} of rDer f2 and bronchoconstriction was determined within 15 min. VO values were averaged every 2 min from the initiation of antigen inhalation, and each average in 2 min periods was used for the comparison of experimental groups.

Determination of plasma antibody levels in hyposensitization experiments

Heparinized blood was collected from the retro-orbital plexus of individual mice. Plasma (about 20 μL·animal^{-1}) was separated from the whole blood and frozen until the determination of antibody titre. Anti-rDer f2 immunoglobulin (Ig)E levels were determined by mouse-rat heterogeneous 72 h passive cutaneous anaphylaxis (PCA) using SD rats at 8 weeks of age as recipients, according to methods described elsewhere [13]. Total IgE concentration was determined by an enzyme-linked immunosorbent assay (ELISA) using a rat anti-mouse IgE monoclonal antibody (MCA419; Serotec Ltd., Oxford, UK) as a capturing antibody and a biotinylated antibody (MCA420B; Serotec Ltd., Oxford, UK) as a detecting antibody. Anti-rDer f2 IgG1, IgG2a, and IgG2b were determined by ELISAs using rDer f2 as a capturing antigen and the following detecting antibodies: biotinylated rat monoclonal antibodies, antimouse IgG1 (#PM-05002D; Pharmingen, San Diego, CA, USA), anti-mouse IgG2a(#PM05022D; Pharmingen), and anti-mouse IgG2b(#PM-05152D; Pharmingen).

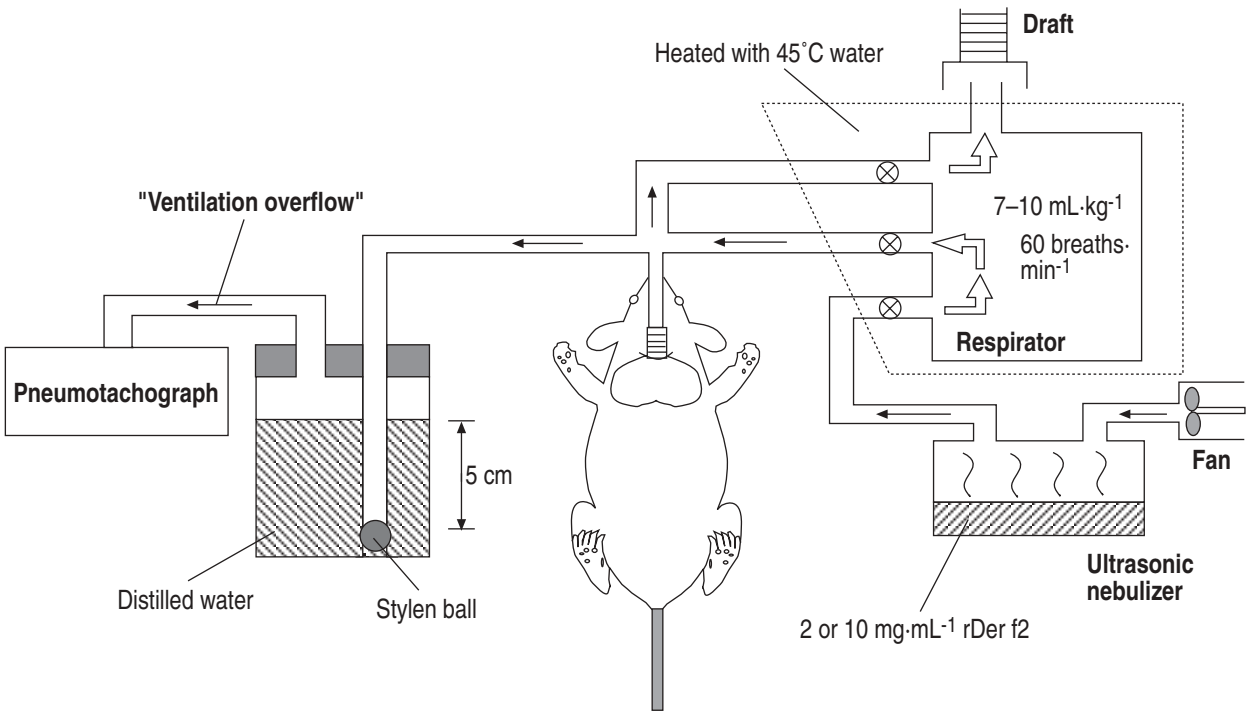

Fig. 1. – Scheme of the Konzett-Rössler method. Recombinant (rDer f2) rDer f2 was inhaled with an ultrasonic nebulizer to induce immediate bronchoconstriction.

