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IgE Responsiveness to *Dermatophagoides farinae* in Young Asthmatic Children: IgE Binding Study Using Recombinant Allergens of Der f 1, Der f 2 and Mutant Proteins of Der f 2

Key Words

Asthma
IgE antibodies
Dermatophagoides farinae
Der f 1, recombinant
Der f 2, recombinant
Der f 2 mutant protein
House dust mite
Radioallergosorbent test

Abstract

IgE reactivities to two recombinant (r) allergens, rDer f 1 and rDer f 2, synthesized by cDNA clones from *Dermatophagoides farinae* (Df) were analyzed using sera of Df-sensitive asthmatic patients of varying ages. Positive RAST responses to rDer f 1 and rDer f 2 were found in 88 and 80%, respectively, with sera from young asthmatic children aged 0–1. Positive RAST rates to these antigens increased to 100% up to 5 years of age. There was a significant correlation between RAST levels of rDer f 1 and rDer f 2 in these young children aged 0–1. IgE reactivities to four mutant proteins of rDer f 2, which have at least one amino acid substitution, were similarly examined in the asthmatic sera. IgE reactivity to D7A, in which Asp7 was replaced by Ala, was reduced to 30–60% compared to the rDer f 2. IgE binding to C8/I19S, in which both Cys8 and Cys119 were replaced by Ser, lacking a disulfide bond between Cys8 and Cys119, was reduced almost to a background level at all ages. In contrast, A72L and A120L, in which Ala72 and Ala120 were substituted for Leu, respectively, almost retained the same IgE binding activity as the rDer f 2 at all ages. These results suggest that Der f 1 and Der f 2 are important antigens associated with early sensitization to house dust mite in young asthmatic children. In addition, the disulfide bond between Cys8 and Cys119 and the N-terminal region including the 7th amino acid residue are considered to maintain IgE epitopes of the Der f 2 allergen.

Introduction

Allergic sensitization to house dust mite (HDM) is believed to play an important role in the development of asthma in children, because a high level of IgE antibodies directed against HDM shows a significant correlation not only to a clinical history of asthma, but also to provocation test results with these antigens [1–4]. In the past 2 decades, the nature of HDM antigens has been extensively studied by

physicochemical and immunochemical techniques, and more than 30 IgE binding components have been identified from body and fecal extracts of *Dermatophagoides farinae* (Df) or *Dermatophagoides pteronyssinus* [1, 5–13]. Of these components, two low molecular weight components, Der 1 and Der 2, have been defined as major allergenic proteins [7–9].

It is well known that asthmatic children are often sensitized to HDM during early childhood [14, 15]. However, lit-

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the information is available as to what antigenic constituents of HDM are associated with the initial sensitization of these children. We have recently obtained recombinant (r) Der f 1 and rDer f 2 antigens produced by cDNA clones selected from Df cDNA libraries [16], and four mutant proteins of the rDer f 2 allergens, which possess at least one amino acid substitution each. In this study, to clarify the role of Der 1 and Der 2 in early sensitization to HDM, we examined the IgE reactivities to rDer f 1 and rDer f 2 by RAST using sera of asthmatic children of varying ages. Furthermore, to gain an insight into epitopes of Der f 2, we compared the binding activity of IgE antibodies to four rDer f 2 mutant proteins in these sera.

Materials and Methods

Antigens

The lyophilized mite body extracts prepared from isolated mite bodies of Df were kindly supplied by Torii (Tokyo, Japan), and used as Df antigen [6]. The Der f 1 and Der f 2 contents of this antigen were 0.53 and 0.48% by weight, respectively. Purified Der f 1 and Der f 2 preparations were isolated from mite body extract according to the method described by Yasuda et al. [9], and designated as native (n) Der f 1 and nDer f 2 in this study.

