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Crucial Commitment of Proteolytic Activity of a Purified Recombinant Major House Dust Mite Allergen Der p1 to Sensitization toward IgE and IgG Responses

Yuko Kikuchi,*† Toshiro Takai,2‡ Takatoshi Kuhara,* Mikiko Ota,*§ Takeshi Kato,* Hideki Hatanaka,‡† Saori Ichikawa,*‡ Tomoko Tokura,* Hisaya Akiba,† Kouichi Mitsuishi,† Shigaku Ikeda,∗ Ko Okumura,*‡ and Hideoki Ogawa∗†

The major proteolytic allergen derived from the house dust mite Dermatophagoides pteronyssinus, Der p1, is one of the most clinically relevant allergens worldwide. In the present study, we evaluate the contribution of the proteolytic activity and structure of a highly purified rDer p 1 to immune responses. Mice were i.p. immunized with three forms of rDer p 1 adsorbed to Alum: one enzymatically active, one treated with an irreversible cysteine protease-specific inhibitor, E-64, and one heat denatured. Immunization with E-64-treated or heat-denatured rDer p 1 elicited much less production of serum total IgE and not only rDer p 1-specific IgE but also IgGs compared with immunization with active rDer p 1. Assays for Ab-binding and its inhibition and structural analyses indicated that E-64-treated rDer p 1 retained its global structure and conformational B cell epitopes. A proliferative response and production of IL-5 by spleen cells restimulated with rDer p 1 were observed on immunization with the active rDer p 1 but not E-64-treated rDer p 1. The cells from mice immunized with heat-denatured rDer p 1 exhibited the highest levels of proliferation and production of IL-5 and IFN-γ. The results indicate that the proteolytic activity of the highly purified rDer p 1 crucially commits to the sensitization process, including both IgE and IgG responses. Additionally, we demonstrated immunogenic differences by functional or structural manipulations of the rDer p 1. The findings have implications for sensitization to this relevant allergen in humans and for the design of modified allergen-vaccines for future allergen-specific immunotherapy.

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House dust mites of two species, Dermatophagoides pteronyssinus and Dermatophagoides farinae, are major sources of allergens associated with allergic diseases such as asthma, rhinitis, and atopic dermatitis (1−4). Group 1 and group 2 allergens are major allergens derived from house dust mites based on the frequency of patients sensitized, amount of specific IgE, and content in mite extract (4–10). The group 1 allergens, Der p 1 from D. pteronyssinus and Der f 1 from D. farinae, belong to the papain-like cysteine protease family (11, 12) and actually exhibit cysteine protease activity (13–16). The cDNAs for Der p 1 and Der f 1 were isolated when cDNA cloning of allergens began (11, 12, 17). They encode signal peptides of 18–aa residues responsible for secretion, 80-residue propeptides, and 222-residue mature portions for Der p 1 and 223 residues for Der f 1. Mature Der p 1 and Der f 1 have 82% sequence identity with each other. At least 12 isoforms with different amino acid sequences for Der

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1 and Der f 1 (28). The highly purified and fully active rDer p 1 and rDer f 1 will be the basis for allergen standardization and the design of safer and more effective allergen-vaccines and be useful for studies in vitro and in vivo to investigate the roles of their function and structure in allergic diseases.

Disruption or modification of the tertiary structure of an allergen is an efficient way to reduce the IgE-binding activity of that allergen when designing safer vaccines for allergen-specific immunotherapy (17, 47–50). In the case of Der p 1, as it modulates biological responses through its biochemical function, proteolytic activity, we considered that the function also could be a target for therapy when designing a novel allergen-vaccine using its recombinant form. In the present study, to obtain information for the modulation of immune responses by changing the biochemical function or tertiary structure of the rDer p 1, we compare immune responses including IgE-eliciting activity in mice immunized with three types of rDer p 1, one enzymatically active, one treated with the protease inhibitor, and one heat denatured. We demonstrate that the efficient in vivo responses, including production of not only IgE but also IgG against the highly purified rDer p 1, are crucially dependent on the cysteine protease activity in mice and that the three types of rDer p 1 differing in function or structure elicit distinctly different immune responses.

