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Characterization of the Recombinant Der f 2 Mutant C8/119S and Evaluation of C8/119S in a rDer f 2-Sensitized Rhinitis Mice Model

Satoshi Koyanagi[1]*, Toshio Murakami[2], Kazuyuki Nakashima[2], Toshihiro Maeda[3], Yoshinobu Miyatsu[1], Keishin Sugawara[1] and Hiroshi Mizokami[1,2,3]

[1]*Biologics Development & Production Department The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1 Kyokushikawabe, Kikuchi, Kumamoto 869-1298, Japan*
[2]*Applied Research Department The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1 Kyokushikawabe, Kikuchi, Kumamoto 869-1298, Japan*
[3]*Clinical Development Department The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1 Kyokushikawabe, Kikuchi, Kumamoto 869-1298, Japan*

Abstract

Immunotherapy is the only curative approach to treat allergy, but carries the risk of anaphylaxis. C8/119S is a mutant of Der f 2, which is one of the causative allergens of perennial allergic diseases, and has been selected to decrease the risk of anaphylaxis in immunotherapy. In this study, the physical properties of C8/119S were determined, and the efficacy of C8/119S was evaluated in a NC/Nga mouse rhinitis model. C8/119S and recombinant Der f 2 (rDer f 2) were expressed in *Escherichia coli*. Purified allergens were analyzed by physicochemical and immunological techniques. In addition, rhinitis was provoked in rDer f 2-sensitized NC/Nga mice by nasal administration of rDer f 2, and C8/119S was administered. After provocation tests with rDer f 2, the number of eosinophils infiltrating the nasal mucosa was determined. C8/119S had a disordered structure, and the binding activity of allergic patients' IgE to C8/119S was decreased compared with rDer f 2. In the NC/Nga mouse rhinitis model, eosinophil infiltration provoked by rDer f 2 was significantly controlled by the administration of C8/119S. Although similar therapeutic effects were also observed with rDer f 2 administration, 11 of 20 animals died during the rDer f 2 treatment period. On the other hand, no deaths occurred during C8/119S treatment. C8/119S appears to be an effective allergen vaccine for immunotherapy in patients with mite allergy and also appears to be safer than wild-type allergen vaccines.

Keywords: Dermatophagoides; Der f 2; C8/119S; Immunotherapy; NC/Nga mouse; Rhinitis; Eosinophil infiltration

Introduction

Anti-allergic or anti-histaminic agents are widely used to treat allergic diseases. However, these drugs can only treat the symptoms of allergic disease. Immunotherapy, in which causative antigens of allergic diseases are injected, is believed to be the only curative approach to treat allergy [1-5]. Therapeutic allergens (allergen vaccines) currently used for this purpose are naturally extracted. Allergen extracts, however, contain not only major allergens but also many allergenic and non-allergenic proteins. These proteins may elicit new allergenicity, and as a result there is always a risk of anaphylaxis.

In contrast to the allergens extracted from natural sources the recombinant allergen offer the advantage to provide a better standardized material and ensures that only the relevant allergy causing proteins are included. However, the risk of anaphylaxis remains with immunotherapy, since the wild-type allergen has strong allergenicity. Anaphylaxis is induced by the binding of allergens with allergic patients' IgE. Research on modified allergens that inhibit binding to IgE has advanced. Various methods of modifying allergens, including amino-acid substitution [6-10], the use of synthetic allergen peptides [11,12], adjuvant conjugation [13,14] and the construction of deletion mutants [15-17] have been reported.

The house dust mites *Dermatophagoides farinae* and *D. pteronyssinus* are major causes of allergic diseases such as perennial allergic rhinitis, allergic asthma, and allergic dermatitis. These mites' causative allergens have been identified [18-23] and demonstrated the importance of group one allergens (Der f 1 and Der p 1) and group two allergens (Der f 2 and Der p 2). High rates of group one and two allergen-specific IgE antibody are detected in allergic patients [24]. C8/119S is a mutant of the major house dust mite allergen Der f 2 [25-27]. Of the 130 amino acids constituting C8/119S, Cys 8 and Cys 119 have each been replaced by Ser residues in order to disrupt one of the IgE epitopes of Der f 2. This modification is intended to reduce binding to IgE in allergic patients, whereas maintenance of T-cell epitopes is considered necessary for the effectiveness of immunotherapy [27]. C8/119S is expected to present a low risk of anaphylaxis and to provide an effective allergen vaccine for immunotherapy.

