

Hyposensitization to allergic reaction in rDer f 2-sensitized mice by the intranasal administration of a mutant of rDer f 2, C8/119S

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SUMMARY

C8/119S is a mutant of recombinant Der f 2 (rDer f 2), and lacks a disulphide bond possessed by wild-type rDer f 2. In humans and mice, C8/119S has a very weak IgE-binding capacity compared with the wild-type, but possesses a T cell reactivity comparable to that of the wild-type. C8/119S may thus be a safe immunotherapeutic agent for house dust mite allergy. The aim of the present study was to evaluate whether the intranasal administration of C8/119S could suppress an immediate allergic reaction in mice sensitized with wild-type rDer f 2, possessing an allergic activity comparable to native counterparts purified from mite extract. Seven-week-old male A/J mice were immunized with wild-type rDer f 2 four times, and then intranasally administered 0.2–2 µg of wild-type, 0.2–20 µg of C8/119S, or PBS alone, three times a week for 4 weeks. Seven days after the last administration, the mice were examined for an immediate allergic reaction. The animals administered 2 µg of C8/119S (C2.0 group) showed significantly reduced immediate bronchoconstriction provoked by the i.v. injection of 1 and 10 µg of wild-type rDer f 2, compared with the PBS-treated mice. Similar results were obtained when we examined mice 10 weeks after the last administration. The reactions in the other groups given wild-type or C8/119S also tended to decrease in severity in comparison with the animals of the PBS group. The allergic phenotypes of the T cells, B cells, and basophils in the C2.0 group were shifted to that of naive mice without immunization. We conclude that C8/119S has hyposensitizing activities in mice sensitized with wild-type rDer f 2. C8/119S may be useful for immunotherapy of house dust mite allergy.

Keywords mite recombinant Der f 2 mutant hyposensitization mouse

INTRODUCTION

Specific immunotherapy for the treatment of patients with respiratory allergy has been conducted for more than 80 years [1], and the use of extracts of several pollens, house dust mites, bee venoms and other substances has been demonstrated to be effective [2–4]. Standardizations of such extracts have been carried out to obtain maximal efficacy and to eliminate side-effects, including systemic anaphylaxis in immunotherapy [5–7]. Many clinically important allergens have been purified from the extracts [8–10], and genes for such allergens have been cloned with a view to their use in immunotherapy [11]. To date, derivatives of several purified and recombinant allergens, including chemically modified allergens [12,13], synthetic peptides [14,15], and mutants of wild-type recombinant allergens [16,17], have also been developed. Since these derivatives often possess weaker IgE-binding capacity than

the original allergens, they will not trigger a chemical mediator release from basophils and mast cells by the cross-linking of IgE–IgE receptor complex on these cells [18], and may be safe immunotherapeutic agents for treating allergic diseases. Recently, clinical trials of allergen-specific immunotherapy using synthetic peptides of short length containing T cell epitopes of a major cat allergen, Fel d 1, have been reported [19,20].

Concerning house dust mite allergens, genes for the allergens Der f 1, 2, 3 found in *Dermatophagoides farinae*, and Der p 1, 2, 3, 5 in *Dermatophagoides pteronyssinus* have been cloned [21–23]. Among others, the product of Der f 2 gene (recombinant Der f 2 (rDer f 2)) has been shown to exhibit allergic activity in humans comparable with native Der f 2 prepared from crude mite extract [24,25]. We previously reported the development of murine allergic models by using rDer f 2 as the sensitizing antigen and hyposensitization to the allergic reaction in rDer f 2-sensitized mice by oral administration of rDer f 2 [26,27]. Takai *et al.* recently reported the construction of a mutant of rDer f 2, C8/119S, which retained complete activity to stimulate T cell proliferation and

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markedly reduced binding capacity to Der f 2-specific IgE in humans [28]. In the present study, to evaluate the suitability of C8/119S as an immunotherapeutic agent for house dust mite allergy, we investigated the effect of the intranasal (i.n.) administration of C8/119S on the allergic reaction in A/J mice immunized with wild-type rDer f 2. For comparison, we also used wild-type rDer f 2 for administering intranasally into sensitized mice.

MATERIALS AND METHODS

Antigen

Wild-type rDer f 2 was kindly supplied by Nikka Whisky Distilling Co., Ltd (Chiba, Japan). C8/119S was prepared in our laboratory as described elsewhere [28]. Two cysteine residues at the 8th and 119th positions in the wild-type were converted to serine residues in C8/119S, so that the number of disulphide bonds was reduced from three to two in C8/119S [28]. Wild-type and mutant rDer f 2 were dissolved in PBS before use.

Animals

Male A/J mice and Sprague-Dawley (SD) rats were purchased from Japan SLC (Shizuoka, Japan). The animals were maintained under controlled conditions with 12 h alternating light/dark cycles. Water and commercial food were available *ad libitum*. They were kept without treatment for at least 1 week after arrival at our laboratory.

Immunization

Seven-week-old A/J mice were immunized with wild-type rDer f 2 by the intraperitoneal (i.p.) injection of 10 µg of wild-type adsorbed in 4 mg of aluminium hydroxide four times at intervals of 2 weeks.