Materials and Methods
Recombinant Der p 1
The amino acid sequence of the pre-precursor form of Der p 1 (pro-Der p 1) 1) clone selected for expression was identical with Der p 1.0102 (18), which was reported previously as “clone (c)” by Chu et al. (51), except for one residue (−95) in the signal sequence (44). For the immunization of mice, we used a recombinant mutant Der p 1 designated Der p 1-N52Q, which has the same m.w. as natural Der p 1 (43, 44). Protein concentrations were determined by means of the Bradford procedure with a protein assay kit (Bio-Rad). LPS levels of the purified Der p 1-N52Q were shown to be <20 ng-LPS/mg-protein by using Endospecy (Seikagaku). As B cell epitopes for Der p 1-N52Q and Der p 1-WT are considered to be equivalent (43), we used the hyperglycosylated Der p 1-WT for the coating of ELISA plates to detect Der p 1-specific Abs as an alternative to Der p 1-N52Q, production level of which in yeast is much lower than that of Der p 1-WT (44, 52), although Der p 1-N52Q was used for coating plates in Figs. 4 and 5A. Der p 1-N52Q is described as rDer p 1 throughout this article.

Proteolytic activity
Cysteine protease activity was measured as described previously (16).

Immunization of mice
Six- to 8-wk-old female CBA/J mice were purchased from Charles River Japan and were maintained in a specific pathogen-free animal facility at Juntendo University throughout the study. Before use, 100 μM rDer p 1 was incubated with 5 mM L-cysteine (37°C, 15 min). Then the enzymatic activity was abolished by addition of E-64 (Peptide Institute) (37°C, 30 min) or by heating (98°C, 30 min). The treatment with E-64 was performed with 62.5 mM activated rDer p 1, 3.13 mM L-cysteine, and 1.56 mM E-64 in PBS, and then the Ag was dialyzed with OVA (grade V; Sigma-Aldrich) and E-64-treated OVA were prepared as described above as controls. The heat denaturation was performed with 10 mM activated rDer p 1 and 0.5 mM L-cysteine after dialysis with PBS. The Ags were incubated with Alum (InjjectAlum; Pierce) and further diluted to appropriate volumes with saline. Mice were given four weekly i.p. injections of 2.5 μg of each Ag (28). Peripheral blood was collected weekly from the retroorbital plexus until 1 wk after the last injection. All sera were stored at −20°C before analysis for Ab content.

ELISA for total IgE and levels of Ag-specific Abs
Serum total IgE was measured by a sandwich ELISA as described previously (28). Ag-specific Abs were detected on plates coated with a 10 μg/ml solution of Ags and blocked with BlockAce (Snow Brand) and developed with biotinylated or HRP-conjugated anti-mouse IgE mAb (clone LO-ME-2; Serotec), HRP-conjugated anti-mouse IgG1 mAb (clone X56; BD Biosciences), HRP-conjugated goat anti-mouse IgG2a Ab (Southern Biotechnology Associates), and HRP-conjugated rabbit anti-mouse IgG2b Ab (Zymed Laboratories). HRP-conjugated Extravidine (Sigma-Aldrich) was used in conjunction with biotinylated anti-mouse IgE mAb for detection of Der p 1-specific IgE. Serum dilutions were 1/10, 1/15,000, 1/1,000, and 1/100 for detecting Ag-specific IgE, IgG1, IgG2a, and IgG2b, respectively. For detecting Der p 1- or OVA-specific IgE, the diluted sera were depleted of IgGs (40) as follows. Sera diluted with PBS containing 0.05% (v/v) Tween 20 (PBST) and 10% (v/v) BlockAce were depleted of IgG with protein G-Sepharose 4FF (Amersham Biosciences) within wells of round-bottom microtiter plates (Corning) with agitation at a speed of 1000 rpm by a Bio-Shaker (Titec) for 30 min at room temperature. The total volume of the suspension, the volume of protein G beads, the volume of original serum added, and the final serum dilution were 78, 18, and 6 μl/well and 1/10, respectively. After centrifugation with a swing rotor at 1900 × g for 2 min, the supernatants were used for assays. Volumes of 50 μl from the supernatants in the wells were moved to wells on other plates, which were coated with allergens and blocked as described above. The dilution of the second Abs and the HRP-conjugated Extravidine were 1/1000 and 1/500, respectively. Binding of the conjugates was visualized and measured as described previously (28).