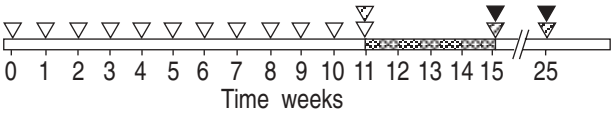

Fig. 2. – Schedule of the oral-hyposensitization experiment. Seven week old A/J mice were immunized with *i.n.* recombinant rDer f2 (rDer f2) 12 times and fed phosphate-buffered saline (PBS) or rDer f2 for 4 weeks, before evoking an antigen induced immediate airway response. ▽ : immunization: 100 mg *i.n.* rDer f2; ▽ : measurement of plasma immunoglobulin (Ig)E and IgG levels; ▼ : determination of airway constriction; ▭ : oral administration of PBS or rDer f2 solution.

Experimental groups for evaluation of oral hyposensitization

Mice sensitized with rDer f2 i.n. were used for the oral-hyposensitization study (fig. 2). On the last day of immunization (12th immunization), animals were divided into three groups: 0.1 mg rDer f2; 1 mg rDer f2; and PBS (control) groups. Several animals with or without immunization were also used for reaction-negative controls in the experiments.

Oral administration of rDer f2 and examination of allergic status

On the day of the last immunization, oral treatment was started. The 0.1 mg·day^{-1} rDer f2 group was fed 0.25 mL of 0.4 mg·mL^{-1} rDer f2 solution, the 1 mg·day^{-1} rDer f2 group was fed 4 mg·mL^{-1} rDer f2 solution and the PBS group was fed PBS alone. These treatments were performed on five sequential days a week for 4 weeks using a syringe fitted with an 18-gauge stainless sound.

Mice were bled on the first and last day of feeding to determine plasma antibody levels. Antigen provocation tests were carried out by the Konzett-Rössler method either 7 days or 10 weeks after the last oral treatments (fig. 2).

Statistical analysis

All the values were expressed as mean±SEM. Antibody levels were log-transformed before analysis. Student's paired t-test was used to compare the difference in the measurements of airway reactions and plasma antibodies between the two groups. Changes in antibody levels before and after the oral treatments were also assessed using the paired t-test. A p-value less than 0.05 was considered significant.

Results

Anti-rDer f2 IgE titre and bronchoconstriction in rDer f2-sensitized mice

Plasma samples were obtained from *i.p.* and *i.n.* sensitized mice 2 weeks after the last immunization. Plasma from five mice were pooled for each immunization group. Anti-rDer f2 IgE titre as determined by PCA was 640 for *i.p.*, and 80 for *i.n.* immunized animals. Sequential *i.n.* administration of rDer f2 without any adjuvant elicited IgE production although the antibody titre was not high. Mice were then examined for the immediate airway constriction provoked by inhalation of 10 mg·mL^{-1} of rDer f2. Naive mice showed no or very weak reactions, and an increase in VO reached 0.21±0.06 mL·min^{-1} at 10 min. In *i.p.* sensitized mice, VO began to increase 2–4 min after the start of the inhalation of 10 mg·mL^{-1} antigen, and an increase in VO was significantly higher than that of naive mice over 14 min (fig. 3). In spite of having lower plasma IgE levels than *i.p.* sensitized mice, *i.n.* sensitized mice began to react within 2 min of the start of challenge, and the increase in VO was significantly higher than that of unaffected mice over the 14 min periods (fig. 3). Although no statistical significance was obtained, an increase in VO in *i.n.* sensitized mice was apparently higher than that of *i.p.* sensitized animals. All the *i.n.* sensitized mice showed maximal reaction within 8–12 min after the initiation of antigen challenge. From the results in naive mice, we regarded the reaction as positive when the increase in VO exceeded 0.25 mL·min^{-1}. No positive reaction was observed in *i.n.* sensitized mice challenged by PBS or 10 mg·mL^{-1} of egg albumin (data not shown), showing that these reactions were antigen-specific.