Experimental groups

On the day of the last (4th) immunization, the mice were divided into six groups: '2 µg wild-type rDer f 2' (referred to as W2.0), '0.2 µg wild-type rDer f 2' (W0.2), '20 µg C8/119S' (C20), '2 µg C8/119S' (C2.0), '0.2 µg C8/119S' (C0.2), and PBS (control). Each group consisted of 7–12 mice. Several animals which were not immunized ('naive mice') were also used as reaction-negative controls in the experiments.

Intranasal administration of wild-type rDer f 2 and C8/119S

On the day of the last immunization, the i.n. treatment was started and performed three times a week for 4 weeks. The mice were lightly anaesthetized with ethyl ether, and a PE-10 polyethylene tube was inserted into the left nasal cavity. Twenty microlitres of antigen solution or PBS were slowly injected into the cavity from a 50-µl microsyringe through the tube. The mice in the W2.0, W0.2, C20, C2.0, and C0.2 groups were given 2 µg of wild-type, 0.2 µg of wild-type, 20 µg of C8/119S, 2 µg of C8/119S, and 0.2 µg of C8/119S per administration, respectively. The control mice were treated with PBS alone. The naive mice were given no treatment.

Determination of plasma antibody levels in hyposensitization experiments

Mice were bled on the first and last days of the i.n. administration for the determination of plasma antibody levels. Heparinized blood was collected from the retroorbital plexus of individual mice. Plasma (about 20 µl/animal) was separated from the whole blood and frozen until the determination of the antibody titre. Total IgE

concentration was determined by ELISA using a rat anti-mouse IgE MoAb (MCA419; Serotec Ltd, Oxford, UK) as a capturing antibody and a biotinylated antibody (MCA420B; Serotec) as a detecting antibody. The levels of anti-wild-type IgG1 and IgG2a were determined by ELISA using wild-type rDer f 2 as a capturing antigen and the following detecting antibodies: biotinylated rat MoAbs, anti-mouse IgG1 (no. PM-05002D; Pharmingen, San Diego, CA) and anti-mouse IgG2a (no. PM-05022D; Pharmingen).

In addition to the measurement of antibody in each plasma sample by ELISA, plasma was pooled for each group to determine anti-wild-type rDer f 2 IgE levels by mouse–rat heterogeneous 72-h passive cutaneous anaphylaxis (PCA) using SD rats at 8 weeks of age as recipients [29]. Plasma was serially diluted (1:80–1:2560), and the dilutions were intracutaneously injected into the back of recipient SD rats. After 72 h, Evans Blue and 0.1 µg of provoking antigen were intravenously injected into the rats to provoke a PCA reaction. After 30 min, the maximum dilution at which a blue spot larger than 5 mm in diameter was induced was determined as the PCA titre.

Determination of airway constriction

Antigen provocation tests were carried out by the Konzett–Rössler method either 7 days or 10 weeks after the last i.n. administration. The immediate airway response was provoked by the i.v. injection of wild-type rDer f 2. The severity of airway constriction was expressed by ventilation overflow (VO) defined by the Konzett–Rössler method [30,31].

Mice were anaesthetized with pentobarbital sodium (50 mg/kg, i.p.) and immobilized with pancuronium bromide (0.5 mg/kg, i.v.). Tracheal cannulation was performed with an 18 G needle connected to an animal respirator. The lung was inflated with a fixed volume of air under 5 cm H₂O pressure at a rate of 60 breaths/min, and the VO was continuously measured by a pneumotachograph. The ventilation volume (7–10 ml/kg) was adjusted so that the VO was about 3 ml/min. When the VO was stabilized, the antigen challenge was started: 0.05 ml of each wild-type rDer f 2 solution, containing 1 µg and 10 µg of the antigen, was sequentially injected into the femoral vein at an interval of 6 min. The VO was recorded for at least 12 min from the administration of the 1 µg wild-type solution. The ΔVO, i.e. the difference between the maximal VO observed after challenge with 1 µg or 10 µg of the antigen and the base VO before the injection of 1 µg of rDer f 2, was used as an index of the degree of immediate airway constriction.

Determination of histamine concentration in the whole blood

Immediately after the measurement of bronchoconstriction provoked by the i.v. injection of 10 µg of wild-type rDer f 2, heparinized whole blood was obtained from the inferior vein of each mouse. The histamine content in the plasma was determined by a fluorescent assay using o-phthaldialdehyde.

Flow cytometric analyses of peripheral blood leucocytes

In the mice of the C0.2, C2.0 and PBS groups, which were not examined for immediate reaction, leucocyte phenotypes were determined. Heparinized blood was collected from the inferior vein of each mouse 7 days after the last i.n. administration.