Sensitive ELISA for Ag-specific IgE and titration of Ag-specific Abs
A more sensitive ELISA for Ag-specific IgE was performed using the commercially available immunoreaction enhancer solutions, CanGetSignal (TOYOBO) (53, 54), without the removal of IgG was performed as follows. Volumes of 50 μl of sera diluted with solution 1 of CanGetSignal were added to wells on plates, which were coated with allergens and blocked as described above. After incubation for 15 h at 4°C and three washes with PBST, HRP-conjugated anti-mouse IgG mAb (clone LO-ME-2; Serotec) diluted with solution 2 of CanGetSignal was added to the plates (1/2000 dilution, 50 μl/well). After incubation for 5 h at room temperature and three washes with PBST, the color was developed for 20 min using tetramethyl benzidine (100 μl/well) (BD-OptEIA kit; BD-biosciences), and the reaction was stopped by adding 2 N sulfuric acid (50 μl/well). The OD at 450 nm, from which that at 570 nm was subtracted, was used as the signal for allergen-specific IgE.

For titration, Ag-specific IgGs were also detected, as well as Ag-specific IgE, with the modification that incubation with the sera and that with the secondary Abs was for 90 min at 37°C. The Ag-specific Ab titers were identified as the reciprocal of the dilutions giving an absorbance of 0.1 calculated from titration curves by using Prism version 4.0 (GraphPad).

Inhibition assay
A pooled serum, which was collected from mice immunized with activated rDer p 1 and diluted (1/20,000) with PBST containing and 10% (v/v) BlockAce, was preincubated with serially diluted inhibitors (rDer p 1 or E-64-treated rDer p 1 dialyzed against PBS) for 30 min at room temperature and then added to plates, which were coated with rDer p 1 or E-64-treated rDer p 1 and blocked. The plates were incubated for 15 h at room temperature. After three washes with PBST, the plates were incubated with HRP-conjugated anti-mouse IgG1 mAb diluted (1/2000) with solution 2 of CanGetSignal for 5 h at 37°C. Binding of the conjugate was visualized and measured. The percentage of inhibition was expressed as the relative reduction of the absorbance in each sample to that when no inhibitors were added.

Circular dichroism
Activated rDer p 1 (125 μg/ml) and the rDer p 1 treated with E-64 were subjected to measurements of circular dichroism spectra after dialysis as described previously (43). Ellipticities at 190–260 nm after smoothing are shown.

Molecular modeling
A model of the Der p 1/E-64 complex was made by superimposing the Mr De rp1 cathepsin K/E-64 complex (55) and removing unwanted portions. The A model of the Der p 1/E-64 complex was shown.

1 Abbreviation used in this paper: pro-Der p 1, precursor form of Der p 1.
heat-inactivated FCS, 0.05 mM 2-ME, and antibiotics. Following homogenization by passage through stainless steel mesh and centrifugation at 500 × g for 5 min at 4°C, cells were resuspended in 1 ml of ACK buffer (8.3 g of NH4Cl, 1 g of K2HCO3, and 37.2 mg of EDTA-2Na in 1 liter) at room temperature for 5 min to deplete RBC. After dilution with 10 ml of the medium, the cell suspensions were centrifuged, resuspended in 10 ml of the medium, centrifuged, and resuspended in 3 ml of the medium at 4°C. Viable cells were enumerated by trypan blue exclusion and the suspensions adjusted to 4 × 106 cells/ml. Aliquots of 100 µl of medium containing rDer p 1 (20 µg/ml) or medium alone were added to 96-well, flat-bottom, tissue culture plates (Corning), and 100-µl aliquots of the cell suspension were added. To determine cytokine production, culture supernatants were collected at 72 h and subjected to ELISA using kits (Genzyme Technic).

