

Heat denaturation affects the ProDer p 1 IgE reactivity and downregulates the development of the specific allergic response

Mauro Magi, MD,^a Lida Garcia, MD,^a Michel Vandenbranden, PhD,^b Rémi Palmantier, PhD,^c and Alain Jacquet, PhD^a *Gosselies, Brussels, and Rixensart, Belgium*

Background: Modified allergens with reduced IgE-binding activity represent an elegant approach to circumvent the risk of anaphylactic reactions in allergen-specific immunotherapy. **Objective:** The current work investigated the effect of heat denaturation on the allergenic properties of recombinant ProDer p 1, a precursor form of the major house dust mite allergen Der p 1.

Methods: The IgE reactivity was estimated by direct and competition ELISA. The immunogenicity of heat-denatured ProDer p 1 was evaluated in naive and Der p 1-allergic mice. **Results:** Heat denaturation in reducing conditions drastically reduced the *in vitro* ProDer p 1 IgE reactivity toward human allergic sera. In naive mice, heat-denatured ProDer p 1 generated mixed T_H1-T_H2 responses characterized by the absence of specific IgE with concomitant rise in specific IgG2a titers and the presence of IL-5 and IFN- γ in splenocyte cultures. In contrast, natural Der p 1 or native ProDer p 1 induced typical strict T_H2-biased allergic responses with strong IgG1 and IgE titers, whereas spleen cells exhibited only high IL-5 secretion. Moreover, native or heat-denatured ProDer p 1 vaccinations prevented airway eosinophil infiltrations after challenge. Although native or heat-treated ProDer p 1 adjuvanted with SBAS1b induced mixed T_H1-T_H2 responses in allergic mice, heat-denatured ProDer p 1, compared with native ProDer p 1, proved to be more effective in redirecting the T_H2-allergic response toward T_H1.

Conclusion: Taken together, our results suggest that variants of Der p 1 with reduced IgE-binding reactivity could represent hypoallergenic molecules suitable for allergen-specific immunotherapy. (*J Allergy Clin Immunol* 2004; 114:545-52.)

Key words: Allergy, T_H1-T_H2 polarization, vaccination, ProDer p 1, denaturation

House dust mite allergens from the genus *Dermatophagoides pteronyssinus* are major causative

Abbreviations used

MPL: Monophosphoryl lipid A

QS21: Saponin adjuvant from *Quillaja saponaria*

factors associated with various allergic diseases such as asthma.¹ Among these allergens, Der p 1 is the most immunodominant antigen because the prevalence of anti-Der p 1 IgE antibodies in patients with allergy is >80%.² The potent allergenicity of Der p 1 is not fully understood, but recent data provided evidence that the proteolytic activity of Der p 1 biases the immune system in favor of the activation of CD4⁺ T_H2 cells.³⁻⁸

Although several Der p 1 T-cell epitopes were extensively characterized,⁹⁻¹³ only few studies were performed to identify its IgE-binding epitopes. B-cell epitopes carried by Der p 1 were shown to be susceptible to thermal and chemical denaturation, and the absence of continuous IgE-binding epitopes in Der p 1 was clearly evidenced by using overlapping peptides covering the entire allergen sequence.¹⁴⁻¹⁶ Taken together, these data indicated that the Der p 1 allergenic determinants are conformational epitopes.

The production of recombinant allergens provides promising tools for allergy diagnosis as well as for the development of novel compounds for the treatment of allergic diseases.^{17,18} Moreover, the modification of the allergen B-cell epitopes represents an approach for the design of hypoallergens that display low or no IgE-binding activity but retain T-cell epitopes to evoke T-cell responses.^{19,20} The use of hypoallergenic modified allergens would increase the safety of immunotherapy by reducing the risks of anaphylactic reactions. We successfully produced at high yield a recombinant precursor form of Der p 1, secreted as ProDer p 1 in Chinese hamster ovary cells.²¹ Because ProDer p 1 was shown to be enzymatically inactive and to display very low histamine releasing capacity, we could consider that ProDer p 1 is less allergenic than Der p 1. However, we recently showed that mice immunizations with Der p 1 or ProDer p 1 induced typical allergic T_H2-biased immune responses characterized by high titers of anti-Der p 1 IgG1, large amounts of specific IgE antibodies, and a weak Der p 1-specific IgG2a response.²²

The aim of the current study was to investigate the effect of heat denaturation on the allergenic properties of ProDer p 1.

From ^aService de Génétique Appliquée, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, Gosselies; ^bLaboratoire de Structure et Fonction des Membranes Biologiques, Université Libre de Bruxelles, Brussels; and ^cGlaxoSmithKline, Rixensart.

Supported in part by GlaxoSmithKline (Rixensart) and by the Walloon Region (Direction Générales des Technologies, de la Recherche et de l'Energie) of Belgium.

Received for publication October 24, 2003; revised April 6, 2004; accepted for publication April 7, 2004.

Available online August 3, 2004.

Reprint requests: Alain Jacquet, PhD, Rue des Professeurs Jeener et Brachet, 12, B-6041 Gosselies, Belgium. E-mail: alain.jacquet@ulb.ac.be.

0091-6749/\$30.00

© 2004 American Academy of Allergy, Asthma and Immunology

doi:10.1016/j.jaci.2004.04.014

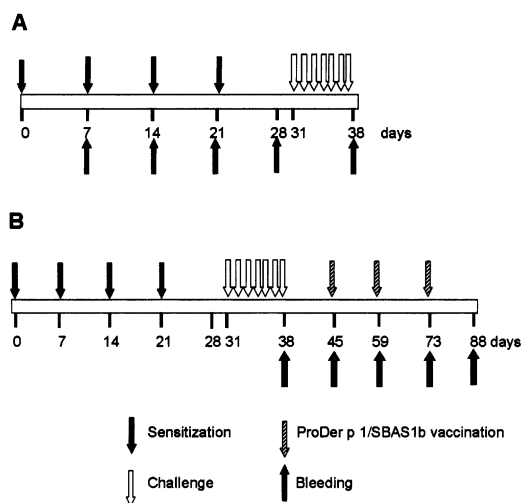


FIG 1. Experimental protocol for the evaluation of the immune responses in naive and allergic mice.