Peripheral blood leucocytes were separated from whole blood by dextran sedimentation for the analysis of CD23 expression on B cells, CD11b (Mac-1), and the IgE molecules on basophils. The leucocytes were washed with PBS and then treated with FITC-conjugated rat anti-mouse B220 (no. 32554A;

Pharmingen) + biotinylated rat anti-mouse CD23 (no. 32554A; Pharmingen), or FITC-conjugated rat anti-mouse CD11b (no. 32554A; Pharmingen) + biotinylated rat anti-mouse IgE (no. 32554A; Pharmingen). Biotinylated antibodies were labelled with Cychrome-conjugated avidin (no. 32554A; Pharmingen). The flow cytometric analyses were performed with the use of FACSscan (Becton Dickinson, San Jose, CA).

Determination of IL-6 and interferon-gamma production from spleen cells

Spleen cells of mice which were subjected to the flow cytometric analyses of peripheral blood leucocytes were examined for cytokine production. Spleen cells were obtained immediately after bleeding, and incubated in serum-free medium, AIM-V (Gibco, Grand Island, NY) in the presence or absence of 10 µg/ml of wild-type rDer f 2 for 72 h. IL-6 and interferon-gamma (IFN-γ) contents of the culture supernatants were determined by ELISA using the following capturing and detecting antibodies: rat monoclonal anti-mouse IL-6 (no. PM-18071D; Pharmingen) and biotinylated rat monoclonal anti-mouse IL-6 (no. PM-18082D; Pharmingen); rat monoclonal anti-mouse IFN-γ (no. PM-18181D; Pharmingen) and biotinylated rat monoclonal anti-mouse IFN-γ (no. PM-18112D; Pharmingen). The detecting antibodies were labelled by avidin-conjugated β-galactosidase (no. A-2300; Vector Labs, Burlingame, CA) for fluorescence development. Standard curves for cytokines were made using recombinant mouse IL-6 (no. PM-19251V; Pharmingen), and IFN-γ (no. PM-19301U; Pharmingen).

Statistical analysis

All of the data obtained are expressed as means with s.e.m. The cell surface molecule and antibody level data were log-transformed before analysis. Student's paired *t*-test was used to compare differences in the measurements of airway reactions, leucocyte analyses, and plasma antibodies between pairs of groups. Changes in antibody levels before and after the oral treatments were also assessed with the paired *t*-test. *P* < 0.05 was considered significant.

RESULTS

Comparison of allergic activity of wild-type rDer f 2 and C8/119S in mice

Before examining the hyposensitizing activity of C8/119S, we compared the allergic activity of C8/119S with that of wild-type rDer f 2 in mice. In humans, the binding activity of C8/119S to Der f 2-specific IgE was 1/100–1/10 of that of wild-type rDer f 2 or native Der f 2, as determined by a skin-prick test and a basophil histamine release assay. Two rats intracutaneously sensitized with serial dilutions of hyperimmune plasma (PCA titre = 640, obtained from sensitized A/J mice) were challenged by either wild-type or mutant rDer f 2. Blue spots appeared at the site of sensitization, as shown in Fig. 1a. The PCA reaction induced by C8/119S was much weaker than that by wild-type rDer f 2. The IgE binding capacity of C8/119S was estimated to be 1/30–1/10 of wild-type.

We then determined the T cell reactivity of wild-type and mutant rDer f 2 using spleen cells that were obtained from mice immunized four times with wild-type rDer f 2. After the spleen cells were cultured in the presence or absence of 10 µg/ml of either wild-type or mutant rDer f 2 for 72 h, the concentrations of IL-6 and IFN-γ in the supernatant of the cell culture were measured by ELISA (Fig. 1b). The cytokine production by sensitized spleen