Expression and Purification of Recombinant Der f 2

Expression and purification procedures of recombinant Der f 2 were described previously [17]. Briefly, *Escherichia coli* BL21 cells harboring a pGEMEX1 (Promega, Wisc., USA) derivative containing each of the cDNAs for Der f 2 under the control of the T7 promoter were cultured overnight in 5 ml of LB medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl/L, pH 7.5) containing 50 µg/ml ampicillin at 30°C. The same medium, 170 ml, was inoculated with 1.7 ml of this preculture in a 500-ml shaking flask. After 2.5 h, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM to induce the T7 promoter. After an additional 4-hour incubation, the cells were harvested by centrifugation, resuspended in 6 ml of TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.5) and disrupted by sonication. Since Der f 2 proteins were produced as inclusion bodies in this expression system, insoluble Der f 2 was collected by centrifugation at 7,000 g for 15 min. It was then solubilized with 50 ml of 6 M urea containing 20 mM Tris-HCl (pH 8.5), and dialyzed against 20 mM Tris-HCl (pH 8.5) for 12 h to obtain the correctly refolded molecule. The dialysate containing refolded Der f 2 was applied onto a DEAE-Toyoprep column (3.5×9.8 cm, Tosoh, Tokyo, Japan) equilibrated with 20 mM Tris-HCl (pH 8.5) and eluted with 20 mM Tris-HCl (pH 8.5) including 50 mM NaCl. The rDer f 2 protein was further purified by means of gel filtration chromatography. This expression system yielded about 10 mg of purified rDer f 2 protein from 1 liter culture.

Expression and Purification of rDer f 1

Transfer vector pBL941 (Invitrogen®, San Diego, Calif., USA) containing each of the cDNAs for Der f 1 under the control of the polyhedrin promoter and viral DNA of baculovirus (Introvigen®) were co-transfected to Sf9 cells (Introvigen®). The clones were screened by colony immunoassay using rabbit anti-Der f 1 antiserum, and several

putative r-virus plaques were picked up. A plaque which was confirmed to expressed Der f 1 by Western blot analysis was selected and the r-virus was amplified. The culture medium containing the r-virus was fractionated by DEAE-sephacel and purified by the hydroxyapatite column. The purified rDer f 1 protein was treated with 200 mM acetate buffer (pH 4) and incubated at 4°C for 2 days and further buffered with PBS.

Synthesis of Mutant Proteins of Der f 2

Four mutant proteins of Der f 2, D7A, A72L, A120L and C8/119S were created by site-directed mutagenesis using PCR [18, 19]. The mutant proteins have at least one amino acid substitution: D7A has Ala instead of Asp7, A72L Leu instead of Ala72, A120L Leu instead of Ala120, and C8/119S Ser instead of Cys8 and Cys119.

Subjects

The sera were obtained from asthmatic children visiting the Pediatric Allergy Clinic of the University Hospital of Tsukuba. The diagnosis of asthma was based on the definition of the American Thoracic Association [20]; patients with two or more episodes of wheezing and shortness of breath during the preceding year were considered to have bronchial asthma. A total of 1,420 sera of asthmatics were examined for IgE reactivities to Df by the Sepharose-RAST methods. The positive RAST sera for Df were divided into six groups according to age, 0–1, 2, 3, 4, 5–10 and 11–20 years. Finally, twenty-five sera were randomly selected from each of the six age groups.

The sera for RAST correlation experiments were separately obtained from 28 asthmatic patients with varying levels of Df-specific IgE antibody.

Sepharose RAST

The Sepharose RAST method was described in detail in a previous article [21]. One milligram of Df antigen and 50 µg of nDer f 1, rDer f 1, nDer f 2, rDer f 2 and the four mutant proteins of Der f 2 were coupled to 200 mg of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the method described by Wide and Bennich [22]. The antigen-coupled particles (ACP) were suspended in RAST buffer obtained from Phadebas RAST kits (Pharmacia, Uppsala, Sweden) at a concentration of 20 mg/ml. Fifty microliters of the ACP suspension were incubated for 3 h at room temperature with 50 µl of the test sera. After washing 3 times with 4 ml of RAST buffer (0.15 mol/l of NaCl solution containing 1% Tween 20), 50 µl of ¹²⁵I-labeled anti-IgE (25,000 cpm) obtained from Phadebas RAST kits (Pharmacia) was added to the tubes, and the mixture was further incubated for 16 h. Excess of labeled anti-IgE was washed away with 0.15 mol/l of NaCl solution containing 1% Tween 20. Radioactivity bound to the ACP was measured in a γ-counter. RAST determinations were done in duplicates, and the mean value was used for the calculation of the RAST binding. The RAST value was expressed as a percent binding: the counts bound to ACP/total counts added × 100.