METHODS

Antigens

Natural Der p 1 was isolated from whole *D pteronyssinus* culture as previously described.²¹ Recombinant ProDer p 1 was purified from Chinese hamster ovary spent culture medium.²¹ Recombinant ProDer p 1 was heat-denatured for 5 minutes at 100°C in the presence of 50 mmol/L β -mercaptoethanol. Endotoxin levels were determined by using the limulus assay (Limulus amoebocyte lysate QCL 1000, Bio Whittaker, Verviers, Belgium). The antigens used for the vaccinations contained <0.2 U/10 μ g.

Immunization protocols

Female Balb/C mice (6 weeks old) were obtained from Harlan (Horst, The Netherlands). For allergenicity studies, groups of 10 naive animals were immunized at weekly intervals for 4 weeks with 5 μ g natural Der p 1 or native or denatured ProDer p 1 adsorbed to 200 μ g alum (Fig 1). As control, 1 group of mice was immunized with adjuvant alone. Mice were bled from the retro-orbital venous plexus on days 7, 14, 21, 28, and 38, and sera were collected. To induce airway inflammation, mice were airway allergen-challenged 10 days after the last sensitization by exposition to aerosolized crude *D pteronyssinus* extract over a 30-minute period for 7 consecutive days. The protein concentration of crude mite extract was 300 μ g/mL.

In a therapeutic approach, mice were T_H2-sensitized with 4 doses of 5 μ g natural Der p 1 adjuvanted with alum at weekly intervals, then challenged as described below (Fig 1). Two weeks after the last airway challenge, the animals were vaccinated 3 times with 5 μ g natural Der p 1 or native or denatured ProDer p 1 formulated with SBAS1b adjuvant, a combination of monophosphoryl lipid A (MPL) and saponin adjuvant from *Quillaja saponaria* (QS21; proprietary formulation of GlaxoSmithKline, Rixensart), at 2-week intervals.²³ Sera were collected on days 38, 45, 59, 73, and 88.

Measurement of ProDer p 1-specific IgG, IgG1, IgG2a, and IgE. Sera were assayed for anti-ProDer p 1 IgG, IgG1, and IgG2a antibodies by ELISA as previously described.²²

IgE-binding activity assay

Immunoplates were coated overnight with Der p 1 or native or denatured ProDer p 1 (500 ng/well) at 4°C. Plates were then washed 5 times with 100 μ L per well of TRIS-buffered saline (TBS)—Tween

buffer (50 mmol/L TRIS-HCl, pH 7.5; 150 mmol/L NaCl; 0.1% Tween 80) and saturated for 1 hour at 37°C with 150 μ L of the same buffer supplemented with 1% BSA (Sigma, Bornem, Belgium). Sera from 22 patients allergic to *D pteronyssinus* diluted 1:8 (all of the individual sera had a RAST value >100 kU/L) were then incubated for 1 hour at 37°C. Plates were washed 5 times with TBS-Tween buffer, and the allergen-IgE complexes were detected after incubation with a mouse anti-human IgE antibody (Southern Biotechnology Associates, Birmingham, Ala) coupled to alkaline phosphatase (dilution 1:7500 in TBS-Tween buffer; Promega, Leiden, the Netherlands). The enzymatic activity was measured by using the p-nitrophenylphosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{410nm} was measured in a Biorad Novapath ELISA reader (Nazareth Eke, Belgium).

For IgE inhibition assays, plates were coated with natural Der p 1 (500 ng/well). A pool of 20 human sera from patients with mite allergy (RAST value >100 kU/L) were preincubated overnight at 4°C with various concentrations (0.004–3.6 μ mol/L) of Der p 1 or native or heat-denatured ProDer p 1 in reduced or unreduced conditions as inhibitors. The sera were then added on ELISA plates, and IgE-binding activity was measured as described above.

Proliferation and cytokine assays

The Der p 1-specific T-cell proliferative responses and the level of IFN- γ and IL-5 in the supernatants were measured as previously described.²²

Briefly, spleen cells from immunized mice were stimulated with serial dilutions of ProDer p 1. After 72 hours, cells were pulsed with 1 μ Ci/well [³H]-thymidine (Amersham, Roosendaal, The Netherlands) for 16 hours. Cells were harvested, and [³H]-thymidine uptake was measured by scintillation counting. Culture supernatants from restimulated splenocytes were assayed for the presence of IFN- γ and IL-5 by using ELISA kits supplied by PharMingen.

Bronchoalveolar lavage

The bronchoalveolar lavages as well as the differential cell counts were performed as described previously.²² Briefly, the lungs from mice were washed, the lavage fluid was centrifuged, and the cells were counted in a Thoma hemocytometer (Sigma). Cytospin preparations from 50- μ L aliquots were stained with May-Grünwald Giemsa stain for differential cell counts.

Statistical analysis

Group data were compared by using the Student *t* test. Differences were considered significant for *P* values $<.05$.

RESULTS

ProDer p 1 denaturation reduces drastically IgE-binding reactivity toward allergic sera

In direct ELISA, we characterized the IgE-binding capacity of recombinant ProDer p 1 after thermal denaturation. Plates were coated with native or denatured ProDer p 1 and incubated with 22 individual sera from house dust mite-sensitized patients. Heat denaturation of ProDer p 1 reduced drastically the IgE-binding reactivity of the Der p 1 precursor ($P < .0001$; Fig 2). The addition of reducing agent during the heat denaturation step amplified the alteration of binding of serum anti-Der p 1 IgE ($P < .0001$). It must be pointed out that the pretreatment of the allergens with a reducing agent (50 mmol/L β -mercaptoethanol) before coating did not influence their IgE-binding capacity (data not shown).

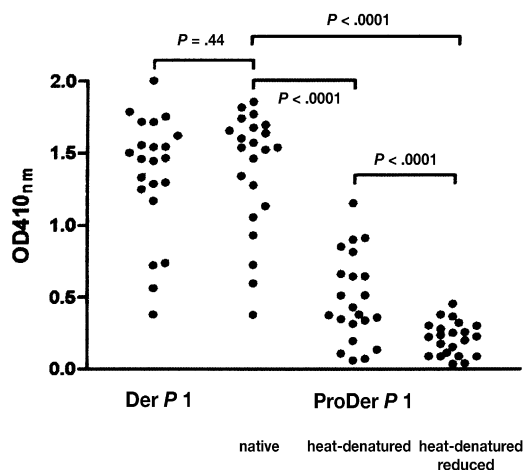


FIG 2. Effect of heat denaturation on the IgE reactivity of recombinant ProDer p 1. The IgE reactivity of natural Der p 1 and different preparations of recombinant ProDer p 1 were evaluated in direct ELISA and by using radioallergosorbent positive sera to *D pteronyssinus*.