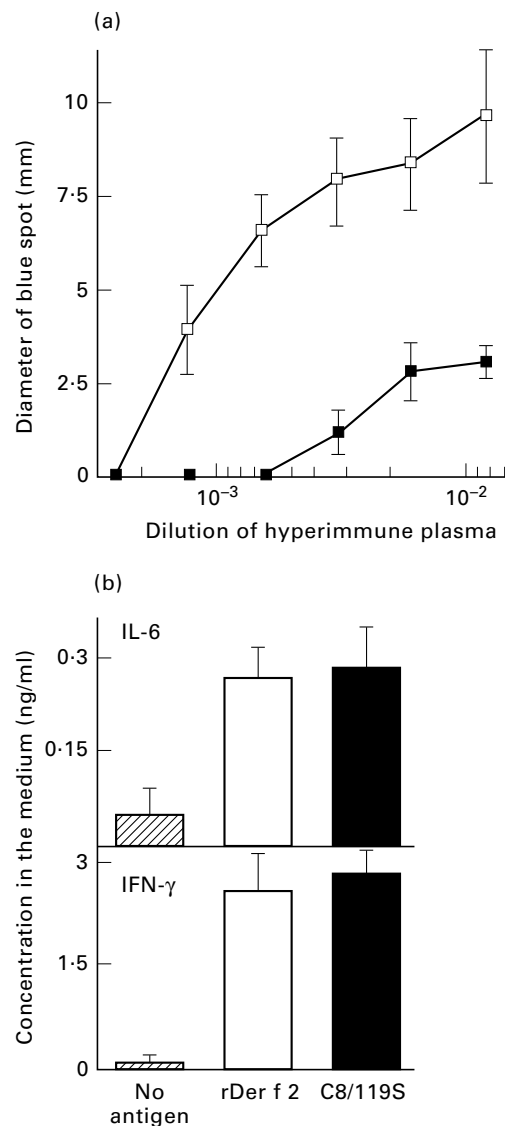


Fig. 1. Comparison of allergic activities of wild-type rDer f 2 and C8/119S in A/J mice immunized with wild-type rDer f 2. (a) Passive cutaneous anaphylaxis (PCA) reaction provoked with wild-type rDer f 2 (□) and C8/119S (■). Two rats intracutaneously sensitized with serial dilutions of hyperimmune plasma were challenged by either wild-type rDer f 2 or C8/119S. (b) IL-6 and IFN-γ production by spleen cells cultured for 72 h in the presence or absence of 10 µg/ml of either wild-type rDer f 2 or C8/119S. Spleen cells were prepared from three sensitized mice. Vertical bars represent s.e.m.

cells after stimulation with C8/119S was comparable to that with wild-type. The concentration of IL-4 could not be determined in this study. IL-6 and IFN-γ production by naive spleen cells after incubation with wild-type rDer f 2 and C8/119S was negligible (data not shown).

Effect of i.n. administration of wild-type rDer f 2 or C8/119S on immediate response

Since we confirmed that C8/119S possessed adequate T cell reactivity and very weak IgE-binding capacity in mice, we performed a hyposensitization study in sensitized mice by using C8/119S and wild-type rDer f 2.

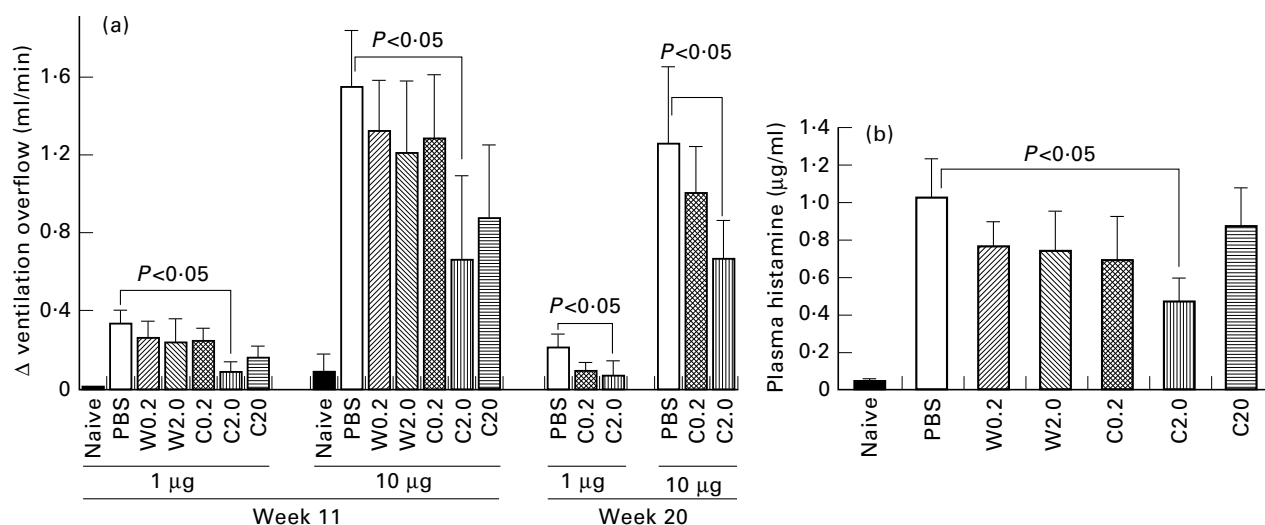


Fig. 2. Comparison of the immediate reaction provoked by the i.v. injection of wild-type rDer f 2 among the experimental groups. (a) Increase in the ventilation overflow (VO) after the injections of 1 and 10 µg of wild-type rDer f 2. Antigen provocations were performed 7 days (week 11) or 10 weeks (week 20) after the last intranasal treatment. The numbers of mice were as follows; PBS, $n = 10$; W0.2, $n = 7$; W2.0, $n = 7$; C0.2, $n = 9$; C2.0, $n = 10$; C2.0, $n = 7$; and naive, $n = 4$ at week 11; PBS, $n = 8$; C0.2, $n = 8$; and C2.0, $n = 8$ at week 20. (b) Plasma histamine concentration immediately after the determination of bronchoconstriction 7 days after the last intranasal treatment. The same mice as in (a) were examined.

