

Der p 2 isoallergens have different allergenicity, and quantification with 2-site ELISA using monoclonal antibodies is influenced by the isoallergens

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Summary

Background Der p 2 isoallergens have been reported and the possibility of different allergenicity has also been suggested. In addition, the quantification with 2-site ELISA may be affected by the isoallergens.

Objectives Two different recombinant Der p 2 (rDer p 2) isoallergens were compared in terms of human IgE responses and the reliability of quantification of them with two-site ELISA kits which use monoclonal antibodies (mAbs) as capture and detection of Der p 2.

Methods Seven different Der p 2 cDNA from the cultured *Dermatophagoides pteronyssinus* (DP) were cloned and polymorphism in nine amino acid residues was found. Two different recombinant isoallergens (rDer p 2A and rDer p 2B) were expressed and compared to their human IgE immune responses by ELISA and the ELISA inhibition test with 23 sera of DP-allergic patients. The reliability of quantification of two different available 2-site ELISA kits, which used mAbs for capture and detection of Der p 2, was evaluated.

Results The ELISA optical density of rDer p 2B-specific IgE (sIgE) was higher than that of rDer p 2A ($P < 0.001$). The ELISA inhibition curve of rDer p 2B sIgE in pool I sera ($n = 5$; high sIgE both to rDer p 2A and rDer p 2B) did not show any differences in the 50% inhibition concentration and maximum inhibitory percentage of rDer p 2A and rDer p 2B sIgE. However, with pool II sera ($n = 5$; markedly higher sIgE to rDer p 2B than rDer p 2A), the 50% inhibitory concentrations (10 $\mu\text{g/mL}$ vs. 40 ng/mL) and maximum inhibitory percentage (61% vs. 99%) of rDer p 2B sIgE with the two recombinant isoallergens were quite different. rDer p 2B could be quantified with two different 2-site ELISA kits, but rDer p 2A was detected by only one kit.

Conclusion We conclude that isoallergens of Der p 2 may have different IgE immune responses. Quantification of Der p 2 with 2-site ELISA kits that adopted mAbs, might be affected by the prevalent form of the isoallergens in reservoir dust.

Key words *Dermatophagoides pteronyssinus*, Der p 2, isoallergen, recombinant allergen

Submitted 24 April 2001; revised 25 June 2001; accepted 24 January 2002

Introduction

Polymorphisms in amino acid sequence have been found for major allergens of the mite and pollens and it may influence the IgE and T cell epitopes recognized by allergic patients [1–4]. Der p 2, which is one of the major allergens of *Dermatophagoides pteronyssinus* (DP), consists of 129 amino acid residues. Studies on the allergenicity and affinities of the monoclonal IgG antibodies (mAbs) would be very important for the clinical application of Der p 2 isoallergens or of their peptides in the development of new therapeutic and diagnostic tools. Several Der p 2 isoallergens have been reported with differences of 3–7 amino acid residues [4–6], moreover, the prevalent form of Der p 2 isoallergens may vary geographically.

some investigators have suggested the possibility of differences in the allergenicity of house dust mite (HDM) group 2 isoallergens [5], and the discrepancy in the affinities of mAbs to recombinant group 2 isoallergens have been demonstrated [6–10]. There are, however, no unequivocal results, which support differences in the allergenicity of Der p 2 isoallergens among the house dust mite atopic patients.

In this study, we evaluated the discrepancies in human IgE allergic responses according to the recombinant Der p 2 (rDer p 2) isoallergens. The reliability of the two different 2-site ELISA kits that use mAbs for quantification of rDer p 2 isoallergens was also evaluated.

Materials and methods

Subjects

We enrolled 23 DP-sensitized respiratory allergic patients, with bronchial asthma and/or allergic rhinitis. Their mean age was 26.2 ± 10.0 years and the male : female ratio was 11:12. All of

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them had DP-specific IgE. Their DP-specific IgEs, which were measured using the CAP test (Pharmacia, Uppsala, Sweden), were higher than 7 KU/L (mean value; 44.5 ± 31.1 KU/L).