The IgE reactivity of denatured ProDer p 1 was further analyzed by competitive inhibition assays (Fig 3) in which pooled serum from patients was preincubated with various concentrations of ProDer p 1 forms. As previously observed, natural Der p 1 was able to inhibit IgE binding to coated Der p 1 more efficiently than native ProDer p 1 used at the same concentrations. The inhibition capacity of heat-denatured ProDer p 1 was reduced compared with that of native ProDer p 1. Der p 1 and native and denatured ProDer p 1 exhibited a 50% inhibition in IgE binding for an allergen concentration of 0.003, 0.03, and 2 $\mu\text{mol/L}$. In contrast, no inhibition of IgE binding to Der p 1 was observed with serum samples incubated with denatured and reduced ProDer p 1 ($P < .001$ compared with other allergens).

ProDer p 1 denaturation in reducing conditions prevents the IgE synthesis and induces the production of IgG2a and IFN- γ in naive mice

Balb/C mice were immunized with denatured and reduced ProDer p 1 and were subsequently exposed to aerosolized house dust mite extracts to provoke airway inflammation. The specific immune responses were compared with that induced after injection with natural Der p 1 or native ProDer p 1. Mice immunized by 4 injections of natural Der p 1 produced high titers of anti-ProDer p 1 IgG and IgG1 as well as large amounts of specific IgE antibodies. In contrast, the anti-ProDer p 1 IgG2a responses were absent (Table I). Similar results were obtained after vaccination with native ProDer p 1 ($P > .05$). Denatured and reduced ProDer p 1 was shown to be less immunogenic than Der p 1 or native ProDer p 1 ($P = .021$). However, modified ProDer p 1 immunizations induced significant production of anti-ProDer p 1 IgG2a ($P < .001$) and, strikingly, prevented the development of

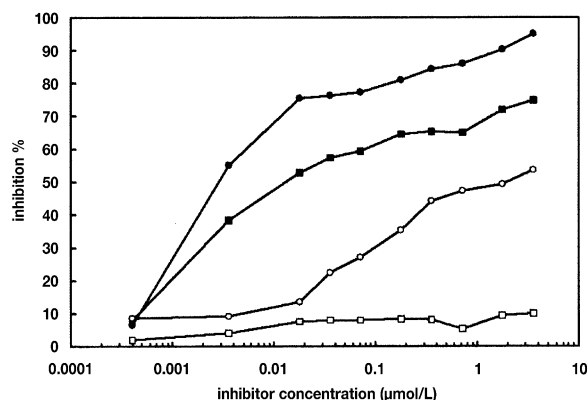


FIG 3. Inhibition of IgE binding to natural Der p 1. IgE binding to immobilized natural Der p 1 was inhibited by preincubation of sera with increasing concentrations of natural Der p 1 (●) and native (■), heat-denatured (○), or reduced, heat-denatured (□) recombinant ProDer p 1. Results are shown as 100% IgE binding in the presence of inhibitor/IgE binding in the absence of inhibitor.

specific IgE antibodies ($P < .001$). The profiles of these humoral immune responses were not modified after bronchoprovocations. Comparison of the IgG1:IgG2a ratio between groups clearly confirmed that immune responses induced by denatured and reduced ProDer p 1 are T_H1 -oriented, even in the presence of a pro- T_H2 adjuvant such as alum.

Splenocytes from denatured and reduced ProDer p 1-vaccinated mice proliferated in a dose-dependent manner after native ProDer p 1 stimulation (data not shown). Moreover, the stimulation index values were comparable with those measured from proliferations of lymphocytes isolated from Der p 1-immunized or native ProDer p 1-immunized animals ($P > .05$). Supernatants from cultured, stimulated lymphocytes were harvested and assayed for IFN- γ and IL-5. A typical T_H2 cytokine profile characterized by high production of IL-5 and low or no secretion of IFN- γ was elicited by native ProDer p 1 or Der p 1 vaccinations ($P > .05$; Fig 4, A). Again, the T_H1 bias of the immune response induced by heat-denatured and reduced ProDer p 1 was confirmed because animals immunized with modified ProDer p 1 secreted respectively large and low amounts of IFN- γ and IL-5 on re-stimulation ($P = .037$ for IL-5 compared with ProDer p 1; $P = .019$ for IFN- γ compared with ProDer p 1). No IFN- γ or IL-5 secretion was produced by mice vaccinated with alum alone.

Prevention of airway eosinophilia by ProDer p 1 vaccinations is independent of the structure of the allergen

Differential cell counts showed the development of an important airway eosinophilia in bronchoalveolar lavage fluids from animals immunized with Der p 1 and challenged with aerosolized house dust mite extracts (Table II). On the other hand, the presence of eosinophils was not observed in bronchoalveolar lavage fluids from

TABLE I. Anti-ProDer p 1 antibodies from naive mice immunized with different antigens*

Antigen	Bleeding	IgG	IgG1	IgG2a	IgE (OD dil 10×)	IgG1/IgG2a
Der p 1	Day 14	173 ± 65	743 ± 311	< 10	0.44 ± 0.41	>74.3
	Day 21	6074 ± 3826	52686 ± 94661	< 10	0.26 ± 0.19	>5268.6
	Day 28	14336 ± 6394	67997 ± 43783	< 10	0.25 ± 0.08	>6799.7
	Day 38	24654 ± 14589	72802 ± 52011	< 10	0.26 ± 0.11	>7280.2
ProDer p 1	Day 14	320 ± 169	855 ± 575	< 10	0.48 ± 0.30	>85.5
	Day 21	3623 ± 3318	16517 ± 12317	< 10	0.47 ± 0.23	>1651.7
	Day 28	18820 ± 9569	62673 ± 2742	< 10	0.34 ± 0.18	>6267.3
	Day 38	24873 ± 11429	116566 ± 53216	< 10	0.28 ± 0.12	>11656.6
Denatured ProDer p 1	Day 14	142 ± 46	95 ± 49	89 ± 80	0.00 ± 0.00	1.06
	Day 21	1824 ± 1013	3844 ± 3854	1244 ± 703	0.00 ± 0.00	3.09
	Day 28	4443 ± 1796	11853 ± 5721†	3872 ± 2121†	0.00 ± 0.00†	3.06
	Day 38	4659 ± 2121†	10522 ± 3799†	5043 ± 3548†	0.01 ± 0.01†	2.08
Alum	Day 14	<50	<50	< 10	0.00 ± 0.00	—
	Day 21	<50	<50	< 10	0.00 ± 0.00	—
	Day 28	<50	<50	< 10	0.01 ± 0.01	—
	Day 38	<50	<50	< 10	0.01 ± 0.01	—