In this study, the physicochemical and immunological properties of C8/119S were demonstrated. To evaluate the efficacy of C8/119S in immunotherapy, we established a Der f 2-sensitized rhinitis mouse model. NC/Nga mice were established as an inbred strain in 1955 and have biological characteristics that include hepatic and renal esterases like those in a DBA/2 strain, high susceptibility to X-irradiation, and high susceptibility to anaphylactic reaction. According to some recent reports, total plasma IgE levels are markedly elevated following allergen administration in these mice [28-30]. The efficacy

***Corresponding author:** Satoshi Koyanagi, Biologics Development & Production Department, The Chemo-Sero-Therapeutic Research Institute (Kaketsuken), 1314-1, Kyokushikawabe, Kikuchi, Kuamoto 869-1298, Japan, Tel: +81-(0)968-37-3100; Fax: +81-(0)968-37-3616; E-mail: koyanagi@kaketsuken.or.jp

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of therapeutic administration of C8/119S was evaluated in the NC/Nga mouse rhinitis model. Eosinophil infiltration of the nasal mucosa provoked by the nasal administration of rDer f 2 was examined.

Materials and Methods

Preparation of C8/119S and rDer f 2

C8/119S and recombinant Der f 2 (rDer f 2) were expressed in *Escherichia coli* (*E. coli*) and recovered as inclusion bodies. The inclusion bodies were dissolved in denaturing buffer, and refolding was performed by diluting the denaturing agent. The recombinant proteins were then purified by anion-exchange chromatography and hydrophobic chromatography, as previously described [31-33].

Characterization assay

SDS-PAGE was carried out as follows. Samples were reduced by treatment with 2-mercaptoethanol or not reduced, and aliquots of the samples, equivalent to 2μg of C8/119S or rDer f 2, were subjected to SDS-PAGE using a ready-made gel (15%; Funakoshi, Tokyo, Japan). After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Peptide mapping was performed as previously described [32]. A 0.96-mg aliquot of C8/119S, which was equivalent to 69 nmol, was used for quantitative analysis of free sulfhydryl groups. The quantity of free sulfhydryl groups was determined by the Ellman method [34]. Circular dichroism (CD) spectroscopy was done and analyzed as follows. Purified C8/119S and rDer f 2 were suspended in 10 mM phosphate buffer (pH 7.2) containing 140 mM sodium chloride (PBS) at a final concentration of 2 mM. CD spectra of the samples were obtained with a Model J-500A (Jasco, Tokyo, Japan). CD spectra were recorded from 200 to 252 nm at a digital resolution of 0.2 nm with a scan speed of 20 nm/min. Data from eight scans were averaged.

Assay of IgE-binding inhibition

Antigens (C8/119S or rDer f 2) were diluted in a tenfold dilution series. Anti-Der f 2 IgE-positive patients' sera and diluted antigens were mixed and incubated prior to apply to an ELISA plate (Nalge Nunc, Roskilde, Denmark). Pre-incubated sera-antigen mixtures were applied to the ELISA plate coated with rDer f 2. And Der f 2 specific IgE bound to the ELISA plate was detected by ELISA method. Then the half maximal (50%) inhibitory concentrations (IC_{50}) of C8/119S or rDer f 2 were calculated and the Wilcoxon's signed rank sum test was performed.