Cloning and production of recombinant isoallergens

We cloned Der p 2 cDNA using RT-PCR based methods using sense and anti-sense primers corresponding to the N- and C-terminal site of Der p 2. The primers were designed on the basis of the reported cDNA sequence of Der p 2 [11]. Messenger RNA was extracted from cultured DP which were obtained from domestic environments in Seoul, Korea. cDNA for Der p 2 (400 bp) was subcloned into pet21a vector (Novagene, Madison, WI, USA). His-tagged Der p 2 peptide (rDer p 2) was expressed in BL21DE3 *E. Coli* transformed by Der p 2 cDNA inserted pET21a vector. The *E. Coli* strains were cultivated in SOC medium (20 mM Glucose, 2% bactotrypton, 0.5%, yeast extract, and 0.5% NaCl) containing 50 mg/mL ampicillin at 37 °C. The same medium (100 mL) was inoculated with 10 mL of this preculture in a 500-mL flask. After 1.5 h, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce the cells, and incubation was continued for 4 h at 30 °C. Cells were harvested by centrifugation, resuspended in TE buffer (100 mM Tris-hcl, 10 mM EDTA, at pH 7.5), sonicated at 7000 g for 15 min, solubilized with 6 M urea containing PBS, and dialysed against PBS for 12 h to obtain the correctly refolded molecules. The dialysate containing refolded Der p 2 was applied and purified through a Ni-NTA agarose affinity column. The sample was purified according to the method provided by Qiagen (Valencia, CA, USA). The protein contents of the recombinant allergens were assayed with a protein assay kit (Bio-Rad, Hercules, CA, USA).

IntraDermal skin test with recombinant isoallergenic Der p 2

IntraDermal skin testing with isoallergenic rDer p 2A (6th clone) and rDer p 2B (7th clone) were performed in 23 DP-sensitized atopic patients and 14 non-atopic control subjects. The recombinant allergens were diluted to a final concentration of 1 μ g/mL in 0.3% phenol saline – 0.03% human serum albumin and used for intraDermal testing. 0.1 mg/mL histamine was used as a positive control (Allergopharma, Reinbek, Germany).

ELISA and ELISA inhibition test

rDer p 2-specific IgE (sIgE) in patients' sera was measured by ELISA. Polystyrene microplates (Costar, Cambridge, MA, USA) were coated with 50 μ L of 1 μ g/mL of recombinant allergen in 0.05 M carbonate buffer (pH 9.6) overnight at 4 °C. Non-specific binding was blocked with 1% bovine serum albumin-PBS-T (137 mM NaCl, 1.8 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 27 mM KCl, and 0.1% Tween-20, at pH 7.4) for 1 h. Fifty μ L of patient's sera was incubated for 1 h at room temperature. After washing three times with PBS-T, 50 μ L of 1:1000 diluted biotin-labelled goat anti-human IgE (Vector, Burlingame, CA, USA) was added to the wells and incubation continued for 1 h at room temperature. The wells were then washed three times with PBS-T and incubated with 1:1000 v/v streptavidin-peroxidase (Sigma, St Louis, MO, USA) for 30 min and again washed. Colorimetric reaction was developed by adding 50 μ L of ABTS solution [25 mg of 2,2-azino-bis-3-ethylbenzthiazoline-sulfonic acid in 50 mM citrate phosphate buffer 50 μ L of

30% H_2O_2] for 5 min. The reaction was stopped by the addition of 50 μ L of 2 N NaN_3 , and the optical densities measured at 410 nm using an automated UV microplate spectrophotometer (Dynatec, Alexandria, CA, USA). All assays were performed in duplicate.

For ELISA inhibition, the non-diluted pooled sera from five atopic patients was preincubated with various concentrations isoallergens (10^{-3} , 3×10^{-3} , 10^{-2} , 3×10^{-2} , 10^{-1} , 3×10^{-1} , 1, 3, 10, 30 μ g/mL) of each recombinant, overnight at 4 °C, and isoallergen sIgE in the preincubated pooled sera was measured with the same methods described above.

Measurement of Der p 2 with two-site ELISA

rDer p 2 concentration was measured using two different 2-site ELISA kits; one was provided by Indoor Biotechnologies Ltd (Cardiff, UK) [12], and the other kit was kindly donated by Yasueda et al. [13]. The former kit uses 1D8 mAb as a capture antibody and 7A1 mAb as a detection antibody, and the latter kit used P2A24 mAb as a coating antibody and P2A91 mAb as a detection antibody. The procedures were performed according to the manufacturer's instructions.