Evaluation of oral-hyposensitization to the asthmatic reaction

We performed a hyposensitization study in the *i.n.* sensitized mice, because a more intense reaction was induced in these than *i.p.* sensitized animals. Mice at 7 weeks of ages were newly immunized for this study, the sensitized animals used in the comparison of *i.p.* and *i.n.* immunizations were excluded. Animals were fed 0, 0.1, or 1 mg·day^{-1} of rDer f2 for 4 weeks, and had their bronchoconstriction evaluated upon antigen provocation 7 days after the last oral treatment. As shown in figures 4 and 5, maximal increase in VO in the group of mice fed 1 mg·day^{-1} rDer f2 was significantly lower than that of PBS-fed animals after inhalation of both 2 and 10 mg·mL^{-1} of rDer f2. In the provocation by 2 mg·mL^{-1} of rDer f2,

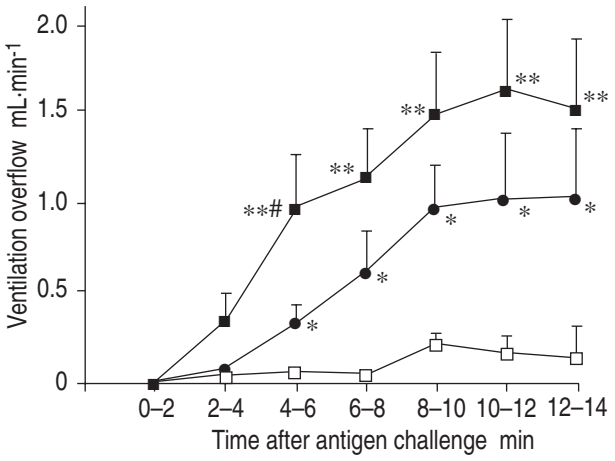

Fig. 3. – Time course of bronchoconstriction after inhalation of 10 mg·mL^{-1} recombinant Der f2. ■: *i.n.* immunized, n=5; ●: *i.p.* immunized, n=5; □: naive, n=5. Values are represented as mean±SEM. *, **: $p<0.05$, $p<0.01$ compared with naive mice. #: $p<0.05$ compared with *i.p.* immunized mice.

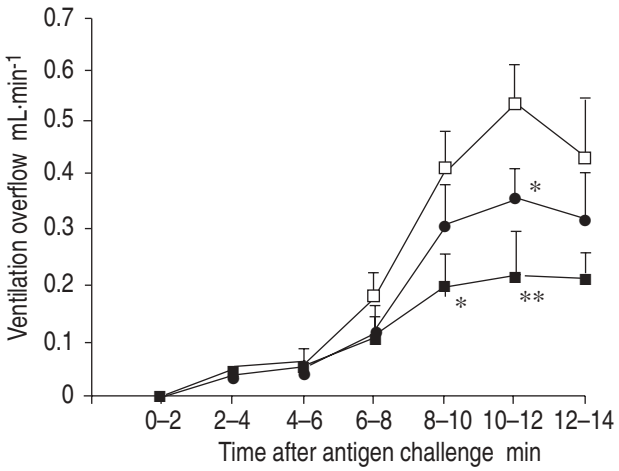

Fig. 4. – Time course of bronchoconstriction after inhalation of 2 mg·mL^{-1} of recombinant Der f2 (rDer f2). ❑: phosphate-buffered saline (PBS)-fed group, n=10; ●: 0.1 mg·day^{-1} rDer f2-fed group, n=9; ■: 1 mg·day^{-1} rDer f2-fed group, n=9. Mice were examined 7 days after the last feeding. Values are presented as mean±SEM. *, **: $p<0.05$, $p<0.01$ compared with the PBS-fed mice.