Therapeutic administration in the NC/Nga mouse rhinitis model

Seven-week-old male NC/Nga mice were purchased from Charles River Japan (Osaka, Japan). The experimental schedule is shown in Figure 1. The rDer f 2 solution was diluted in PBS to obtain a 1 mg/ml concentration. A 1 mg/ml rDer f 2 solution was mixed with an equal volume of 20 mg/ml of Alum (Cosmo bio, Tokyo, Japan) and intraperitoneally administered at 100 μl/head to the control group (n=20), the C8/119S group (n=20), and the rDer f 2 group (n=20). Administration was performed on days 0, 7, 14 and 21. To induce rhinitis, 0.1 mg/ml rDer f 2 solution in PBS was nasally administered at 10 μl/head to the control group, C8/119S group, and rDer f 2 group every day from day 22 to day 35. In the non-treatment group (n=10), PBS was nasally administered at 10 μl/head. Nasal administration was performed under nembutal anesthesia.

Allergens (C8/119S or rDer f 2) were diluted with PBS to 0.2 mg/ml for therapeutic administration. C8/119S solution was subcutaneously administered at 100 μl/head to the C8/119S group, and rDer f 2 solution was administered in the same fashion to the rDer f 2 group three times per week for four weeks (from day 35 to day 61). PBS was subcutaneously administered at 100 μl/head to the control group on the same timeline. On day 62, all animals were nasally administered 0.1 mg/ml rDer f 2 solution in PBS (10 μl/head) under nembutal anesthesia for provocation testing. In the non-treatment group, PBS was administered in the same fashion instead of rDer f 2.

Animals were euthanized on day 64, and their heads were fixed with 10% neutral formalin solution. Fixed heads were decalcified, and the blocks except for brains were cut out from the nasal cavity to the occiput. Strip preparations were trimmed to observe the nasal septum, nasal mucosa, and turbinate bones. Two strip preparations per animal were obtained, and were stained with Congo red (Yoneyama, Osaka, Japan). Eosinophils in two right and left nasal mucous membrane specimens each were counted, and the total value (4 locations in total) was considered the number of infiltrating eosinophils. Mean values and S.E.s of the number of infiltrating eosinophils were calculated, and the Aspin-Welch test was performed.

Results

Characterization of C8/119S

C8/119S expressed in *E. coli* was purified by anion-exchange chromatography and hydrophobic chromatography. Purity was evaluated by SDS-PAGE analysis. C8/119S and rDer f 2 were each displayed as a single band with a molecular weight of approximately 15 kDa (Figure 2). The Der f 2 molecule has three disulfide bonds (Figure 3A) [23], while C8/119S is a mutant that lacks the Cys8-

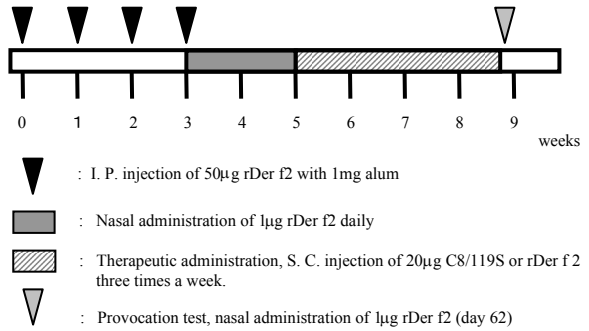

Figure 1: Experimental schedule for the NC/Nga mouse rhinitis model. rDer f 2-sensitized NC/Nga mice were nasally administered 1 μg of rDer f 2 to provoke rhinitis, and mice with rhinitis were subcutaneously injected with 20 μg of C8/119S or rDer f 2 to treat rhinitis. The provocation test was performed with nasal administration of 1 μg of rDer f 2.

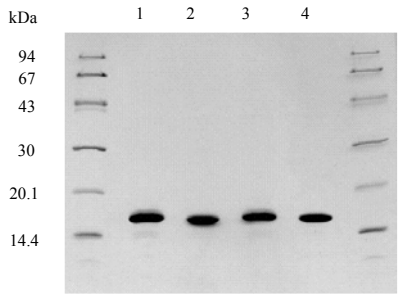

Figure 2: SDS-polyacrylamide gel electrophoresis. Lane 1, reduced purified C8/119S; lane 2, non-reduced purified C8/119S. Lane 3, reduced purified rDer f 2; lane 4, non-reduced purified rDer f 2. The gel was stained by Coomassie Brilliant Blue.