The cutoff point between RAST positivity and negativity was defined as 350% of the background binding produced by 2% human serum albumin (HSA). The results were regarded as positive when the percentage binding of the sample serum was higher than the cutoff value. In order to verify the specificity of this assay, RAST determinations were done in 30 sera from patients with allergic rhinitis showing positive skin test results to criptomera and orchard grass, and negative skin test to Df, and two IgE myelomatous sera (2,000 IU/ml). None of the sera exceeded the cutoff value.

Table 1. Amount of antigens required for 50% RAST inhibition

RAST	Inhibitors	50% RAST inhibition ng/ml
rDer f 1	rDer f 1	25
	nDer f 1	29
	rDer f 2	>2,000
	nDer f 2	>2,000
nDer f 1	rDer f 1	36
	nDer f 1	44
rDer f 2	rDer f 2	26
	nDer f 2	28
	rDer f 1	>2,000
	nDer f 1	>2,000
nDer f 2	rDer f 2	32
	nDer f 2	42

RAST Inhibition Test

The serum pool from Df-sensitive asthmatic children were diluted to 1:32 with PBS containing 2% HSA, and subjected to RAST inhibition experiments. Serial 5-fold dilutions of each of rDer f 1, nDer f 1, rDer f 2 and nDer f 2 solutions (2,000 ng/ml) were prepared, and 100 μ l of each was incubated with diluted sera for 3 h at room temperature. The serum inhibitor mixture was then subjected to the RAST procedure. The RAST count produced by 2% HSA was regarded as a background binding. Percentage inhibition was calculated using the following formula: % inhibition = $[1 - (\text{RAST binding with inhibitor} - \text{background binding}) / (\text{RAST binding without inhibitor} - \text{background binding})] \times 100$.

Semilogarithmic paper was used for plotting percentage inhibition versus dose of inhibitor added. The concentration of the antigen solution required for 50% RAST inhibition was read from the inhibition curve.

IgE Binding Study for Der f 2 Mutant Proteins

The positive RAST sera for rDer f 2 were serially diluted from 1:4 to 1:1,024, and subjected to the Sepharose RAST for rDer f 2 proteins. The endpoint titer of the serum dilution producing RAST positivity was determined. Twenty sera having a RAST endpoint titer of 1:32 or more were selected from each of the six groups of different ages. All sera were adjusted to have the same RAST titer of 1:32 with PBS containing 2% HSA, and subjected to RAST for the mutant proteins of Der f 2. The response parameter was expressed as a % control: % binding of RAST to a mutant Der f 2 protein/% binding to the rDer f 2 $\times 100$.

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney test and linear regression. The difference was considered to be significant when the p value was less than 0.05.

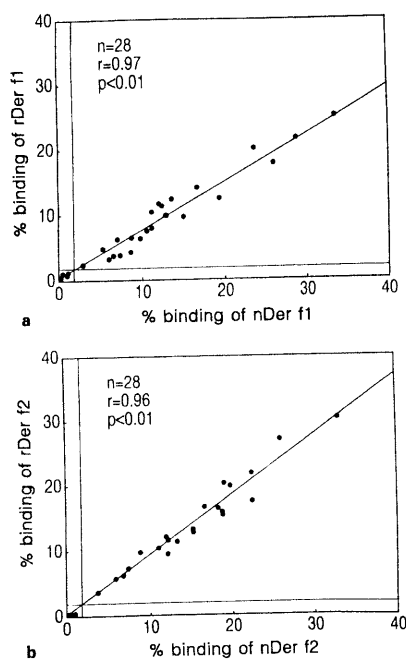


Fig. 1. Comparison of RAST levels between nDer f 1 and rDer f 1 (a), and between nDer f 2 and rDer f 2 (b). The RAST levels were expressed as % binding: RAST counts to specific antigen/total count added $\times 100$. The lines indicate the cutoff level between RAST positivity and negativity for each antigen.