Results

Isoallergens of Der p 2

The seven clones of Der p 2 were analysed and seven different isoallergens found (Table 1). Nine differences in amino acid residues were identified, such as, Val₆/Leu₆, Pro₂₆/Thr₂₆, Phe₃₅/Tyr₃₅, Val₄₀/Leu₄₀, Thr₄₇/Ser₄₇, Tyr₇₅/Ser₇₅, Met₁₁₁/Leu₁₁₁, Asp₁₁₄/Asn₁₁₄ and Ala₁₁₈/Gly₁₁₈. The frequencies of the residue difference are summarized in Table 2. The 7th clone shared seven of the nine dominant amino acid residues (Pro₂₆, Phe₃₅, Leu₄₀, Ser₄₇, Tyr₇₅, Leu₁₁₁, and Asn₁₁₄), whereas, the 6th clone shared only two of the dominant amino acid residues (Tyr₇₅ and Ala₁₁₈). rDer p 2 as produced was based on the 6th clone (rDer p 2A) and the 7th clone (rDer p 2B), these probably represent the minor and predominant forms of Der p 2 isoallergens in Korea.

Intradermal skin reactivity of rDer p 2 isoallergens

All 23 DP sensitized allergic patients showed skin responsiveness to rDer p 2A and rDer p 2B by intradermal skin test, while none of the 14 non-atopic subjects showed positive responses. The mean weal sizes of rDer p 2A and rDer p 2B were similar (17.0 ± 4.4 vs. 17.9 ± 4.9 mm).

sIgE to rDer p 2 isoallergens

When the ELISA optical density of rDer p 2 sIgE in DP-allergic patients was compared, a significant correlation was found between rDer p 2A and rDer p 2B sIgE levels ($r = 0.716$, $P < 0.001$), but the geometric mean optical density for rDer p 2B sIgE was significantly higher than that of the rDer p 2A in respiratory allergic patients (0.386 vs. 1.001, $P < 0.001$) (Fig. 1).

ELISA inhibition test

DP-allergic patients were categorized into two groups according to their ELISA OD values of rDer p 2-specific IgE. In group 1, the OD value of rDer p 2B sIgE did not exceed twice

Table 1. Amino acid sequences of 8 isoallergenic rDer p 2A and rDer p 2B

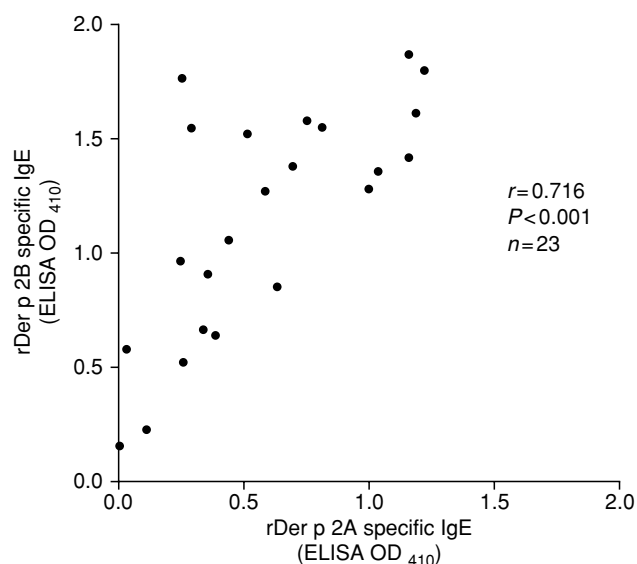
	10	20	30	40	50
	DQVDVKDCAN	HEIKKVLVPG	CHGSEPCIIH	RGKPFQLEAV	FEANQNTKTA
1	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
2	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
3	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
4	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
5	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
6	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
7	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
	60	70	80	90	100
	KIEIKASIDG	LEVDPGIDP	NACHYMKCPL	VKGQQYDIKY	TWNVPKIAPK
1	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
2	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
3	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
4	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
5	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
6	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
7	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●
	110	120	130		
	SENVVVTVKV	MGDDGVLACA	IATHAKIRD>		
1	●●●●●●●●●●	L●●●●●●●●●●	●●●●●●●●●●>		
2	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●>		
3	●●●●●●●●●●	L●●●●●●●●●●	●●●●●●●●●●>		
4	●●●●●●●●●●	L●●●●●●●●●●	●●●●●●●●●●>		
5	●●●●●●●●●●	L●●●●●●●●●●	●●●●●●●●●●>		
6	●●●●●●●●●●	●●●●●●●●●●	●●●●●●●●●●>		
7	●●●●●●●●●●	L●●●●●●●●●●	●●●●●●●●●●>		

rDer p 2A; 6th clone, rDer p 2B; 7th clone.