For estimating proliferative responses, the cultures in triplicate wells were pulsed with 1 µCi/well [3H]thymidine (PerkinElmer Japan) for the next 16 h and harvested using a Micro 96 Harvester (Skatron). Incorporated radioactivity was measured using a microbeta counter (MicroBeta Plus; Wallac). Data are shown as stimulation indexes that were calculated as the ratio of the counts per minute of culture wells containing rDer p 1 to that of wells containing medium alone. The mean of triplicate wells was used as the data for one mouse.

Statistical analysis

Student's t test (two-tailed) was used to evaluate the significance of the differences, although the Mann-Whitney U test was used to analyze the Ab titration data. A value of p < 0.05 was regarded as statistically significant. Data shown are representative of three independent immunization experiments.

Results

Inactivation of proteolytic activity of rDer p 1 by E-64 or heating

By an assay using a synthetic fluorogenic substrate, we confirmed that the proteolytic activity of the E-64-treated rDer p 1 and heat-denatured rDer p 1 was inactivated (Fig. 1A). On SDS-PAGE, rDer p 1 and E-64-treated rDer p 1 showed single bands of similar mobility (Fig. 1B). Their mobilities were higher in nonreducing conditions on SDS-PAGE than in reducing conditions, indicating molecular compactness due to the formation of intramolecular disulfide bonds. The two also showed similar elution volumes in size exclusion column chromatography (our unpublished observation). The results indicated that E-64-treated rDer p 1 remained monomeric. On the other hand, heat-denatured rDer p 1 showed a broad-ranged smear band of high m.w. under nonreducing conditions while a major band with a mobility equivalent to rDer p 1 and E-64-treated rDer p 1 was detected under reducing conditions (Fig. 1B), indicating that the heat-denatured rDer p 1 was polymerized by the formation of intermolecular scrambled disulfide bonds after disruption of the correct intramolecular disulfide bonds by heating in the presence of l-cysteine.

E-64-treated rDer p 1 and heat-denatured rDer p 1 elicited much less production of total IgE and Der p 1-specific Abs compared with active rDer p 1

While mice immunized with the enzymatically active rDer p 1 produced total IgE (Fig. 2A, left) and Der p 1-specific IgE (Fig. 2, B, left, and C), those immunized with E-64-treated rDer p 1 and heat-denatured rDer p 1 produced as little IgE as control mice and significantly decreased levels of Der p 1-specific IgE. The level of Der p 1-specific IgE at 4 wk was determined to be significantly higher on immunization with active rDer p 1 than on immunization with E-64-treated rDer p 1 by the Mann-Whitney U test (p = 0.0022) in Fig. 2B and more clearly in Figs. 2C and 3A, where an ELISA with higher sensitivity using immunoreaction enhancer solutions was applied as described in Materials and Methods. The total IgE level peaked at 2 wk (Fig. 2A, left), preceding the elevation in the level of Der p 1-specific IgE (Fig. 2B, left). Der p 1-specific IgGs were also at significantly low levels on immunization with E-64-treated rDer p 1 and heat-denatured rDer p 1 compared with active rDer p 1 (Figs. 2, D–F, left, and 3B). Compared with active rDer p 1, geometric means of titers for Der p 1-specific IgE, IgG1, IgG2a, and IgG2b decreased to <1/20, <1/100, <1/15, and <1/30, respectively, on immunization with E-64-treated rDer p 1 and further on immunization with heat-denatured rDer p 1 (Fig. 3). In terms of the Ab titers following immunization with active rDer p 1, the IgG subclasses tested ranked as follows: IgG1 > IgG2b > IgG2a.