Results

Comparison of IgE Reactivities to Native and Recombinant Antigens

As shown in figure 1a, there was a close correlation between RAST levels to nDer f 1 and rDer f 1 in 28 sera with varying levels of Df-specific IgE ($r = 0.97$; $p < 0.01$). Four subjects with negative RAST levels to nDer f 1 were also negative for RAST to rDer f 1, while the remaining 24 subjects with positive RAST to nDer f 1 were also positive for RAST to rDer f 1. The RAST levels were similarly compared between nDer f 2 and rDer f 2 using the same sera (fig. 1b); there was a strong correlation between the RAST levels to the two antigens ($r = 0.96$; $p < 0.01$). Five subjects

Table 2. Positive RAST response to rDer f1 and rDer f2 in six groups of Df-sensitive asthmatic children

Age group years	Subjects with positive RAST to, %		
	rDer f1	rDer f2	rDer f1 or rDer f2
0-1	88	80	96
2	100	92	100
3	100	92	100
4	100	92	100
5-10	92	92	92
11-20	100	100	100

had negative RAST to nDer f1 and rDer f1, while the remaining 23 showed positive RAST to both antigens. Thus, the recombinant antigens had almost the same IgE binding capacity as the native ones in both Der f1 and Der f2, although native antigens showed slightly higher RAST reactivity than recombinant antigens on a weight basis.

RAST inhibition tests were performed using both recombinant and native antigens. As shown in table 1, the amounts of rDer f1 required for 50% inhibition of RAST for both rDer f1 and nDer f1 were quite similar to those of nDer f1. The amounts of rDer f2 required for 50% RAST inhibition for both rDer f2 and nDer f2 were also similar to those of nDer f1. In contrast, both rDer f2 and nDer f2 failed to inhibit rDer f1 RAST by 50% even at higher concentrations, and vice versa.

Comparison of RAST Reactivities to rDer f1 and

rDer f2 in Six Groups of Df-Sensitive Asthmatic Children

As shown in table 2, a positive RAST response to rDer f1 was found in 22/25 (88%) of Df-sensitive children aged 0-1. The rate of RAST positivity increased to 25/25 (100%) in older age groups, except for 23/25 (92%) in the age group of 5-10. When the RAST reactivities to rDer f2 were similarly compared between the six groups, a positive RAST response was found in 20/25 (80%) of children aged 0-1. The positive RAST rates were from 23/25 (92%) to 25/25 (100%) thereafter. On the other hand, a positive RAST response to at least one of rDer f1 and rDer f2 was found in 24/25 (96%) in the youngest age group.

The RAST levels were compared between rDer f1 and rDer f2. As shown in figure 2a and b, there were weak, but significant correlations between the two antigens in children aged 0-1 ($r=0.24$; $p<0.05$) and aged 2 ($r=0.65$; $p<0.05$). A significant correlation was also found in children aged 3 years ($r=0.31$; $p<0.05$), 4 years ($r=0.59$; $p<0.05$), 5-10 years ($r=0.76$; $p<0.05$) and 11-20 years ($r=0.58$; $p<0.05$).

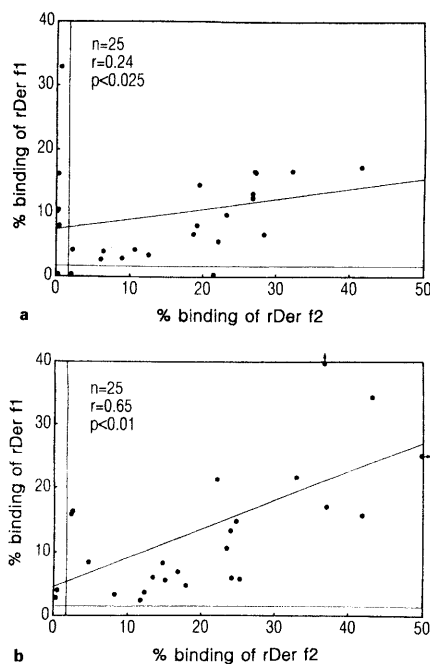


Fig. 2. Comparison of RAST levels for rDer f1 and rDer f2 in children aged 0-1 (**a**) and children aged 2 (**b**). The RAST levels were expressed as % binding. The lines indicate the cutoff level between RAST positivity and negativity for each antigen.

Comparison of IgE Reactivities to Four rDer f2 Mutant Proteins in Six Groups of Children of Different Ages

To further investigate IgE binding epitopes of Der f2, we examined the reactivity of IgE antibodies to four mutant proteins of rDer f2, D7A, A72L, A120L and C8/I19S, in asthmatic subjects of different ages. The amino acid sequences of Der f2 and four mutant proteins of Der f2 are shown in figure 3. Here, the IgE reactivities to the mutant proteins were compared with that to rDer f2, and the results were expressed as a percent control. As shown in figure 4, the mean percent controls of D7A in subjects aged 0-1, 2, 3, 4, 5-10 and 11-20 were 28.7, 43.8, 49.1, 56.7, 58.5 and 59.5%, respectively. The average value for all subjects was 49.4%. IgE reactivities to this antigen in children aged 0-1 were significantly lower than those in any other group ($p<0.01$).