*Specific IgG titers were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD410_{nm} value. For IgE titers, results are expressed as OD410_{nm} values for a 1:10 dilution of sera. Results are expressed as the means of individual titers ± SDs from 10 mice.

†*P* < .001 compared with Der p 1—vaccinated or native ProDer p 1—vaccinated mice.

mice vaccinated with alum or native or denatured ProDer p 1, indicating that ProDer p 1 maintains its capacity to prevent the development of airway eosinophilia after challenge even after its thermal denaturation.

Vaccination of allergic mice with denatured and reduced ProDer p 1 redirects the unique T_H2 immune response toward a mixed T_H1-T_H2 response

To determine whether denatured and reduced ProDer p 1 immunization was able to shift a typical allergic T_H2-biased immune response to a nonallergic T_H1-biased response, Der p 1—sensitized and challenged animals were immunized with native or modified ProDer p 1. In our model, the proteins were adjuvanted with the pro-T_H1 SBAS1b adjuvant to encourage the redirecting of the immune response.

As expected, a decline of the specific IgG and IgG1 titers was observed in Der p 1—sensitized animals vaccinated with the adjuvant alone together with the maintenance of the specific IgE response (Table III). Allergic mice receiving native ProDer p 1 immunotherapy developed a specific T_H1-T_H2 immune response as reflected in a significant increase of specific IgG2a (*P* < .001 compared with allergic mice vaccinated with adjuvant alone or not vaccinated) and IgE responses (*P* < .01) and no detectable modification of the specific IgG1 titers (*P* > .1). The typical T_H2 allergic response was also redirected by vaccination with adjuvanted denatured and reduced ProDer p 1. However, the production of specific IgG2a antibodies after this treatment was more intense than that induced after native ProDer p 1 vaccinations

(*P* < .001). Moreover, no significant increase of the specific IgE response could be demonstrated after denatured and reduced ProDer p 1 immunotherapy (*P* > .1).

The mixed T_H1-T_H2 response after native ProDer p 1 immunotherapy was confirmed by the production of both IL-5 and IFN-γ by splenocytes restimulated with native ProDer p 1. Again, the T_H1 bias was more pronounced after denatured and reduced ProDer p 1 immunotherapy (Fig 4, B). This treatment elicited, respectively, a higher and a weaker production of IFN-γ (*P* = .047) and IL-5 (*P* = .025) compared with that measured after native ProDer p 1 vaccination of allergic mice. The IL-5:IFN-γ ratio decreased from 2.5 in the native ProDer p 1—treated group to 0.6 in the modified ProDer p 1—treated animals.

DISCUSSION

Because the proteolytic activity of Der p 1 has a direct influence on the immune system to drive the activation of CD4⁺ T_H2 cells,³⁻⁸ vaccination with ProDer p 1 might be suggested for immunotherapies of house dust mite allergies. Indeed, ProDer p 1 was shown to be enzymatically inactive and a low histamine release inducer, contrary to natural Der p 1.²¹ Thermal denaturation of ProDer p 1 could represent an efficient way to strengthen the hypoallergenic character of ProDer p 1, because we and others demonstrated that the allergenicity of Der p 1 was heat-sensitive.¹⁴

By direct ELISA and competition experiments, we clearly showed that thermal denaturation of ProDer p 1 influences drastically its IgE-binding capacity. Similar results were also obtained with natural Der p 1 (data not

shown) and also were previously reported for food allergens: the major potato allergen Sol t 1,²⁴ the class I chitinases PvChI from green bean, Prs a 1 from avocado,²⁵ or ovalbumin.²⁶

Although the affinity of native ProDer p 1 for specific IgE was not reduced in the presence of a reducing agent (β -mercaptoethanol), the reduction of the disulfide bonds of heat-denatured Der p 1 or ProDer p 1 further altered its allergenicity. These results suggested that the disulfide bonds of Der p 1 could be accessible only after a drastic structural change and could influence moderately the IgE-binding reactivity of the allergen.

Gel filtration chromatography profile analysis showed that, unlike natural Der p 1 or native ProDer p 1, heat-denatured and reduced Der p 1 or ProDer p 1 was polymerized solubly in physiologic conditions, forming oligomers of different sizes ranging from 120 kd to >600 kd (data not shown). The allergen polymerization into high molecular weight molecules was previously observed with heat-treated Der f 2,²⁷ ovalbumin,^{26,28} Sol t 1,²⁴ and Ara h 1.²⁹ By attenuated total reflectance–Fourier transform infrared spectroscopy, we demonstrated that thermally induced ProDer p 1 aggregation is associated with transformation of α -helical into β -sheet structures (data not shown). These β structures are likely composed of intermolecular antiparallel β -sheets, as previously observed.³⁰ This structural modification of ProDer p 1 could hide or disrupt B-cell epitopes recognized by IgE. This suggestion would be consistent with our results obtained by ELISA competition studies.

To confirm that heat-denatured and reduced ProDer p 1 is hypoallergenic, we studied its immunogenicity in dust mite–allergic and naive mice. To our knowledge, this is the first study in which the administration of denatured group 1 dust mite allergen was evaluated in mice. Because unadjuvanted Der p 1 or ProDer p 1 injections resulted in very weak antibody response (data not shown), the proteins were adjuvanted with alum.