Table 2. Incidence of amino acid variation in rDer p 2 isoallergens in Korea

Amino-acid residue	Polymorphism	Incidence
6	K/L	5/2
26	P/T	5/2
35	F/Y	6/1
40	V/L	3/4
47	T/S	2/5
75	Y/S	6/1
111	M/L	2/5
114	D/N	3/4
118	L/G	6/1

that of the rDer p 2A sIgE, and in group 2, the OD value of rDer p 2B sIgE exceeded three times that of the rDer p 2A. The inhibition patterns of rDer p 2B sIgE were compared in pooled sera of group 1 ($n=5$) and group 2 ($n=5$), which were incubated with different concentrations of rDer p 2A and rDer p 2B, respectively. In the case of the pooled sera of group 1 patients, the inhibition curves of rDer p 2B sIgE with rDer p 2A and rDer p 2B were similar. The 50% inhibitory concentrations of rDer p 2A and rDer p 2B were the same (0.02 µg/mL) and the maximum inhibition percentages of rDer p 2B sIgE were similar (96% vs. 99%) (Fig. 2a). However, the inhibition curves of rDerp2b sIgE with rDer p 2A and rDer p 2B were quite different in the pooled sera of group 2 patients. The 50% inhibitory concentrations of rDer p 2A and rDer p 2B were 10 µg/mL and 40 ng/mL, respectively, and the maximum inhibition percentages of rDer p 2B sIgE with rDer p 2A and rDer p 2B were 61% and 99% (Fig. 2b).

**Fig. 1.** ELISA OD of anti-rDer p 2A IgE and anti-rDer p 2B IgE in DP-sensitized respiratory allergic patients.

Two-site ELISA measurements of rDer p 2

rDer p 2B was detected with both two different 2-site ELISA kits, one using 1D8 and 7A1 mAbs, and another using P2A24 and P2A91 mAbs. rDer p 2A was detected with the kit using P2A24 and P2A91 mAbs but was not detected with the kit using 1D8 and 7A1 mAbs (Fig. 3).