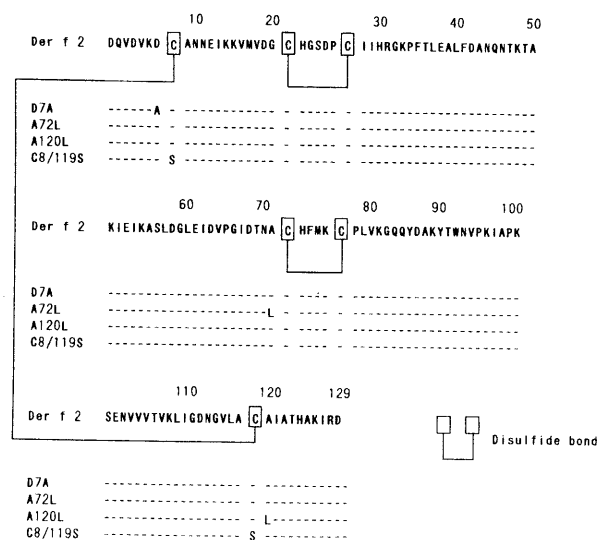


Fig. 3. The amino acid sequences of rDer f2 and four mutant proteins of Der f2 determined from cDNA clones.

Figure 5 shows the IgE reactivities to A72L. As can be seen, the average percent controls in subjects aged 0–1, 2, 3, 4, 5–10 and 11–20 were 76.8, 76.9, 90.8, 96.3, 92.0 and 86.9%, respectively. The mean percent control for all subjects was 86.6%. The IgE reactivity to A72L in young asthmatic children aged 0–1 and 2 was significantly lower than in patients aged 3, 4, 5–10 and 11–20. Figure 6 shows the IgE reactivities to A120L, in which the average percent controls of A120L in subjects aged 0–1, 2, 3, 4, 5–10 and 11–20 were 91.0, 87.9, 93.4, 99.0, 86.9 and 89.0%, respectively. The mean percent control for all subjects was 91.2%. The IgE reactivities to A120L were not significantly different in each of the six groups. The IgE reactivities to C8/119S are shown in figure 7. As can be seen, the average percent controls of C8/119S proteins in subjects aged 0–1, 2, 3, 4, 5–10 and 11–20 were 6.1, 8.2, 11.0, 13.5, 13.4 and 11.8%, respectively. The average value for all subjects was 10.6%.

Discussion

Our correlation experiments have shown that there are strong correlations between RAST levels for nDer f1 and rDer f1, and between nDer f2 and rDer f2. Furthermore,

RAST inhibition tests have demonstrated that the recombinant antigens have almost similar inhibitory activities to native antigens. These findings suggest that recombinant allergens may be useful alternatives for native allergens in monitoring allergy.

Our results showed that positive RAST responses to rDer f1 and rDer f2 were found in 88 and 80% of sera from randomly selected DF-sensitive young asthmatic children aged 0–1, and those to rDer f1 or rDer f2 in 96%. This suggests that both Der 1 and Der 2 are important allergens associated with initial sensitization to HDM during early childhood. Recently, Calkhoven et al. [14] reported that more than half of the newly generated IgE antibodies in the first IgE response to HDM in young children were directed toward components distinct from the major allergens, Der p1 and Der p2. This is in contrast to our results, which may be explained by differences in the study design, as well as the clinical characteristics of the subjects used.