Animals were intranasally given 0–20 µg of either wild-type or C8/119S three times a week for 4 weeks, and had their bronchoconstriction evaluated upon antigen provocation 7 days after the last administration. As shown in Fig. 2a, the sequential i.v. injection of 1 and 10 µg of rDer f 2 to mice in the PBS group provoked immediate airway constriction in a dose-dependent manner, whereas no such reaction was observed in the naive mice. The responses were weaker in the W0.2 and W2.0 groups, given 0.2 and 2.0 µg/administration of wild-type rDer f 2, respectively, than in the PBS group. However, no significant difference compared with the PBS group was obtained, and the values of the W0.2 and W2.0 groups were comparable. This was not the case for the C0.2 and C2.0 groups, given 0.2 and 2.0 µg/administration of C8/119S, respectively. The immediate reaction in the C2.0 group was significantly lower than that in the PBS group, and the C0.2 group exhibited a value intermediate between those of the PBS and

C2.0 groups. Since the IgE-binding capacity of C8/119S was much lower than that of wild-type, we prepared a C20 group given 20 µg/administration of C8/119S. The airway response in the C20 group was not suppressed as clearly as in the C2.0 group, however (Fig. 2a). The histamine level in the plasma immediately after measurement of the bronchoconstriction was almost proportional to the increase in VO after the i.v. injection of 10 µg of wild-type (Fig. 2b). To determine the continuance of the hyposensitization, the animals in the PBS, C0.2 and C2.0 groups were examined for bronchoconstriction 10 weeks after the last administration. The results were similar to those from day 7 (Fig. 2a).

Changes in plasma IgE, IgG1 and IgG2a levels

The total IgE, wild-type rDer f 2-specific IgG1 and IgG2a levels before and after the administration period are shown in Fig. 3. The levels of total IgE and that of anti-wild-type IgG1 were slightly

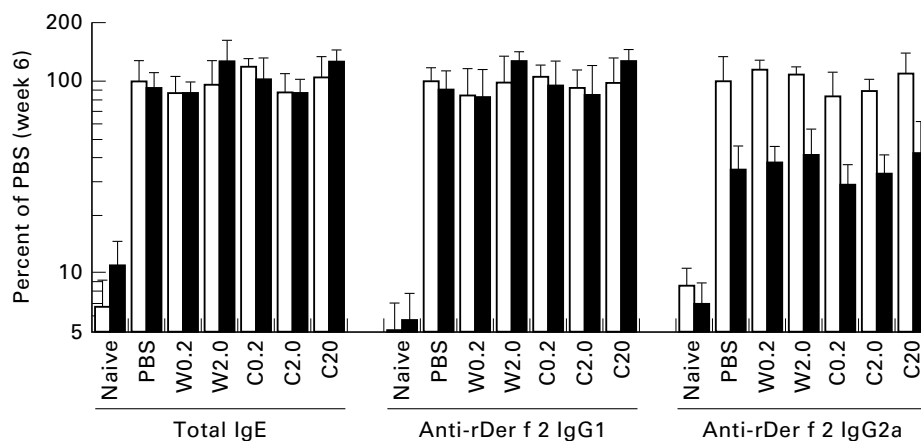


Fig. 3. Plasma total IgE concentration, anti-wild-type rDer f 2 IgG1, and IgG2a levels before (□) and after (■) intranasal treatments in mice that were examined for immediate reaction 7 days after the last treatment. Regarding the total IgE concentration, '100%' was 2.35 µg/ml.

decreased and anti-wild-type IgG2a was markedly decreased after the administration in all groups except the W2.0 and C2.0 groups. In these groups, the total IgE and anti-wild-type IgG1 levels were slightly increased after 4 weeks of i.n. administration. The anti-wild type PCA titre was 320 in all groups over the experimental period. No significant difference was observed in antibody levels among the groups or between before and after the administration. The antibody levels did not correlate with the immediate reaction expressed by the increase in VO, or with plasma histamine levels (data not shown). Neither a drop in the level of reagenic antibody nor an increase in the level of blocking antibody were found.

Flow cytometric analysis of peripheral blood leucocytes

Mice in the PBS, C0.2 and C2.0 groups in which no immediate reaction was provoked were examined for the phenotypes of peripheral blood leucocytes and spleen cells 7 days after the last i.n. administration.

B cells were distinguished from the other cell types by B220 expression and cellular morphology. CD23 expression on B cells in the PBS group mice as determined by flow cytometry was two-fold higher than that in the naive mice. The CD23 values of the C2.0 group were significantly lower than those of the PBS group, while the C0.2 group showed values comparable to those of the PBS group (Fig. 4).

Basophils were distinguished by surface IgE and cellular morphology. Since the naive A/J mice had a small amount of IgE in their plasma, their basophils could also be captured by higher signals for IgE molecules, in seven of nine animals examined. The basophils counted by flow cytometry were about 0.7% of whole leucocytes in the PBS, C0.2 and C2.0 groups (without significant differences among them), and 0.3% in the naive mice. These values were comparable to the basophil counts determined by histochemical analysis (data not shown). The

CD11b and IgE molecules on basophils were 2–4-fold more numerous in the PBS group than in the naive mice, while the numbers of these molecules in the C0.2 and C2.0 groups were decreased compared with the PBS group, in a dose-dependent manner (Fig. 4). No significant difference was obtained between the PBS and C8/119S-administered groups, however. The plasma total IgE levels in the PBS, C0.2 and C2.0 groups were comparable, and thus the numbers of IgE molecules on the basophils were considered to reflect the numbers of IgE receptors, except in the naive mice.