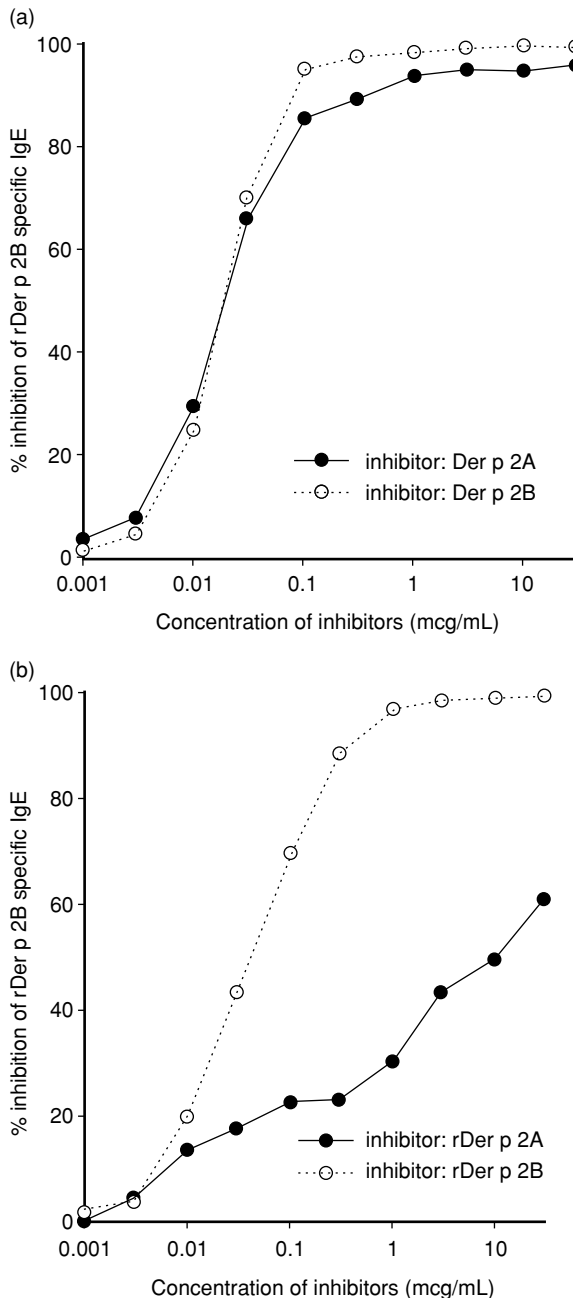


Fig. 2. ELISA inhibition curves of rDer p 2B-specific IgE with rDer p 2A and rDer p 2B antigens. (a) and (b) showed the inhibition curves with the pooled sera ($n = 5$) of patients with similar levels of Der p 2A and rDer p 2B-specific IgE (group 1) and the patients with more than three times higher level of Der p 2B-specific IgE than that of Der p 2A (group 2), respectively.

Discussion

In this study, we demonstrate that specific human IgE immune responses and affinities of mAbs might be different according to the Der p 2 isoallergens. Nine different residues in the full amino acid sequences of Der p 2 were associated with seven different Der p 2 isoallergens in this work. When these were compared with each other, Leu₆, Thr₂₆, Phe₃₅, Leu₄₀, Ser₄₇, Tyr₇₅, Leu₁₁₁,

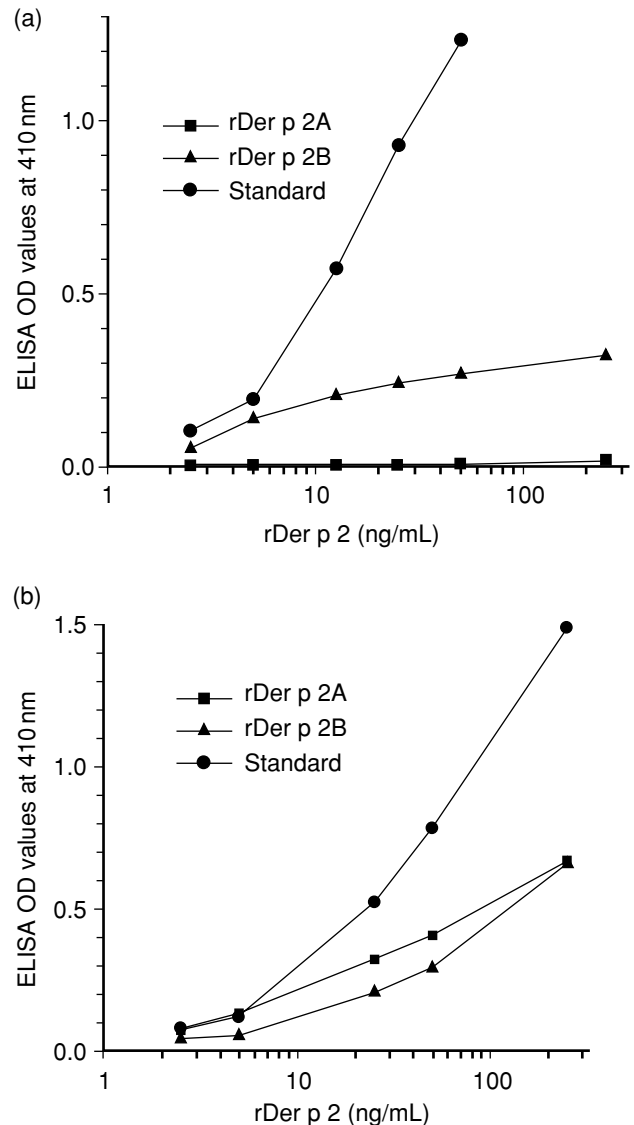


Fig. 3. Measurement of rDer p 2A (■), rDer p 2B (▲) and standard native Der p 2 (●) concentration with two different two-site ELISA kits; one from Indoor Biotechnologies (a) and the other from Yasueda et al. [13] (b), respectively.