It has been shown that the Der 2 antigen is a unique protein lacking amino acid sequence homology with other mite antigens such as Der 1 [23, 24]. In fact, we found with the RAST inhibition tests that neither IgE reactivities to rDer f1 were inhibited by rDer f2, nor those to rDer f2 by rDer f1. Despite this, our results have demonstrated that there is a

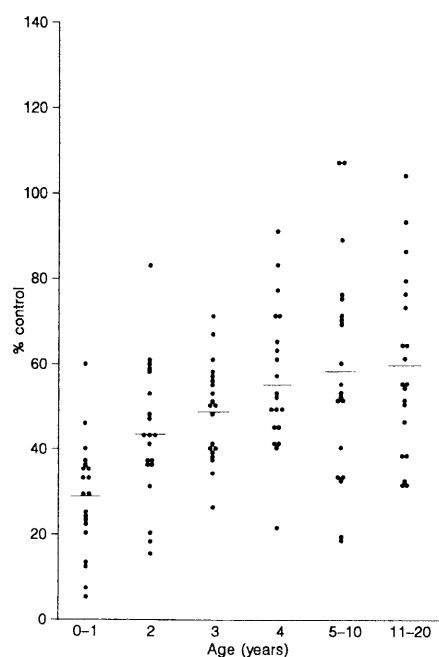


Fig. 4. IgE reactivity to D7A. The RAST reactivity to D7A was compared with that to rDer f2, and the results were expressed as % control: % binding to D7A divided by that to rDer f2 $\times$ 100. The differences were significant between the group aged 0-1 and those aged 2, 3, 4, 5-10, and 11-20, and between the group aged 2 and those aged 4, 5-10, and 11-20.

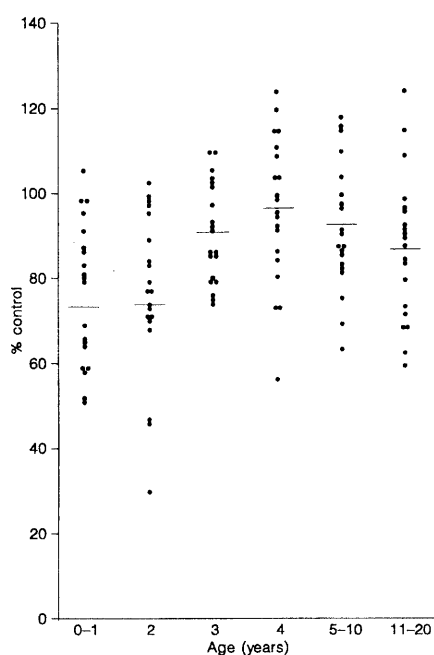


Fig. 5. IgE reactivity to A72L. The RAST reactivity to A72L was compared with that to rDer f2, and the results were expressed as % control. The differences were significant between the group aged 0-1 and those aged 3, 4 and 5-10, and between the group aged 2 and those aged 3, 4 and 5-10.

significantly correlation between RAST levels for rDer f1 and rDer f2 even in asthmatic children aged 0-1. Yasueda et al. [25] have reported that the ratio of Der f1:Der f2 in dust samples from Japanese houses ranged from 2:1 to 1:2, suggesting a quantitative similarity of the Der f1 and Der f2 contents in the dust. It is therefore likely that the sensitization to two different mite antigens, Der f1 and Der f2, may occur almost simultaneously during early childhood.

The Der f2 protein consists of 129 amino acid residues, including three disulfide bonds between Cys8 and Cys119, between Cys21 and Cys27 and between Cys73 and Cys78 [23, 24, 26]. In this study, IgE reactivities to four Der f2 mutant proteins produced by an *E. coli* expression system

were examined. We found that the IgE reactivity to D7A, in which Asp7 is replaced by Ala, is reduced to 30-60% of that to the rDer f2, and that to C8/119S, in which both Cys8 and Cys119 are replaced by Ser, is reduced almost to the background level. In contrast, A72L and A120L, mutant proteins in which Ala72 and Ala120 are substituted for Leu, respectively, retained almost the same IgE binding activity as that of rDer f2.

Nishiyama et al. [27] have previously shown that the IgE binding activity of three mutant forms of rDer f2, in which amino acid changes are found at positions 76, 88, 111 and 125, is not significantly different from that of the native Der f2. All mutant proteins in their study had three disulfide

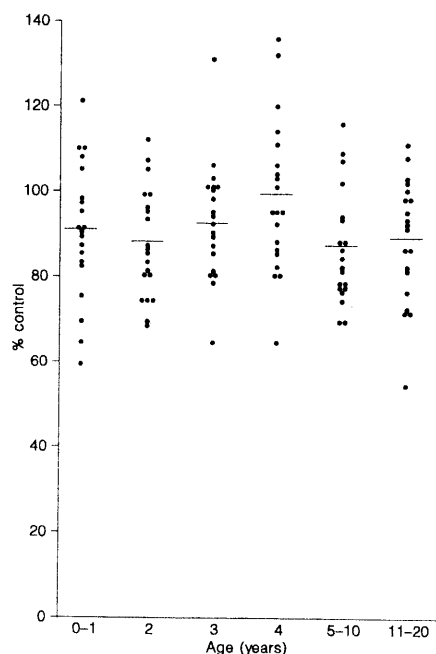


Fig. 6. IgE reactivity to A120L. The RAST reactivity to A120L was compared with that to rDer f2, and the results were expressed as % control. There were no significant differences in the average % control between each of the six groups.