We also examined the expressions of CD44, CD49d and CD117 on basophils. No differences were observed in CD44 and CD49d between the naive and sensitized mice, and CD117 was not detected on the basophils (data not shown).

IL-6 and IFN- γ production by spleen cells

Spleen cells obtained from mice in the PBS, C0.2 and C2.0 groups and naive mice were cultured with or without 10 μ g/ml of wild-type rDer f 2 for 72 h. The concentrations of IL-6 and IFN- γ in the supernatant of the cultures without antigen were <0.1 ng/ml and 1 ng/ml in all groups, respectively (data not shown). The IL-6 and IFN- γ levels after stimulation with wild-type rDer f 2 in the PBS group were two to four times higher than in the naive mice (Fig. 5). IL-6 and IFN- γ production in the C8/119S-administered groups was decreased in a dose-dependent manner, and a significant difference was obtained in IL-6 production between the PBS and C2.0 groups. IL-4 in the culture supernatant could not be determined.

DISCUSSION

Immunotherapy with crude extracts of allergenic substances has been widely performed in the treatment of allergic diseases in the

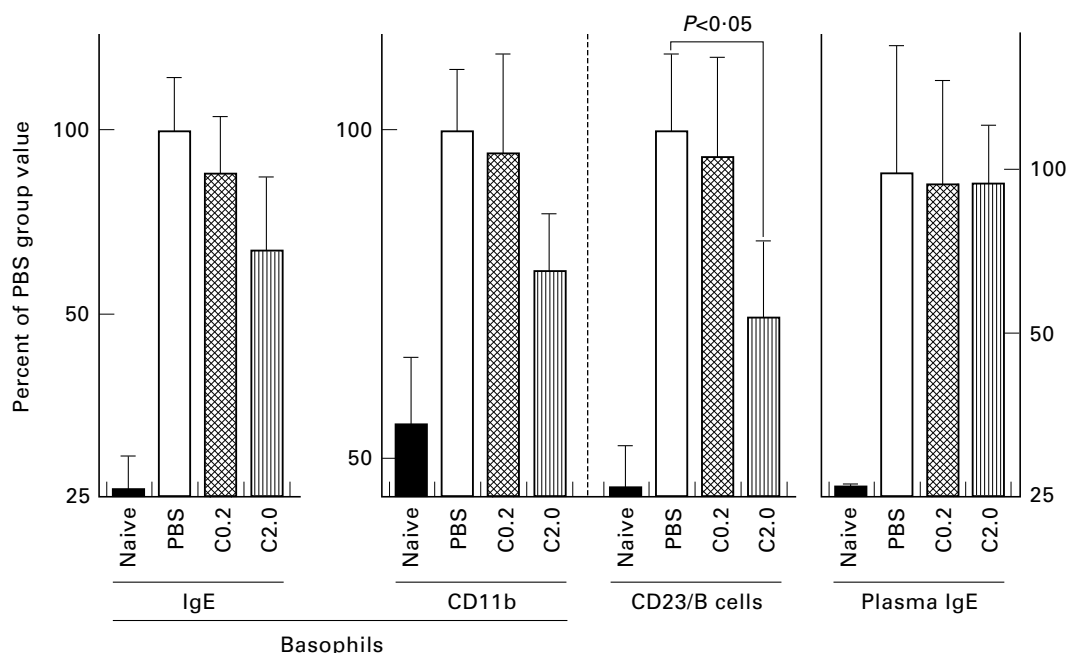


Fig. 4. Plasma total IgE content, IgE and CD11b molecules on basophils, and CD23 expression on B cells. The numbers of mice were as follows: PBS, $n = 10$; C0.2, $n = 10$; C2.0, $n = 9$; and naive, $n = 7$. Data from naive mice whose basophils could not be captured by FACS analysis were omitted.

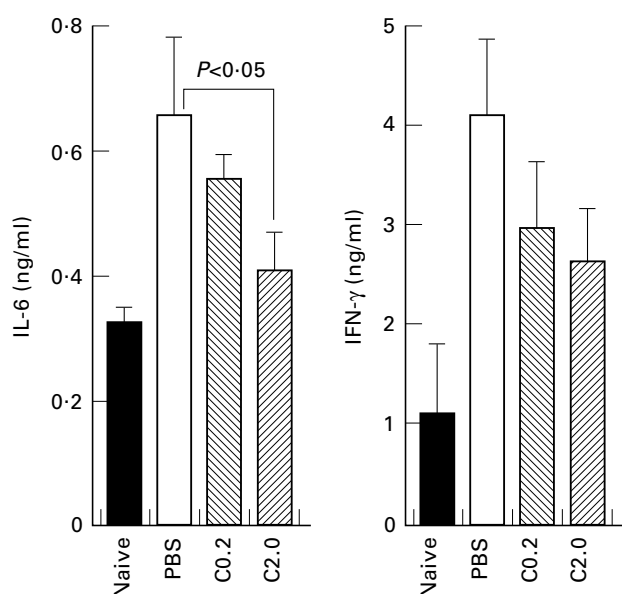


Fig. 5. IL-6 and IFN- γ production by spleen cells cultured for 72 h in the presence of 10 μ g/ml of wild-type rDer f 2. Spleen cells were prepared from mice that were examined for phenotypes of basophils and B cells.