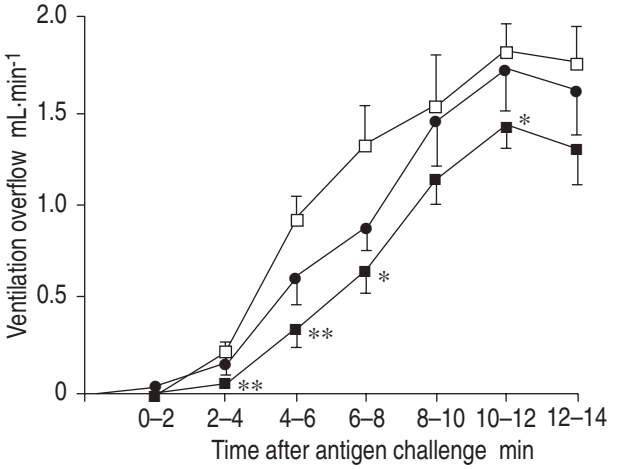

Fig. 5. – Time course of bronchoconstriction after inhalation of 10 mg·mL^{-1} of recombinant Der f2 (rDer f2). ❑: phosphate-buffered saline (PBS)-fed group, n=14; ●: 0.1 mg·day^{-1}-fed group, n=11; ■: 1 mg·day^{-1} rDer f2-fed group, n=12. Mice were examined 7 days after the last feeding. Values are presented as mean±SEM. *, **: $p<0.05$, $p<0.01$ compared with the PBS-fed mice.

although nine out of 10 PBS-fed mice showed positive reactions, only one 1 mg·day^{-1} rDer f2-fed mouse did. Thus, it was assumed that the concentration of rDer f2 at which airway constriction was provoked was elevated by oral administration of rDer f2. The maximal VO increase after inhalation of 2 mg·mL^{-1} of rDer f2 in the 0.1 mg·day^{-1} rDer f2-fed group was also significantly lower than that of PBS-fed animals, and only four out of nine 0.1 mg·day^{-1} animals reacted positively to a challenge with 2 mg·mL^{-1} of antigen. With regards to the reaction provoked by 10 mg·mL^{-1} of rDer f2, no significant difference to the PBS-fed group was observed. Suppression of the immediate reaction in sensitized mice seemed to be dependent on the dose of rDer f2. To determine the continuance of the hyposensitization, other sensitized animals fed PBS or 1 mg·day^{-1} of rDer f2 for 4 weeks were examined 10 weeks after the last feeding. Results were similar to those on day 7, showing that suppression of airway constrictive response after oral administration of rDer f2 was long-lasting (fig. 6).

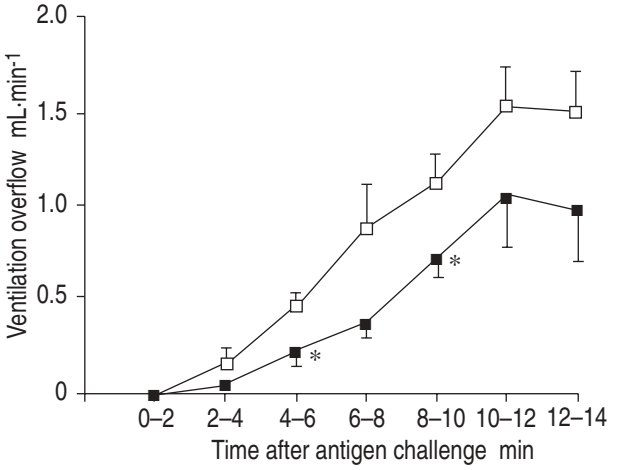

Fig. 6. – Time course of bronchoconstriction after inhalation of 10 mg·mL^{-1} of recombinant Der f2 (rDer f2). ❑: phosphate-buffered saline (PBS)-fed group, n=8; ■: 1 mg·day^{-1} rDer f2-fed group, n=8. Mice were examined 10 weeks after the last feeding. Values are presented as mean±SEM. *: $p<0.05$ compared with the PBS-fed mice.