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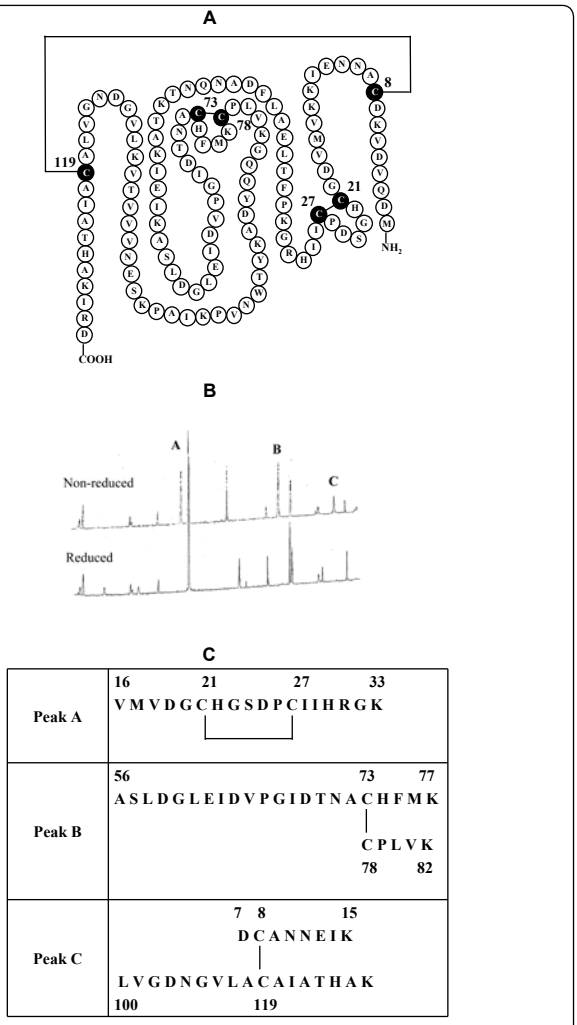

Figure 3: (A) Structural model of mite allergen Der f 2. Der f 2 has three disulfide bonds (Cys21-Cys27, Cys73-Cys78 and Cys8-Cys119). The solid lines show the disulfide bonds. rDer f 2 is comprised of 130 amino acids, and its deduced molecular weight is 15 kDa. A methionine is added at the N-terminal of rDer f 2. Peptide mapping analysis of rDer f 2 **(B)**. Samples derived from non-reduced treatment and reduced treatment were analyzed. Peak A was derived from a Cys21 –Cys27 disulfide bond-related peptide fragment, peak B from a Cys73 –Cys78 disulfide bond-related peptide fragment, and peak C from a Cys8 –Cys119 disulfide bond-related peptide fragment. **(C)** Amino acid sequences of peaks A, B and C. The solid lines show the disulfide bonds.

Cys119 disulfide bond. In order to confirm that Der f 2 and C8/119S had the intended disulfide bond structures, respectively, a peptide map analysis was conducted. A comparison of peptide map patterns under reduced and non-reduced conditions demonstrated three peaks of disulfide bond-related peptide fragments, peaks A, B, and C, with Der f 2 (Figure 3B) and two peaks of disulfide bond-related peptide fragments, peaks A and B, with C8/119S [32]. These peaks were fractionated and identified by amino acid sequencing (Figure 3C). The quantity of free sulfhydryl groups in C8/119S and rDer f 2 was determined. Free sulfhydryl group was not detected in either of the recombinant allergens. All intramolecular Cys residues formed disulfide bonds.

The secondary structure of C8/119S was compared with that of rDer f 2 by CD spectroscopy (Figure 4). The structure of C8/119S differed from that of rDer f 2. The composition ratio of the β sheet structure in C8/119S decreased remarkably compared with rDer f 2,