airway, and many affirmative results concerning the therapy have been obtained in clinical and experimental studies. Despite the beneficial effect of immunotherapy, however, several problems remain in the use of crude extracts. First, although only a few allergens that sensitize patients are necessary in the immunotherapy, crude allergens often contain unnecessary substances [32,33]. The effect of the administration of these substances on patients' health is still unclear. Second, allergens in the extract have strong IgE-binding activity, and side-effects such as systemic anaphylaxis may be caused by the injection of a crude extract if the dose of the extract is incorrect or the patient's physiologic status is not good [34,35]. The international standardization of allergen extracts has ameliorated these problems to some extent, but not completely. Recent advances in biochemistry and immunology have further improved the situation. Several recombinant forms of clinically important allergens with high purity are now available in large quantities [11,25], and derivatives of wild-type allergens including synthetic peptides and mutants of recombinant allergens have been developed from the original allergens. Several of these derivatives have been shown to contain T cell epitope and possess little or no IgE-binding capacity [16,20]. Since it seems that interactions between T cells and injected allergens play a pivotal role in the hyposensitization of patients to allergens, such derivatives have the potential to be safe immunotherapeutic agents [36].

Concerning house dust mite allergens, the genes for group 1 (Der f 1, Der p 1) and 2 (Der f 2, Der p 2) major allergens, both of which have been known to sensitize 60–80% of patients with house dust mite allergy [37,38], have been cloned, and T and B cell epitopes of these allergens have been well studied [39–41]. C8/119S, a mutant of rDer f 2, was recently shown to possess complete activity to stimulate T cell proliferation and markedly reduced binding capacity to Der f 2-specific IgE in humans [28]. These features were shared with mice. Since C8/119S has an entire sequence of Der f 2 with 129 amino acid residues, unlike a

synthetic peptide with 10–20 residues, C8/119S can contain each of the T cell epitopes known to vary considerably from patient to patient [42,43]. In the present study, we examined the hyposensitizing activity of C8/119S in A/J mice sensitized with wild-type rDer f 2.

In previous studies, we showed that the oral administration of 1 mg/day of rDer f 2 to mice immunized with rDer f 2 for 4 weeks significantly suppressed the immediate airway constriction and neutrophil influx into airways [26,27]. In these experiments, each mouse was orally given 20 mg of rDer f 2, but preparing such an amount of C8/119S is currently impossible. We thus chose the i.n. administration of wild-type and mutant rDer f 2 in the present study. Mice immunized with wild-type rDer f 2 were intranasally given 0.2–20 μ g of either wild-type or C8/119S for 4 weeks, and then examined for their allergic reaction. The immediate reaction provoked by the i.v. injection of wild-type was suppressed in all groups that received wild-type rDer f 2 or C8/119S compared with the PBS group, and the decrease in the allergic reaction was most prominent in the C2.0 group. The VO increase and plasma histamine concentration after challenge with 10 μ g of rDer f 2 in the C2.0 group were almost equivalent to the values after i.v. challenge with 2–3 μ g of rDer f 2 in sensitized mice without hyposensitizing treatment (data not shown). These results indicate that the provocative concentration for the allergic reaction of the C2.0 group was increased at least several-fold by the administration of C8/119S. This decrease in the sensitivity to allergen after immunotherapy was comparable to that in several clinical studies [44,45].

It seemed that i.n. hyposensitization with C8/119S and wild-type had an optimum dose showing maximal effects. Higher doses did not always produce better results. In a range within 0.02–0.2 μ g/administration, the hyposensitizing activity of C8/119S was higher than that of wild-type in our murine allergic model. The increase in plasma IgE levels in the W2.0 and C2.0 groups, which received the largest amount of antigens, may reflect the local 'sensitization'. In a previous study, we showed that i.n. injection of 0.1 mg of rDer f 2 once a week for 12 weeks could induce systemic IgE production and airway hyperresponsiveness [27]. The outcome of i.n. immunotherapy may depend on the balance of local 'sensitization' and 'hyposensitization' in A/J mice immunized with wild-type rDer f 2. A clear difference in the immediate reaction between the W2.0 and C2.0 groups, both of which received 2 μ g/administration of antigen, suggested roles for mast cells and basophils in the local sensitization. These cells have high-affinity IgE receptors on the cellular surface, and can be activated by the interaction between antigens and IgE molecules on the receptors, followed by the secretion of both chemical mediators and Th2-type cytokines [18,46–48]. Wild-type rDer f 2 may react to IgE and induce Th2-type cytokines/mediators more strongly than does C8/119S. These cytokines could shift the balance from 'hyposensitization' to 'sensitization', which may account for the unsuccessful immunotherapy in the W2.0 group. To confirm this hypothesis, a comparison of cytokine-inducing activity from mast cells and basophils of wild-type and mutant rDer f 2 should be performed. The suppression of the airway response was maintained for over 10 weeks after stopping the i.n. administration of C8/119S, suggesting that the effect of hyposensitization was long-lasting.