The current study demonstrated that the regulation of ProDer p 1 specific IgE and IgG2a responses was dependent of the antigen conformation in naive mice, because the absence of IgE and the presence of IgG2a occurred only after vaccination with denatured and reduced ProDer p 1. The allergen denaturation in reducing conditions also induced a shift in cytokine profile characterized by an enhanced production of IFN- γ and decreased IL-5 release, confirming that a T_H1 - T_H2 response preferentially T_H1 -oriented was generated by denatured and reduced ProDer p 1. The incomplete shift from T_H2 to T_H1 immune response after denatured ProDer p 1 administration could be attributed to the use of alum, a strong T_H2 -inducing adjuvant. Nevertheless, our data correlated with the results obtained with other aggregated antigens such as ovalbumin.^{31–33}

In this study, ProDer p 1 was used as the plate-coated antigen in ELISA for the detection of specific antibodies and also as the restimulating antigen in lymphoproliferative assays, because the source of natural Der p 1 was too limited. Moreover, the autocatalytic pro-

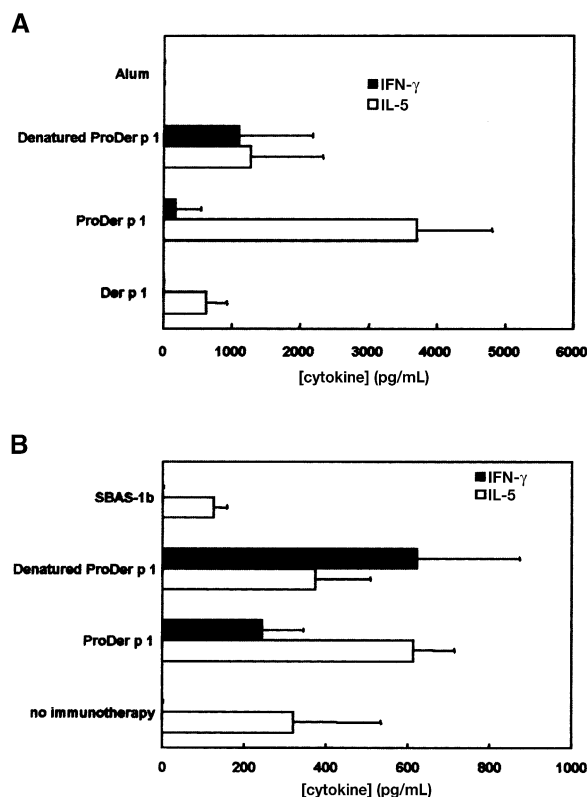


FIG 4. IL-5 and IFN- γ concentrations in supernatants from ProDer p 1–restimulated splenocytes. The production of IL-5 and IFN- γ by ProDer p 1–activated splenic cells from vaccinated naive (A) or allergic (B) mice was measured as described in the section Materials and Methods. Results are expressed as the means $\pm$ SDs from 10 mice.

cessing of ProDer p 1 generated mature Der p 1 at a very low yield (data not shown). Therefore, antibodies directed to the proregion and T cells responsible for peptides within the prosequence were not negligible. ELISA and lymphoproliferative assays performed with natural Der p 1 as coated and restimulating antigen, respectively, resulted in weaker specific antibody titers and cytokine production (data not shown). However, if the intensity of the humoral and cellular responses was modulated, the T_H1 - T_H2 polarization was not modified.

Sequence polymorphisms have been found in Der p 1 cDNA clones, and different isoforms can contribute in different ways to the antigenic and allergenic behavior of Der p 1.^{13,34,35} Notably, the Ala/Val polymorphism at residue 124 within the immunodominant region clearly affects T-cell responses. In our experiments, ProDer p 1 displayed the same polymorphism as natural Der p 1, with, notably, an alanine residue at position 124 instead of a valine residue, with the ProDer p 1 cDNA originating from the mite cultures used for the natural Der p 1 isolation.³⁶ It must be pointed out that peptide with alanine at position 124 stimulated more effectively T-cell responses of mice immunized with Der p 1.¹³

TABLE II. Cellular composition in bronchoalveolar lavage fluids after challenge with aerosolized house dust mite extracts*

Antigen	Cell number ($\times 10^5/\text{mL}$)	Cell percentage			
		Macrophages	Lymphocytes	Neutrophils	Eosinophils
Der p 1	42.2 $\pm$ 8.6†	4.8 $\pm$ 2.9	66.6 $\pm$ 15.5†	4.6 $\pm$ 4.1	23.5 $\pm$ 16.11†
ProDer p 1	14.4 $\pm$ 3.3	6.0 $\pm$ 1.4	85.8 $\pm$ 4.5	2.5 $\pm$ 0.9	5.6 $\pm$ 3.4
Denatured ProDer p 1	16.4 $\pm$ 9.3	5.1 $\pm$ 1.2	88.1 $\pm$ 3.8	1.4 $\pm$ 0.9	5.1 $\pm$ 3.3
Alum	13.9 $\pm$ 3.7	4.5 $\pm$ 2.7	92.4 $\pm$ 5.4	1.3 $\pm$ 1.6	2.0 $\pm$ 1.5

*Values are expressed as the means $\pm$ SDs from 10 mice. Total cell counts were performed in a Thoma chamber.

† $P < .05$ compared with other groups of vaccinated mice.

TABLE III. Modulation of the IgG, IgG1, IgG2a, and IgE ProDer p 1-specific responses in allergic mice vaccinated with adjuvanted native or denatured ProDer p 1*