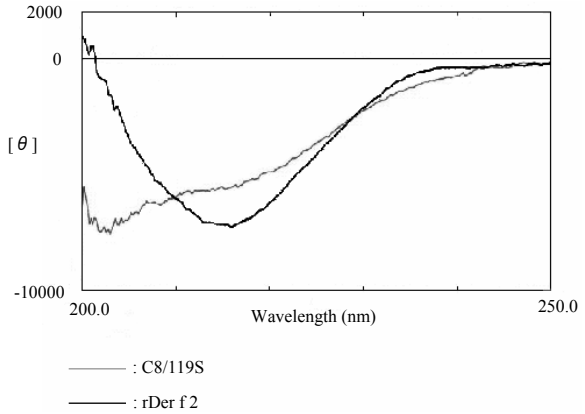

Figure 4: CD spectroscopy analysis of purified C8/119S and rDer f 2. C8/119S and rDer f 2 were suspended in PBS at a final concentration of 2 mM. CD spectra were recorded from 200 to 252 nm at a digital resolution of 0.2 nm with a scan speed of 20nm/min. Gray and black lines show the average signals of eight scans of C8/119S and rDer f 2, respectively.

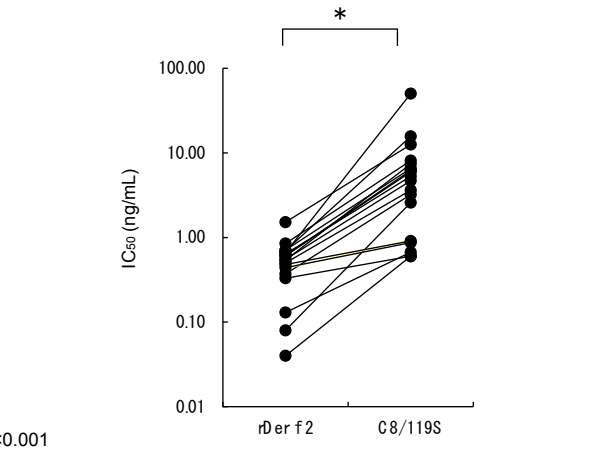

Figure 5: Anti-Der f 2 IgE-positive patients' sera were evaluated by IgE-binding inhibition assay. Half maximal (50%) **i**nhibitory **c**oncentration (IC_{50}) was calculated and the Wilcoxon's signed rank sum test was performed.

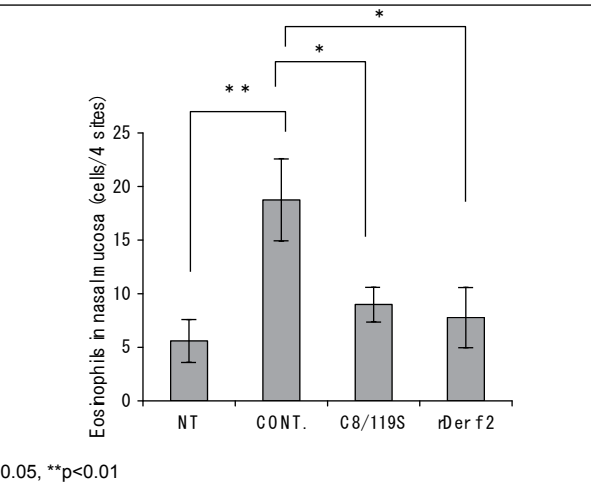

Figure 6: Effects of therapeutic administration of C8/119S and rDer f 2 in a NC/Nga mouse rhinitis model. The non-treatment group (NT, n=10), control group (CONT, n=20), C8/119S administration group (C8/119S, n=20), and rDer f 2 administration group (rDer f 2, n=9) were evaluated. Mean values and S.E.s of the number of infiltrating eosinophils were calculated, and the Aspin-Welch test was performed.

Secondary structure	C8/119S	rDer f 2
α-Helix	18 %	12 %
β-Sheet	25 %	37 %
Random coil	57 %	51 %

Analysis of the secondary structure was performed with SSEAX4 software (Jasco)

Table 1: Types of secondary structures in C8/119S and rDer f 2.