Asn₁₁₄, and Gly₁₁₈ were found to be the prevalent polymorphic residues of Der p 2 in Korea. rDer p 2B shared seven of nine dominant amino acid residues and rDer p 2A shared only two sites of dominant residues. Therefore, we postulated that rDer p 2B would represent the prevalent form and rDer p 2A was the less prevalent form of Der p 2 in Korea. In this study, the IgE immune response to the prevalent isoallergen was found to be more profound than that to the less prevalent isoallergen. Our results imply that selecting prevalent isoallergens is important in terms of the regional development of new diagnostic and treatment modalities, using recombinant Der p 2 or its peptides. In this study, intradermal skin test was done and found that rDer p 2 would be a reliable diagnostic allergen with high sensitivity and specificity. Our results are consistent with the reports of Lynch [14, 15] who demonstrated that rDer p 2 would

be one of reliable diagnostic reagent for the DP-sensitized allergic patients. IgE epitopes of Der p 2 has been known as conformational structure [16, 17]. The conformational structure of rDer p 2 and native Der p 2 are very similar and the human IgE affinity of rDer p 2 was not different from native Der p 2 [8, 18]. These results suggest that rDer p 2 can be used for development of diagnostic and therapeutic new modality. But contrary to the ELISA test, we could not find differences in the weal size of intradermal test. Although intradermal test is more sensitive than serologic test, weal size do not well reflect the quantity of specific IgE. The skin responses is markedly affected by individual histamine responsiveness and releasability of mast cell and several different the grading systems, such as weal size, weal area, or allergen histamine ratio, have been used. End-point titration may complement the reliability of the quantification of sIgE but the clinical reliability is also limited [19].

Several isoallergens of Der p 2 have been reported worldwide, and the polymorphic amino acid sequences of rDer p 2A and rDer p 2B are similar to these previous reports. The polymorphism of Val₄₀/Leu₄₀, Thr₄₇/Ser₄₇, and Asp₁₁₄/Asn₁₁₄ were reported by Chua [5], and Hakkaart [6] also reported the polymorphism of Pro₂₆/Ser₂₆ (Pro was substituted with Thr in this study), Thr₄₇/Ser₄₇ and Asp₁₁₄/Asn₁₁₄. Recently Smith et al. [4] also reported the polymorphism of Val₄₀/Leu₄₀, Thr₄₇/Ser₄₇, Ala₉₈/Thr₉₈, Met₁₁₁/Leu₁₁₁, Asp₁₁₄/Asn₁₁₄, Val₁₁₆/Ala₁₁₆, and Ile₁₂₇/Leu₁₂₇ in Perth, Australia. In this study, we showed additional polymorphisms of Der p 2, such as Val₆/Leu₆, Phe₃₅/Tyr₃₅, Tyr₇₅/Ser₇₅, and Ala₁₁₈/Gly₁₁₈. Therefore, it is likely that the polymorphisms of amino acid sequences in Der p 2 isoallergens may be extensive and different world-wide. The IgE epitopes of Der p 2 are known as a conformational structures [16, 17], and its three disulphide bonds (residues 21–27, 73–78 and 8–119) and residues 45–48 are important for the recognition of human IgE [8, 9, 20, 21]. So, at least the polymorphism of Pro₂₆/Ser₂₆ and Thr₄₇/Ser₄₇ might be important factors determining the IgE response. But the polymorphisms such as Phe₃₅/Tyr₃₅, Met₁₁₁/Leu₁₁₁, Asp₁₁₄/Asn₁₁₄ and Ala₁₁₈/Gly₁₁₈ might be important for sIgE epitopes and we could not excluded the possibility. Our results show that, in addition to the disulphide bonds, the substitution of individual amino acid residues influence IgE binding affinities.

In this study, we also demonstrated that the reliability of the 2-site ELISA kits is dependent on isoallergens. These findings are consistent with that of others. According to their study, the bindings of mAbs, such as 1D8 and other several mAbs were influenced by the polymorphism at position 114, and replacement of aspartic acid with asparagine ensure that the mAbs reactivities but not other substitutions, such as 26th and 47th residues, had no effect on the affinities of mAbs [6, 10]. The affinity of 7A1 mAb to Der p 2 was markedly affected by the mutants of 45–48th or 22–26th residue mutants [9]. We therefore thought that the Pro₂₆/Ser₂₆, Thr₄₇/Ser₄₇, Asp₁₁₄/Asn₁₁₄ polymorphisms might affects the affinities of 1D8 and 7A1 mAbs which used as the capture and detection mAbs of 2-site ELISA kit in this study.

Taken together, we believe that these results demonstrate the extent to which the variability of human IgE immune response and mAbs affinities of isoallergens would be different according to their subtypes. We also believe that these results must be considered before the clinical application of rDer p 2A and Der p 2-specific mAbs as diagnostic or therapeutic tools.

Acknowledgement

This study was supported by the Korea Science and Engineering Foundation, 1997 (KOSEF-97-04-03-02-01-3).

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