Antigen	Bleeding	IgG	IgG1	IgG2a	IgE (OD dil 10 $\times$)	IgG/IgG2a
Der p 1-sensitized and challenged (day 38)		31679 $\pm$ 19488	148140 $\pm$ 75138	< 10	0.81 $\pm$ 0.32	>14814
ProDer p 1						
	Day 45	29385 $\pm$ 13727	114624 $\pm$ 41001	< 10	0.76 $\pm$ 0.27	>11462
	Day 59	22907 $\pm$ 5656	143215 $\pm$ 41231	2833 $\pm$ 1336	1.92 $\pm$ 0.67	50.6
	Day 73	29459 $\pm$ 9235	156234 $\pm$ 48293	8895 $\pm$ 6321	1.79 $\pm$ 0.57	27.3
	Day 88	26268 $\pm$ 9539	145892 $\pm$ 46664	9099 $\pm$ 5601	1.71 $\pm$ 0.58	16.0
Denatured ProDer p 1						
	Day 45	29158 $\pm$ 17216	104481 $\pm$ 57256	< 10	0.74 $\pm$ 0.36	>1651.7
	Day 59	27856 $\pm$ 5899	98108 $\pm$ 60463	360 $\pm$ 90	1.08 $\pm$ 0.55	272.5
	Day 73	50368 $\pm$ 16536	126483 $\pm$ 55863	36276 $\pm$ 13324	1.00 $\pm$ 0.43	3.5
	Day 88	41650 $\pm$ 13636	102072 $\pm$ 54591	34186 $\pm$ 12499	1.07 $\pm$ 0.47	2.9
SBAS-1b						
	Day 45	32033 $\pm$ 16605	143469 $\pm$ 63246	< 10	0.77 $\pm$ 0.22	>14346
	Day 59	18919 $\pm$ 8668	93392 $\pm$ 35604	< 10	0.62 $\pm$ 0.18	>9339
	Day 73	15395 $\pm$ 8017	62435 $\pm$ 37058	< 10	0.73 $\pm$ 0.34	>6243
	Day 88	9987 $\pm$ 4190	32980 $\pm$ 17069	< 10	0.57 $\pm$ 0.20	>3298

*Specific anti-ProDer p 1 IgG, IgG1, and IgG2a titers were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD_{410 nm} value. Results are expressed as the means of individual titers $\pm$ SDs from 10 mice. For IgE titers, results are expressed as OD_{410 nm} values for a 1:10 dilution of sera.

To compare the effectiveness of native or denatured ProDer p 1 for their capacity to switch typical T_H2 allergic response to T_H1 nonallergic immunity in allergic mice, the allergens were formulated with a combination of monophosphoryl lipid A and QS21 for 3 reasons. First, adjuvanted ProDer p 1 has been found to be more immunogenic than nonadjuvanted allergen, whatever the type of adjuvant used. Second, the combination of QS21 with MPL was able to enhance humoral and cellular immune responses and polarization of the T-cell response to the type 1 subtype by stimulation of IL-12 production and B7-1 expression.²³ Third, incorporation of MPL adjuvant in the immunization of rats prevented a secondary specific IgE response.³⁷

Immunization of allergic mice with native or modified adjuvanted ProDer p 1 switched the strong T_H2 immune response to a mixed T_H1-T_H2 response. Interestingly, immunotherapy using denatured and reduced ProDer p 1 induced a more pronounced T_H1 bias than vaccination with native ProDer p 1 in allergic mice, as evidenced by the comparison of the IgG1:IgG2a and IL-5:IFN- γ ratios. The weak T_H1 polarization observed after native ProDer p 1 administration likely reflects more the influence of the adjuvant than the effect of the recombinant allergen itself.

In another experiment, we clearly observed that the immunotherapeutic potential of heat and reduced ProDer p 1 vaccinations was not related only to the recombinant product. Indeed, similar results were obtained after the immunotherapeutic treatment of allergic mice with thermally denatured Der p 1. As in the case of ProDer p 1, comparison of the IgG1:IgG2a and IL-5:IFN- γ ratios indicated that vaccination with heat-denatured Der p 1 more markedly induced the T_H2-T_H1 shift than injections with a native form of Der p 1 (data not shown).

Whereas it was reported that MPL, like LPS, can enhance the generation of a specific immune response without being associated directly with the antigen,³⁸ treatment with SBAS1b adjuvant alone did not influence the T_H2-polarized immune responses.

During long-term immunotherapy in human beings³⁹ and mice,⁴⁰ the specific IgE was shown to increase during the first period of the immunotherapy. Thereafter, a clear decline of the IgE concentration was observed in conjunction with the concomitant rise in allergen-specific IgG and particularly of IgG2a isotype. During the current short-term immunotherapy, we detected an increase of specific IgE only after native ProDer p 1 administration. Remarkably, this increase of specific IgE was not

observed with denatured and reduced ProDer p 1 vaccination. The increase of ProDer p 1 specific IgG2a after therapeutic vaccination might block IgE-facilitated allergen presentation, mimicking the protective functions of IgG4 in human beings.

Several studies reported that the efficacy and safety of long-term immunotherapy were dependent on the dose of the allergens used.^{41,42} Moreover, it was recently demonstrated that restimulation of T cells from patients allergic to house dust mite with high allergen concentrations increased the number of IFN- γ -producing T cells and induced apoptosis of CD4⁺IL-4⁺ T cells and T-cell anergy.⁴³ These results suggested that high allergen exposure might induce tolerance by shifting the production of allergen-specific antibodies from IgE to IgG4. In this context, we also addressed the influence of the modified allergen dose on the induction of the T_H2-T_H1 shift in allergic mice. Groups of allergic mice vaccinated 3 times at 2-week intervals with different doses of heat-denatured Der p 1 or ProDer p 1 (1, 5, or 25 μ g) developed a mixed T_H1-T_H2 response (data not shown). Significant and dose-dependent differences were encountered in the specific IgG1 and IgG2a titers but not in the IgE antibody concentrations. In the same time, the ProDer p 1-induced proliferation of spleen cells from vaccinated mice as well as the secretion of IFN- γ and IL-5 did not increase with the injected allergen concentration. Consequently, the comparison of the IgG1:IgG2a and IL-5:IFN- γ ratios showed that the T_H1-T_H2 polarization was not allergen dose-related but was dependent on the conformation of the allergen.

The influence of the 3-dimensional structure of the allergen is critical in the development of the distinct T-cell cytokine profile by preferential usage of different antigen-presenting cells.^{44,45} The IgE-mediated antigen presentation through IgE bound to Fc ϵ receptors on B and dendritic cells facilitates allergen presentation to T cells and promotes the induction of T_H2-type cytokine and IgE formation. Consequently, heat denaturation in reducing conditions of ProDer p 1 might inhibit the IgE-facilitated antigen presentation of the allergen, resulting in a reduction of T_H2 polarization of the immune response. Immunotherapy in patients with allergy has been suggested to induce allergen-specific T_H1 cells that might suppress the effector function of allergen-specific T_H2 cells and alter allergen-specific IgE. Although reduced T_H2-type cytokine production by allergen-specific T cells after immunotherapy has been demonstrated in most studies,⁴⁶ increased T_H1-type cytokines are not always concomitant.^{47,48} The current study suggested that immunotherapy with SBAS1b-adjuvanted ProDer p 1 could trigger the induction of ProDer p 1-specific T_H1-type cells to counterbalance the T_H2 cell-induced allergic reaction. However, treatment of allergic mice with the adjuvanted recombinant allergen might also generate CD8⁺ regulatory T cells, as previously shown during allergen immunotherapy in human beings and animals; this area is also a source of IFN- γ , leading to the rise in IgG2a levels.^{49,50}

In conclusion, this report demonstrated that ProDer p 1 conformation plays a critical role in its allergenicity. Aggregation of ProDer p 1 by thermal denaturation is an efficient way to obtain a hypoallergenic variant of ProDer p 1 with a dramatic reduction of IgE reactivity. Denatured ProDer p 1 may be a valuable tool for improved immunotherapy for the treatment of house dust mite allergy.