Injection of E-64 with OVA did not affect levels of the Abs (Figs. 2, A, B, and D–F, right, and 3), suggesting that the reduction of total IgE and Der p 1-specific Abs on immunization with E-64-treated rDer p 1 was caused by elimination of the proteolytic activity of rDer p 1 and not by a nonspecific effect of excess of E-64 injected with rDer p 1. On OVA immunization, total IgE and OVA-specific IgE levels increased in a time-dependent manner (Fig. 2, A and B, right).

B cell epitopes were maintained in E-64-treated rDer p 1 but disrupted in heat-denatured rDer p 1

In plates coated with E-64-treated rDer p 1 (Fig. 4, immobilized Ag: rDer p 1 + E-64), the Ab-binding pattern was similar to that in rDer p 1-coated plates (Fig. 4, immobilized Ag: rDer p 1), indicating that the B cell epitope structure of rDer p 1 was maintained after E-64-treatment. Der p 1-specific IgGs in sera from rDer p 1-immunized mice failed to bind plates coated with heat-denatured rDer p 1, indicating that the B cell epitopes of rDer p 1 were disrupted by the heating (Fig. 4, immobilized Ag: rDer p 1 + heat). The results indicated that the reduction in the production of Der p 1-specific Abs on immunization with E-64-treated rDer p 1 or heat-denatured rDer p 1 (Figs. 2, B–F, and 3) was not due to a failure to detect Abs recognizing structures unique to the modified conformation of E-64-treated rDer p 1 or heat-denatured rDer p 1.

E-64-treated rDer p 1 retained its global structure

The rDer p 1 without and with E-64 treatment completely inhibited each other in the solid phase assay for IgG1 binding at higher concentrations of inhibitors (Fig. 5A). E-64-treated rDer p 1 exhibited a similar circular dichroism spectrum to rDer p 1, showing similarity in their secondary structure (Fig. 5B). E-64 is a low m.w. irreversible cysteine protease-specific inhibitor and covalently
binds to the sulfur atom of the cysteine residue at the catalytic center of cysteine proteases. In papain family cysteine proteases, the binding of E-64 to the catalytic cysteine, which is located at the bottom of the substrate binding cleft, has been reported to induce only a small structural changes (55–58). This is considered to be the case with Der p 1, and E-64 covers only a small area of the Der p 1 in the model of Der p 1/E-64 complex (Fig. 5C). The results indicated that E-64-treated rDer p 1 retained almost all the B cell epitopes, which were reported to be highly conformation dependent (59–61), and the global tertiary structure of rDer p 1, although a
the statistically significant differences were indicated (the differences between treatments without and with E-64 or heating, and mice). Student's t test (two-tailed) was used to evaluate the significance of means.

FIGURE 4. ELISA using plates coated with the immunized rDer p 1 derivatives used for immunization. A, Schematic procedure. The rDer p1, Der p 1-N52Q, was used for coating plates after the treatments shown. The rDer p 1 used for coating was not treated with l-cysteine. E-64-treated rDer p 1 and heat-denatured rDer p 1 used for coating were prepared by the same procedures for preparation of those for immunization. B, Levels of IgGs specific to the plate-coated Ags were measured in mouse sera at 4 wk. Serum dilution factors were 15,000, 100, and 100 for detecting Ag-specific IgG1, IgG2a, and IgG2b, respectively. The data shown represent the means ± SEM of the values for six mice except for the vehicle control (five mice). Student’s t test (two-tailed) was used to evaluate the significance of the differences between treatments without and with E-64 or heating, and the statistically significant differences were indicated (*, p < 0.05; **, p < 0.01; and #, p < 0.001).

relatively small reduction in Ab-binding affinity occurred with the E-64-modification (Fig. 5A, upper panel).