Figure 7 shows the total IgE concentration and anti-rDer f2 IgG1, IgG2a, and IgG2b levels before and after the feeding. IgE and anti-rDer f2 IgG subclass antibodies were not detected in plasma from naive mice (data not shown). No significant difference was observed in antibody levels either among the groups or between, before and after the administration in each group. The plasma anti-rDer f2 PCA titre was also unaffected by antigen treatments, remaining at 80 for the entire experimental period in all groups. Relationships between the hyposensitization to allergic reaction and changes in plasma antibody levels could not be confirmed in this study.

Side-effects induced by rDer f2 administration

No anaphylactic response was observed in mice given rDer f2 orally over the experimental period in hyposensitization studies.

Discussion

Crude mite extract prepared from Dermatophagoides mites has been used widely in the diagnosis and immunotherapy of, and in many *in vitro* studies on, house-dust mite allergy [17–19]. However, since mite extracts contain many allergens at various concentrations, it is difficult to obtain biochemically and immunologically standardized mite extract. Antigenic proteins and substances that can activate the complement or platelet activating factor [1–3, 20, 21], and that are not necessary to the immunotherapy or diagnosis, may cause additional inflammation or allergic reactions after injection of crude mite extract. Thus, purified mite allergens are more suitable than crude mite extract. To date, genes for several important allergens have been cloned, and several recombinant allergens have been shown to possess allergic activities comparable with native allergens [22]. Recombinant allergens were used to sensitize animals in order to examine the activity eliciting the production of antigen-specific IgE antibodies [23, 24]. In humans, immunotherapy by T-cell-reactive peptides

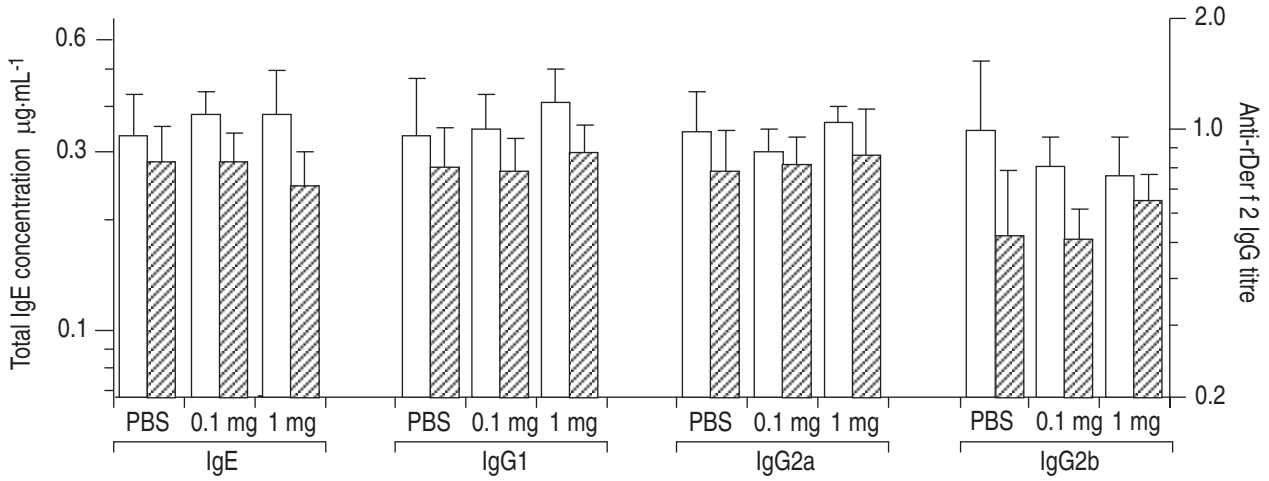

Fig. 7. – Total plasma immunoglobulin (Ig)E concentration, anti-recombinant (Der f2) IgG1, IgG2a, and IgG2b levels. ☐ : before; ▨ : after oral feeding. PBS: phosphate-buffered saline; 0.1 mg: 0.1 mg rDer f2; 1 mg: 1 mg rDer f2. No significant difference was observed in antibody levels either among or between group before and after the administration.

derived from a cat allergen, Fel d I [25], and diagnosis of hypersensitivity using recombinant allergens, Phl p 1 in timothy grass pollen and Bet v 1 in birch pollen, have been reported [26, 27]. Among the major house-dust mite allergens, genes for Der f 1, 2, 3 found in *D. farinae*, and Der p 1, 2, 3, 5 in *Dermatophagoides pteronyssinus* have been cloned [28–31]. In particular, the recombinant protein of Der f2 has been shown to possesses an IgE-binding and allergic activity comparable with the native Der f2 protein by radioallergosorbent test (RAST) of allergic sera and skin-prick test of mite-allergic patients [5].