Serum No.	IC_{50} (ng/mL)		Ratio of IC_{50}
	rDer f 2	C8/119S	(C8/119S) / (rDer f 2)
1	0.54	7.39	13.69
2	0.04	0.60	15.00
3	0.08	2.59	32.38
4	0.64	5.30	8.28
5	0.70	15.68	22.40
6	0.71	6.55	9.23
7	0.66	50.26	76.15
8	0.50	3.63	7.26
9	0.55	4.69	8.53
10	0.61	5.97	9.79
11	0.37	3.22	8.70
12	0.43	0.94	2.19
13	1.52	12.58	8.28
14	0.85	8.11	9.54
15	0.61	6.21	10.18
16	0.33	0.60	1.82
17	0.48	0.91	1.90
18	0.44	0.87	1.98
19	0.13	0.67	5.15
Average			13.29
S.D.			16.94

Table 2: The IC_{50} values of C8/119S and rDer f 2 in 19 Der f 2 positive patients' sera.

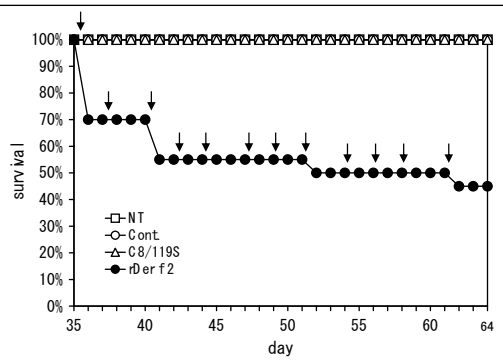

Figure 7: Time course of the ratio of survival of mice with rhinitis during the administration period. Open squares, open circles, open triangles, and closed circles show the non-treatment group, control group, C8/119S administration group, and rDer f 2 administration group, respectively. Arrows show the times of therapeutic administration.

and the ratio of random coiled structures in C8/119S was higher than that in rDer f 2 (Table 1). C8/119S exhibited a disordered structure compared with rDer f 2.

IgE-binding to C8/119S was compared with that to rDer f 2 using the sera of 19 anti-Der f 2 IgE positive patients (Table 2). The IC_{50} of C8/119S were significantly higher than that of rDer f 2 in all sera. When the average of IC_{50}s of C8/119S in all sera was compared with that of rDer f 2, the value of C8/119S was 13.29 times higher than that of rDer f 2. And the ratios of the IC_{50} of C8/119S to rDer f 2 were five times or more in 15 of 19 sera. It was confirmed that IgE-binding to C8/119S decreased compared with rDer f 2 (Figure 5).

C8/119S administration in the NC/Nga mouse rhinitis model

The number of infiltrating eosinophils in the provocation test after administration to the NC/Nga rhinitis mouse model was 18.78±3.85 cells/4 locations in the control group. This number was significantly higher than that in the non-treated group (5.60±1.99 cells/4 locations). On the other hand, the number of infiltrating eosinophils was 9.00±1.61 cells/4 locations in the C8/119S administration group, and significantly lower than that in the control group. In the rDer f 2 administration group, the number of infiltrating eosinophils (7.78±2.82 cells/4 locations) was also significantly lower than that in the control group (Figure 6). However, 11 of 20 animals in the rDer f 2 administration group died during the administration period. In contrast, no deaths were observed in the C8/119S group (Figure 7).

Discussion

In this study, we aimed to confirm whether the C8/119S produced on an industrial scale had the potency to become an effective allergen vaccine. First of all, the characters of C8/119S and rDer f 2 were analyzed and compaired. In the SDS-PAGE analysis, these two allergens were detected at approximately 15 kDa as a single band. The sizes of these molecules were thought to be almost equal based on the SDS-PAGE analysis. In contrast, the CD spectrums showed quite different patterns in C8/119S and rDer f 2. It was confirmed that the composition ratio of the β sheet structure in C8/119S decreased remarkably, and that the characteristic secondary structure of Der f 2 was destroyed. The difference in secondary structures is supposed to originate in the difference in the intramolecular disulfide bonds in these two allergens. We then performed peptide map analysis to confirm that the intramolecular disulfide bonds were correctly constructed in these allergens. The peptide peaks related to the disulfide bonds, which were Cys21-Cys27, Cys73-Cys78, and Cys8-Cys119, were observed in Der f 2, while the peptide peaks related to disulfide bonds, which were Cys21-Cys27 and Cys73-Cys78, were observed in C8/119S [32]. C8/119S and rDer f 2 were both thought to have correct disulfide bonds. For both molecules, C8/119S and rDer f 2, no free sulfhydryl groups could be detected and all Cys residues from the correct disulphide bonds.