We thank Louis Delhay for excellent animal handling, and Michèle Haumont, Lina Mazzu and Margarita Jurado for their excellent technical assistance.

REFERENCES

1. Platt-Mills TAE, Chapman MD. Dust mites: immunology, allergic disease and environmental control. *J Allergy Clin Immunol* 1987;80:755-75.
2. Krilis S, Baldo BA, Sutton R, Basten A. Antigens and allergens from the common house dust mite *Dermatophagoides pteronyssinus*. I: demonstration of multiple allergens by immunochemical and biologic analysis. *J Allergy Clin Immunol* 1984;74:132-41.
3. Shakib F, Gough L. The proteolytic activity of Der p 1 selectively enhances IgE synthesis: a link between allergenicity and cysteine protease activity. *Clin Exp Allergy* 2000;30:751-2.
4. Gough L, Schulz O, Sewell HF, Shakib F. The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999;190:1897-902.
5. Schulz O, Sewell HF, Shakib F. Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med* 1998;187:271-5.
6. Ghaemmaghami AM, Robins A, Gough L, Sewell HF, Shakib F. Human T cell subset commitment determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN-gamma. *Eur J Immunol* 2001;31:1211-6.
7. Wan H, Winton HL, Soeller C, Gruenert DC, Thompson PJ, Cannell MB, et al. Quantitative structural and biochemical analyses of tight junction dynamics following exposure of epithelial cells to house dust mite allergen Der p 1. *Clin Exp Allergy* 2000;30:685-98.
8. King C, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J Immunol* 1998;161:3645-51.
9. Yssel H, Johnson KE, Schneider PV, Wideman J, Terr A, Kastelein R, et al. T cell activation-inducing epitopes of the house dust mite allergen Der p 1: proliferation and lymphokine production patterns by Der p I-specific CD4⁺ T cell clones. *J Immunol* 1992;148:738-45.
10. O'Brien RM, Thomas WR, Tait BD. An immunogenetic analysis of T-cell reactive regions on the major allergen from the house dust mite, Der p I, with recombinant truncated fragments. *J Allergy Clin Immunol* 1994;93:628-34.
11. Higgins JA, Thorpe CJ, Hayball JD, O'Hehir RE, Lamb JR. Overlapping T-cell epitopes in the group I allergen of *Dermatophagoides* species restricted by HLA-DP and HLA-DR class II molecules. *J Allergy Clin Immunol* 1994;93:891-9.
12. Harris SJ, Roth JF, Savage N, Woodrow SA, Hemingway IK, Hoyne GF, et al. Prediction of murine MHC class I epitopes in a major house dust mite allergen and induction of T1-type CD8⁺ T cell responses. *Int Immunol* 1997;9:273-80.
13. Jamnicki AG, Thomas WR. Stimulatory and inhibitory epitopes in the T cell responses of mice to Der p 1. *Clin Exp Allergy* 2002;32:942-50.
14. Lombardero M, Heymann PW, Platts-Mills TA, Fox JW, Chapman MD. Conformational stability of B cell epitopes on group I and group II *Dermatophagoides* spp. allergens: effect of thermal and chemical denaturation on the binding of murine IgG and human IgE antibodies. *J Immunol* 1990;144:1353-60.
15. Greene WK, Thomas WR. IgE binding structures of the major house dust mite allergen Der p I. *Mol Immunol* 1992;29:257-62.
16. Collins SP, Ball G, Vonarx E, Hosking C, Shelton M, Hill D, et al. Absence of continuous epitopes in the house dust mite major allergens