In the present study, with a special reference to the therapeutic application of rDer f2, we established a murine allergic model and examined the effect of oral-hyposensitization therapy. Immunization and antigen challenge experiments clearly showed that *i.n.* administration of rDer f2 caused specific IgE production and airway responsiveness, and inhalation of rDer f2 could provoke immediate airway constriction, showing that rDer f2 possesses a high immunological activity in mice. However, since plasma anti-rDer f2 PCA titre of *i.n.* sensitized mice was much lower than that of *i.p.* sensitized mice, more intense sensitization will be necessary to develop better murine models for allergic asthma of the human, in which patients usually have very high serum IgE titre to sensitizing allergen. The hyposensitization study clearly showed that oral treatment of sensitized mice with 0.1–1 mg·day^{-1} of rDer f2 for 4 weeks significantly suppressed the allergic bronchoconstriction without any visible side-effects. The suppressive effect seemed to be dose-dependent. We estimated the provocative concentration of mice fed 1 mg·day^{-1} to be at least several fold higher than that of control animals, although more detailed studies are necessary to confirm this. To obtain better results by administration of 0.1 mg·day^{-1} of the antigen, oral treatment should be longer than 4 weeks. The suppression in airway response was kept over 10 weeks after termination of the oral administration of rDer f2, suggesting that the effect of hyposensitization was long-lasting. The data obtained in the present study give rise to optimism that oral immunotherapy with rDer f2 in house-dust mite allergy might be of benefit.

Studies in humans and experimental models have shown that oral hyposensitization therapy can suppress allergic reactions ranging from asthma and rhinitis to auto-immune diseases [32–36]. In mice, antigen-specific IgE production and cellular immunity-mediated diseases such as autoimmune encephalomyelitis and diabetes were suppressed by oral administration of responsible antigen after onset [37–39]. As mechanisms for the oral hyposensitization, energy or deletion of responsible cells [40–42], and active suppression, that is, generation of cells which secrete suppressive cytokines have been proposed [43, 44]. In clinical studies, relationships between hyposensitization to atopic disease and Thl/Th2 bias are highlighted. Th2 type cytokines, interleukin (IL)-4, 5, 6, and 13, which are considered to play a crucial role in type I allergy, were downregulated [45–48], and Th1 cytokines, interferon (IFN)-γ and IL-12, were sometimes upregulated after successful immunotherapy with subcutaneous, oral, or *i.n.* injection of allergen [49]. Although immediate reactions are known to be triggered by the release of mediators from mast cells or basophils, and developed by contraction of smooth muscle cells and mucus secretion by epithelium, the influence of immunotherapy on the function of these cells in airway tissues are not clear.

Since immunotherapy often reduces the reactivity of the skin and the release of histamine from peripheral basophils, the release of mediators from mast cells in the airway might be also suppressed [50, 51]. This hypothesis was supported by the observations that the concentration of mast cell-derived tryptase in the nasal lavage fluid after allergen provocation was decreased by successful immunotherapy [52]. In addition, increases in the concentration of histamine or methacholine causing a positive reaction after immunotherapy suggested the restoration of airway barrier and reduction of smooth muscle reactivity [53, 54]. Difficulties arise in studying the function of cells in the airway tissues of mice, owing to their small size. Thus, in further studies of the mechanisms of hyposensitization, immunological changes in the airway cells after immunotherapy should be examined in rats or guinea-pigs.

In conclusion, the present study clearly shows that oral administration of recombinant Der f2 to sensitized mice

reduces the contractile response to the provoking antigen without side-effects, and helps pave the way for clinical trials.

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