The difference of C8/119S with regard to the secondary structure is certainly due to the destruction of disulfide bond Cys8-Cys119. This difference in protein structure greatly influenced the IgE antibody binding. In the analysis of 19 patients' IgE-binding inhibition, the IgE-binding to C8/119S significantly decreased compared with rDer f 2. The anaphylaxis could be induced when IgE antibodies on the surface of the mast cells bind to allergens, and chemical mediators are discharged from the mast cells. It is supposed that the risk of anaphylaxis is lowered if binding of the therapeutic allergen to IgE could be avoided or is at least hindered. Therefore the risk of anaphylaxis with C8/119S is lower than that with wild-type allergens.

On the other hand, allergen specific immunotherapy has the potential to improve patients' symptoms by modifying the immune response [35]. If the allergen doesn't have a T-cell epitope, which allows an immune response to be induced, therapeutic efficacy cannot be expected in the immunotherapy. C8/119S is a mutant of Der f 2 in which Cys8 and Cys119 are substituted with Ser residues to decrease IgE binding to C8/119S. If the T-cell epitopes of C8/119S are destroyed by the amino-acid substitution, it cannot be efficacious. The efficacy of C8/119S as an allergen vaccine has been demonstrated in a rhinitis mouse model. The rDer f 2-sensitized rhinitis mouse model was based on NC/Nga mouse which show a markedly elevated total plasma IgE level following allergen administration. The rDer f 2-sensitized mice were nasally administered with rDer f 2 to induce rhinitis. A provocation test was performed with nasal administration

of rDer f 2 and the number of eosinophils infiltrating through the nasal mucosa was evaluated. The number of infiltrating eosinophils in rDer f 2-sensitized mice (control group) was significantly higher than that in the non-treated group, thus confirming that rhinitis was specifically induced by rDer f 2. In the C8/119S administration group, the level of eosinophils infiltration was significantly controlled in the rhinitis provocation test. Thus rhinitis symptoms could be controlled by C8/119S administration in the NC/Nga rhinitis mice model. It has been indirectly proved for C8/119S that T-cell epitopes are maintained in mice and therefore C8/119S appears to be an effective promising allergy vaccine.

This therapeutic efficacy was observed even with wild-type rDer f 2, although more than 50% of animals died during the rDer f 2 treatment period. All deaths were observed the day after administration. It therefore appeared that there was a relationship between these deaths and rDer f 2 administration. The cause of these deaths might be an anaphylaxis induced by rDer f 2 administration. On the other hand, no deaths were observed with C8/119S administration. Assay of allergic patients' IgE binding to recombinant allergens revealed that IgE binding to C8/119S decreased from 1/10 to 1/100 compared with rDer f 2, as previously mentioned. The differences of the secondary structure and thus IgE-reactivity are certainly the reasons for the different outcome of C8/119S and rDer f 2 with regard to the number of deaths in response to their administration as allergen vaccines. It appears that the risk of anaphylaxis in immunotherapy was decreased using C8/119S. C8/119S might therefore be safer than wild-type rDer f 2 for use in immunotherapy.

It is possible to cure allergies by carefully administering small amounts of wild- type allergens, avoiding anaphylaxis. However, a long period of updosing is necessary until the effective higher allergen doses are reached. Recently, rash immunotherapy, in which comparatively large amounts of allergens are administered within a short period, is becoming a main current of immunotherapy. During rash immunotherapy patients are usually hospitalized in a medical facility which provide the relevant equipment and rescue medication. C8/119S has the potential to be administered in higher doses over a short period because the risk of anaphylaxis appears to be lower than with wild-type allergens. Not only the risk of anaphylaxis but also a long treatment period is problematic in current immunotherapy. C8/119S is an allergen vaccine that offers the possibility of a safer and shorter immunotherapy.

C8/119S thus appeared to be an effective allergen vaccine and safer than current allergen vaccines for wild-type major mite allergens. Its safety and efficacy are expected to be demonstrated in further studies, including clinical trials.

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