Concerning the mechanisms underlying the hyposensitization in immunotherapy, the decrease in the T cell response to allergen and the correction of the Th1/Th2 bias in allergic patients have been highlighted, instead of the drop in reagenic antibodies and the

increase of blocking antibodies. The Th2-type cytokines IL-4, IL-5, IL-6 and IL-13, considered to play a crucial role in type 1 allergy, were down-regulated [49–51], while Th1 cytokines IFN- γ and IL-12 were changed or unchanged after successful immunotherapy [52,53]. In the present study, we also observed the down-regulation of IL-6 and IFN- γ production by splenocytes in the C2.0 group, which indicated the suppression of T cell reactivity to the sensitizing antigen, wild-type rDer f 2. Although we could not determine any changes in IL-4, we found that the expression of CD23, one of the activation markers on B cells, was down-regulated in the C2.0 group, as in several clinical studies on immunotherapy [54,55]. CD23 expression on B cells has been reported to be higher in allergic patients compared with healthy people, and can be up-regulated by stimulation with IgE and IL-4 *in vitro* [56–58]. Since several studies showed that the rise of CD23 expression during the pollen season in patients with pollinosis and the drop in the expression after immunotherapy were independent of serum IgE levels [55,59], changes in CD23 may reflect the balance of IL-4 and its antagonists when IgE levels are essentially constant. Thus, the suppression of CD23 expression without decrease in plasma IgE in the C2.0 group may reflect the suppression of the activity of IL-4, though the direct measurement of cytokine production from T cells would be necessary to confirm this.

Immediate reactions are known to be triggered by the release of mediators from mast cells or basophils, and thus functional changes in these cells as well as T cells must accompany successful immunotherapy. It has been shown that the expression of high-affinity IgE receptors or several function-molecules on basophils is up-regulated in allergic patients or after stimulation *in vitro* in human and mouse studies [60–63]. The histamine release from basophils was sometimes decreased after immunotherapy [64,65]. We also found in the C2.0 group that the plasma histamine concentration after antigen challenge was suppressed. In addition, the numbers of IgE and CD11b molecules on peripheral blood basophils were increased after immunization, and tended to decrease after the i.n. treatments with C8/119S in the A/J mice. Although the histamine detected in plasma was considered to be derived from both basophils and mast cells in the circulatory system [66], our findings suggest the improvement of allergic phenotypes of basophils after i.n. administration of C8/119S. We could not determine the histamine release from isolated basophils *in vitro*, because of the insufficient numbers and/or histamine content of the basophils. An assay must be performed to obtain direct evidence of the functional changes in basophils.

Several studies showed that Th2 cytokine-producing cells and IgE-secreting cells were increased in the airway tissues of allergic patients or intranasally sensitized animals [67–72]. Although the influence of Th1 cytokines on the phenotypes of mast cells/basophils is still unclear, Th2-type cytokines, including IL-4 and IL-6, are known to up-regulate the growth and functions of mast cells/basophils *in vitro*, and IgE itself enhances the expression of high-affinity IgE receptor on these cells *in vitro* [73–78]. It has recently been found that the density of IgE receptor on mast cells and Th2 cytokine deposition in the patients' nasal mucosa were significantly elevated compared with that of healthy people, suggesting a close relationship between the activity of mast cells and Th2 cytokines in the airway *in vivo* [79,80]. Thus, Th2 cytokines can contribute to the enhancement of the immediate airway constriction as well as to the promotion of the influx of eosinophils and other cells into the airway [81,82]. In contrast, it is

expected that suppression of Th2 cytokines after immunotherapy will give rise to the down-regulation of the expression of mast cells' IgE receptors, as seen in basophils in our study, or other phenotypic changes in mast cells.

In the present study, we challenged sensitized mice with wild-type rDer f 2 by i.v. injection. Although the down-regulation of CD23 expression on B cells and the IL-6 production by splenocytes suggest the suppression of Th2 cytokines in the C8/119S-treated mice, we do not have any information on the immunological status of the airway tissue. For a confirmation of the hyposensitization in airway tissue after the administration of C8/119S, further studies to induce airway constriction by local allergen challenge and to examine changes in the cytokine production and phenotypes of mast cells after hyposensitization are needed. Although we have developed murine allergic models which react to wild-type rDer f 2 [26,27], a high concentration of rDer f 2 (>10–20 mg/ml) is needed to provoke an allergic reaction by the antigen inhalation challenge in these models. A hyposensitization study with an airway challenge using a large number of mice requires a significant amount of wild-type rDer f 2, which is currently rather expensive. We are attempting to develop an experimental model showing allergic reactions in the airway, including an eosinophilic inflammation, after the inhalation or i.n. injection of a lower concentration of wild-type rDer f 2.

In conclusion, the findings obtained in the present study give rise to optimism that i.n. immunotherapy with C8/119S in house dust mite allergy is of benefit in a murine allergic model.

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