- Der p 1 from *Dermatophagoides pteronyssinus* and Der f 1 from *Dermatophagoides farinae*. *Clin Exp Allergy* 1996;26:36-42.
17. Gregoire C, Chapman MD. Recombinant allergens. *Clin Rev Allergy Immunol* 2001;21:215-27.
 18. Chapman MD, Smith AM, Vailes LD, Pomes A. Recombinant allergens for immunotherapy. *Allergy Asthma Proc* 2002;23:5-8.
 19. Ferreira F, Wallner M, Breiteneder H, Hartl A, Thalhamer J, Ebner C. Genetic engineering of allergens: future therapeutic products. *Int Arch Allergy Immunol* 2002;128:171-8.
 20. Akdis CA, Blaser K. Regulation of specific immune responses by chemical and structural modifications of allergens. *Int Arch Allergy Immunol* 2000;121:261-9.
 21. Massaer M, Mazzu P, Haumont M, Magi M, Daminet V, Bollen A, et al. High-level expression in mammalian cells of recombinant house dust mite allergen ProDer p 1 with optimized codon usage. *Int Arch Allergy Immunol* 2001;125:32-43.
 22. Jacquet A, Magi M, Haumont M, Jurado M, Garcia L, Bollen A. Absence of immunoglobulin E synthesis and airway eosinophilia by vaccination with plasmid DNA encoding ProDer p 1. *Clin Exp Allergy* 2003;33:218-25.
 23. Moore A, McCarthy L, Mills KH. The adjuvant combination monophosphoryl lipid A and QS21 switches T cell responses induced with a soluble recombinant HIV protein from Th2 to Th1. *Vaccine* 1999;17:2517-27.
 24. Koppelman SJ, Van Koningsveld GA, Knulst AC, Gruppen H, Pigman IG, de Jongh HH. Effect of heat-induced aggregation on the IgE binding of patatin (Sol t 1) is dominated by other potato proteins. *J Agric Food Chem* 2002;50:1562-8.
 25. Sanchez-Monge R, Blanco C, Perales AD, Collada C, Carrillo T, Aragoncillo C, et al. Class I chitinases, the panallergens responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating. *J Allergy Clin Immunol* 2000;106:190-5.
 26. Kim MJ, Lee JW, Yook HS, Lee SY, Kim MC, Byun MW. Changes in the antigenic and immunoglobulin E-binding properties of hen's egg albumin with the combination of heat and gamma irradiation treatment. *J Food Prot* 2002;65:1192-5.
 27. Korematsu S, Tanaka Y, Hosoi S, Koyanagi S, Yokota T, Mikami B, et al. C8/119S mutation of major mite allergen Derf-2 leads to degenerate secondary structure and molecular polymerization and induces potent and exclusive Th1 cell differentiation. *J Immunol* 2000;165:2895-902.
 28. Koch C, Jensen SS, Oster A, Houen G. A comparison of the immunogenicity of the native and denatured forms of a protein. *Acta Pathol Microbiol Immun Scand* 1996;104:115-25.
 29. Koppelman SJ, Bruijnzeel-Koomen CA, Hessing M, De Jongh HH. Heat-induced conformational changes of Ara h 1, a major peanut allergen, do not affect its allergenic properties. *J Biol Chem* 1999;274:4770-7.
 30. Van Stokkum IH, Linsdell H, Hadden JM, Haris PI, Chapman D, Bloemendal M. Temperature-induced changes in protein structures studied by Fourier transform infrared spectroscopy and global analysis. *Biochemistry* 1995;34:10508-18.
 31. Salgado J, Casadevall G, Puignero V, Queralto J. Characterization of allergoids from ovalbumin in vitro and in vivo. *Immunobiology* 1996;196:375-86.
 32. Yang X, Gieni RS, Mosmann TR, HayGlass KT. Chemically modified antigen preferentially elicits induction of Th1-like cytokine synthesis patterns in vivo. *J Exp Med* 1993;178:349-53.
 33. Speidel K, Osen W, Faath S, Hilgert I, Obst R, Braspenning J, et al. Priming of cytotoxic T lymphocytes by five heat-aggregated antigens in vivo: conditions, efficiency, and relation to antibody responses. *Eur J Immunol* 1997;27:2391-9.
 34. Chua KY, Kehal PK, Thomas WR. Sequence polymorphisms of cDNA clones encoding the mite allergen Der p 1. *Int Arch Allergy Immunol* 1993;101:364-8.
 35. Smith W, Hales BJ, Jarnicki AG, Thomas WR. Allergens of wild house dust mites. environmental Der p 1 and Der p 2 sequence polymorphisms. *J Allergy Clin Immunol* 2001;107:985-92.
 36. Jacquet A, Haumont M, Massaer M, Daminet V, Garcia L, Mazzu P, et al. Biochemical and immunological characterization of a recombinant precursor form of the house dust mite allergen Der p 1 produced by *Drosophila* cells. *Clin Exp Allergy* 2000;30:677-84.
 37. Wheeler AW, Marshall JS, Ulrich JT. A Th1-inducing adjuvant, MPL, enhances antibody profiles in experimental animals suggesting it has the potential to improve the efficacy of allergy vaccines. *Int Arch Allergy Immunol* 2001;126:135-9.
 38. Gustafson GL, Rhodes MJ. A rationale for the prophylactic use of monophosphoryl lipid A in sepsis and septic shock. *Biochem Biophys Res Commun* 1992;182:269-75.
 39. Van Oosterhout AJM, Van Esch B, Hofman G, Hofstra CL, Van Ark I, Nijkamp FP, et al. Allergen immunotherapy inhibits airway eosinophilia and hyperresponsiveness associated with decreased IL-4 production by lymphocytes in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 1998;19:622-8.
 40. Djurup R. The subclass and nature of the IgG antibody response in patients undergoing allergen specific immunotherapy. *Allergy* 1985;40:469-86.
 41. Ewbank PA, Murray J, Sanders K, Curran-Everett D, Dreskin S, Nelson HS. A double-blind, placebo-controlled immunotherapy dose-response study with standardized cat extract. *J Allergy Clin Immunol* 2003;111:155-61.
 42. Andre C, Perrin-Fayolle M, Grosclaude M, Couturier P, Basset D, Cornillon J, et al. A double-blind placebo-controlled evaluation of sublingual immunotherapy with a standardized ragweed in patients with seasonal rhinitis: evidence for a dose-response relationship. *Int Arch Allergy Immunol* 2003;131:111-8.
 43. Gardner LM, O'Hehir RE, Rolland JM. High dose allergen stimulation of T cells from house dust mite allergic subjects induces expansion of IFN-gamma+ T cells, apoptosis of CD4+IL4+ T cells and T cell anergy. *Int Arch Allergy Immunol* 2004;133:1-13.
 44. Akdis CA, Blesken T, Wymann D, Akdis M, Blaser K. Differential regulation of human T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants. *Eur J Immunol* 1998;28:914-25.
 45. Akdis CA, Blaser K. Bypassing IgE and targeting T cells for specific immunotherapy of allergy. *Trends Immunol* 2001;22:175-8.
 46. Akoum H, Tsicopoulos A, Vormg H, Wallaert B, Dessaint JP, Joseph M, et al. Venom immunotherapy modulates interleukin-4 and interferon-gamma messenger RNA expression of peripheral T lymphocytes. *Immunology* 1996;87:593-8.
 47. Ebner C, Siemann U, Bohle B. Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from Th2 to Th1 in T cell clones specific for Phl p 1, a major grass pollen allergen. *Clin Exp Allergy* 1997;27:1007-15.
 48. Jutel M, Pilcher WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in a decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995;154:4187-94.
 49. Kemeny DM, Noble A, Holmes BJ, Diaz-Sanchez D, Lee TH. The role of CD8+ T cells in immunoglobulin E regulation. *Allergy* 1995;50:9-14.
 50. Draghi M, Jarman ER, Grifantini R, Galli-Stampino L, Lamb JR, Valiante NM, et al. Different profile of CD8+ effector T cells induced in Der p 1-allergic and naive mice by DNA vaccination. *Eur J Immunol* 2002;32:3720-8.