Discussion

We immunized mice with three types of rDer p 1 with functional or structural differences. The activated rDer p 1 has full cysteine protease activity and the tertiary structure of natural Der p 1 (16, 28, 43, 44), E-64-treated rDer p 1 is monomeric and similar in the global structure to rDer p 1 but enzymatically inactivated, and heat-denatured rDer p 1 is structurally disrupted and lacking enzymatic activity. Immunization with active rDer p 1 elicited particularly high levels of production of total IgE and Der p 1-specific IgG1 while that with inhibitor-treated rDer p 1 or heat-denatured rDer p 1 elicited much less production of total IgE and Ag-specific IgE and IgG Abs. The proliferative response of rDer p 1-restimulated spleen cells and production of IL-5 by the cells were significant on immunization with active rDer p 1. The cells from mice immunized with heat-denatured Der p 1 exhibited the highest levels of proliferation and production of IL-5 and IFN-γ. Thus, immunization with functional or structural derivatives of rDer p 1 induced distinctly different immune responses. To our surprise, E-64-treated rDer p 1 induced remarkably little production of both IgE and IgGs despite the maintenance of the global structure and conformational B cell epitopes. This finding is important because it strongly suggests that the IgE- and IgG-eliciting activity of rDer p 1 is dependent on its cysteine protease activity differing from the partial reduction in the IgE-eliciting activity of natural Der p 1 caused by E-64 treatment without affecting IgG production (40, 41).

Gough et al. (40) reported that E-64-treated natural Der p 1 adsorbed to Alum exhibited a partial reduction in IgE-eliciting activity (50–70% reduction of the absorbance in ELISA), and no reduction in Der p 1-specific IgG1 and IgG2b-eliciting activity compared with active Der p 1 while, in the present study, E-64-treated rDer p 1 elicited remarkably low levels of production of total IgE and Der p 1-specific IgE, IgG1, IgG2a, and IgG2b. They and we used the same mouse strain, CBA/J. The differences between our results and theirs might be attributable to differences in doses, timing, Ags used, strengths of the relative proteolytic activity (16, 28, 44) and purity of the Ags (16), or protocols for E-64 treatment. We emphasize that complete inactivation of the proteolytic activity is critical for elimination of the in vivo IgE-eliciting activity on the basis of our observations that E-64 treatment was not able to erase IgE-eliciting activity when the reactions were conducted with lower concentrations of E-64 and rDer p 1 (our unpublished observations), which is considered to decrease the probability of molecular collisions between E-64 and Der p 1 and to decelerate the speed of formation of E-64-rDer p 1 complexes. The cysteine protease activity of the house dust mite group 1 allergens has been reported to cleave some cell surface molecules (22–24, 26–28) and protease inhibitors (25, 26, 28, 30) and to modulate functions of various types of cells (21, 22, 27, 31–39). E-64-treated rDer p 1 with no proteolytic activity is considered to lose such activities and, in the present study, actually exhibited a remarkable reduction in Ab-eliciting activity. We conclude that the cysteine protease activity of the highly purified rDer p 1 is crucial for activity in vivo to elicit production of not only IgE but also IgGs differing from the partial and IgE-selective effects of natural Der p 1 suggested so far. We confirmed that the CBA/J strain used in the present study shows a highly sensitive response in terms of the production of total IgE compared with BALB/c and C57BL/6 against immunization with another recombinant mite group 1 allergen Der f 1 and that the Ab production is dependent on the proteolytic activity of the Ag in the three mouse strains (our unpublished observations).
It appears that rDer p 1 has a greater effect on total IgE than OVA, and, by contrast, the specific IgE to Der p 1 based on the absorbance in ELISA was less than that to OVA. One possible explanation for the results is that the efficiency of the immobilization of Der p 1-WT could be lower than OVA. However, interestingly, the total IgE level peaked at 2 wk, preceding the elevation in the level of Der p 1-specific IgE. Therefore, other explanations also could be envisaged, such as the fact that the ratio total: specific IgE could be influenced by the nature of the administered protein, although whether the total IgE induced before elevation of Der p 1-specific IgE is truly nonspecific has not been clarified yet.