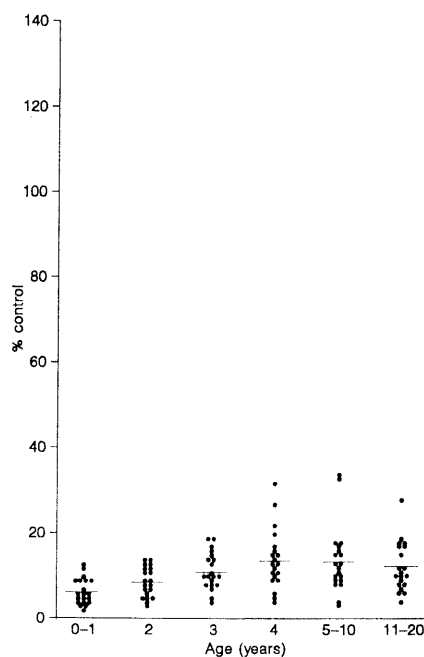


Fig. 7. IgE reactivity to C8:119S. The RAST reactivity to C8:119S was compared with that to rDer f2, and the results were expressed as % control. % control of C8:119S was significantly lower in children aged 0-1 than in older children, although C8:119S lost much of its antigenicity to IgE in every age group.

bonds and showed a CD spectrum profile identical to that of nDer f2 proteins. The same authors also demonstrated that the Der f2 mutant proteins that lost one disulfide bond between Cys21 and Cys27 kept the same binding activity to IgE as that of rDer f2, while the loss of the disulfide bond between Cys73 and Cys78 decreased the IgE binding activity to some extent, and the mutant protein that lost one disulfide bond between Cys8 and Cys119 showed a marked decrease in activity [unpubl. data]. Chua et al. [28] demonstrated that large overlapping peptides of Der p2 (P1-69, P42-117 and P69-129) hardly bound any IgE from HDM allergic patients, suggesting that the B cell epitopes of Der 2 were highly conformational. It is therefore likely that since Cys8 and Cys119 are located far from each other, the

disruption of the disulfide bond between Cys8 and Cys119 has a greater effect on conformation of the Der f2 protein than the disruption of the bond between Cys21 and Cys23 and between Cys73 and Cys78. The possibility remains that the disruption of Cys8 and Cys119 leads to a loss of conformational epitope located somewhere else on the molecule.

Our results have shown that IgE reactivity to D7A, in which Asp7 is replaced by Ala, is reduced to 30-60% of rDer f2. This may be due to the fact that the location around the N-terminal region including the 7th amino acid residue forms an important IgE binding epitope of Der f2, or that the change in the 7th amino acid residue may indirectly influence the formation of the disulfide bond between Cys8

and Cys119, though our data also suggest that the change in the 120th amino acid does not affect the disulfide bond between Cys8 and Cys119.

It is interesting to note our finding that IgE reactivity to D7A was very low in young asthmatic children aged 0–1, but increased with increasing age. Two explanations can be offered for this phenomenon. One is that more diverse B cell clones recognizing new epitopes expressed on Der f2 emerge by the continuous stimulation of Df and/or booster effect due to the frequent exposure. Another explanation is affinity maturation. Experimental studies have revealed that the affinity of antibodies for specific antigens increases

when animals are repeatedly immunized with a low dose of antigens [29]. In this study, since asthmatic children in the older age groups have been exposed to a low dose of antigen for a longer time, it is likely that the increasing IgE reactivity to D7A depending upon age may be due to increased affinity of IgE antibodies.

In conclusion, our results suggest that both Der f1 and Der f2 are important antigens associated with early sensitization to HDM in young children. In addition, the region including the 7th amino acid and the disulfide bond between Cys8 and Cys119 are considered to maintain IgE epitopes of Der f2 allergen.

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