Our results clearly demonstrated that the tertiary architecture of the Der p 1 molecule is itself not enough to induce efficient production of both IgE and IgGs and that the proteolytic activity is crucial for eliciting positive immune responses in naive mice. This might imply that the commitment of the biochemical function of this molecule to the sensitization process makes this protein one of the most clinically relevant allergens. Although whether the biochemical functions of other major allergens have such a critical commitment to sensitization is an issue unsolved as yet (62, 63), we consider that studies based on this question might be important in elucidation of the pathogenesis of allergic diseases, prevention of their development, and therapy (50, 64, 65). Interestingly, people who do not become allergic to house dust mites do not make IgG specific for major house dust mite allergens (62, 63, 66, 67). The close link between allergenicity and immunogenicity of the major mite allergens in humans is similar to our results in mice where substantial increases in IgE, IgGs, and T cell responses were observed on immunization with active rDer p 1 but not on immunization with E-64-treated rDer p 1. Immune responses in people nonallergic to mites might be prohibited by physical and biochemical barrier systems, including intrinsic protease inhibitors produced at interfaces between the body and environment (29, 35), and/or might be actively regulated to achieve immunological tolerance against major allergens. The latter possibility that active regulation works in mice immunized with variants without the cysteine protease activity but retaining the tertiary global structure should be examined in a future study.

Heat-denatured rDer p 1, which is structurally disrupted and lacking enzymatic activity, elicited almost no production of IgE and Ag-specific Abs similar to E-64-treated rDer p 1. However, spleen cells from mice immunized with heat-denatured rDer p 1 proliferated more rapidly and produced much higher levels of cytokines than cells from active rDer p 1-immunized mice in response to restimulation with correctly folded rDer p 1, indicating that the heat-denatured rDer p 1 was not ignored by the immune system and Der p 1-specific T cells developed during the immunization. Disruption or modification of the tertiary structure of allergens is an effective way to reduce the IgE-binding activity of allergens when designing safer vaccines for allergen-specific immunotherapy (17, 47–50), and the change in the tertiary structure should be examined in a future study.

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cellular responses using mature rDer p 1. Such rDer p 1 derivatives, which contain the mature sequence only, might have an advantage over the pro-Der p 1 derivatives in not eliciting the production of Abs or cellular responses against the prodomain.

In the case of Der p 1, the biochemical function of the cysteine protease activity also could be considered a target for therapy when designing a novel allergen-vaccine, along with the allergen’s structure (16, 28, 43, 44). Interestingly, it was reported that a cysteine protease of the nematode *Nippostrongylus brasiliensis* elicits IgE and IgG1 responses in infected rats (71) and that IgE and IL-5 responses against highly purified papain, a potent occupational allergen (73), were eliminated or reduced by E-64 treatment of the Ags in mice as well as rDer p 1 in the present study. These results suggest that a common mechanism might determine the immune response to the cysteine proteases of allergens or parasite Ags. Our in vivo experimental system using the highly purified recombinant mite cysteine protease-allergens, which retain a structure and function similar to the natural counterparts (16, 28, 43, 44), will be useful for exploration of the mechanism involved and evaluation of recombinant allergen-vaccines for allergen-specific immunotherapy.

In summary, we investigated effects of E-64 treatment or heat denaturing of a highly purified and enzymatically active rDer p 1 on immune responses in vivo. The results suggest that the cysteine protease activity of rDer p 1 crucially contributes to in vivo immune responses, including the production of not only IgE but also IgG against the tertiary architecture of this most clinically relevant allergen. Additionally, we showed that when functionally or structurally modified, rDer p 1 induced distinctly different immune responses. Our system would be useful for analysis of the mechanism determining whether sensitization or tolerance to major mite allergens develops in humans and that underlying the cysteine protease-dependent positive immune responses, and for evaluation of the potential of functional or structural derivatives as allergen-vaccines for specific immunotherapy. Further analysis of prophylactic or therapeutic approaches through the administration of functional or structural derivatives may contribute to immunotherapy for IgE/Th2-mediated allergic diseases.

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**Disclosures**

T. Takai, inventor, and the Chemo-Sero-Therapeutic Research Institute have a pending patent with the title “Modification of Group 1 Mite Allergen.”

**References**


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