

IgE and monoclonal antibody binding by the mite allergen Der p 7

H.-D. SHEN, K. Y. CHUA*, W. L. LIN, H. L. CHEN, K.-H. HSIEH†, and W. R. THOMAS‡

Department of Medical Research, Veterans General Hospital-Taipei, *Institute of Immunology and †Department of Paediatrics, National Taiwan University, College of Medicine, Taipei, Taiwan, Republic of China and ‡Institute for Child Health Research, Perth, Western Australia, Australia

Summary

Background The recently characterized group 7 house dust mite allergens give positive skin-test reactions in 53% of allergic patients.

Objective This study was performed to compare the IgE binding activity of natural and recombinant Der p 7, to measure the binding in allergic sera in comparison to major allergen Der p 2 and characterize the response by competitive inhibition with monoclonal antibodies.

Methods IgE anti Der p 2 and Der p 7 antibodies against the recombinant allergens and monoclonal binding activities were measured by a solid phase radioimmune assay.

Results A competitive binding assay showed that rDer p 7 inhibited 91% of IgE-binding to natural Der p 7 in 2 sera and 73% in a further two. The IgE binding of rDer p 2 and Der p 7 from 41 sera was then compared. Of the sera 88% and 46% respectively showed positive binding. All of the 19 sera which bound Der p 7 also bound Der p 2 but 11 (58%) had bound IgE to Der p 7 as high or higher than the binding to Der p 2. These sera were mostly high responders to both allergens. A panel of six monoclonal antibodies produced against either rDer p 7 or rDer f 7 was used for epitope analysis. All of these reacted with each allergen by enzyme linked immunosorbent assay (ELISA) and immunoblotting. Two patterns of cross inhibition of monoclonal antibody binding were observed and of five monoclonal antibodies tested, four could inhibit the binding of IgE (WH9, WH22, WP8 and HD19) while one (WH14) could not.

Conclusions Although Der p 7 only reacts with 50% of allergic sera it often has a high IgE binding activity and may be more important than the major Der p 2 allergen in a high percentage of subjects. The combined competitive inhibition experiments show the IgE response is directed at several specificities.

Keywords: mite allergen, Der p 7, IgE, monoclonal antibody, recombinant allergen

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Introduction

cDNA cloning has defined the allergen Der p 7 of *Dermatophagoides pteronyssinus* [1] and the corresponding Der f 7 in *D. farinae* which demonstrate 86% amino

acid sequence identity [2]. The recombinant Der p 7 (rDer p 7) expressed as a fusion protein with glutathione S-transferase (GST) has been shown to induce an immediate skin-test reactivity in 16 (53%) of 30 asthmatic children [1] but the IgE-binding reactivity of rDer p 7 in comparison with that of natural allergen and its significance in relation to other mite allergens remains to be determined. In order to do this it is necessary either

Correspondence: Dr W. R. Thomas, Institute for Child Health Research, PO Box 855, West Perth 6872, Australia.

to examine the natural allergen or to compare the recombinant and natural allergen. In the present study, the ability of rDer p 7 to inhibit the IgE-binding of natural Der p 7 was first analysed. Here we show that rDer p 7 inhibits IgE-binding to natural Der p 7 by over 90%. The recombinant allergen was then used to show that while only 50% of patients have IgE-binding to Der p 7 the level is as high or higher than the binding to the allergen Der p 2 which is a major specificity in the antibody titre to house dust mite extracts.

To further characterize the allergen the cross-inhibition of a panel of monoclonal antibodies has been studied as well as their ability to inhibit IgE-binding. The results show Der p 7 has at least several binding sites for IgE and that the monoclonals are suitable for purification and two-site binding assays for the quantitation of allergen and antibody assays.

Materials and methods

Preparation of mite extracts

D. pteronyssinus from Commonwealth Serum Laboratories, Parkville, Australia (now CSL-Ltd) were extracted by homogenizing the lyophilized mites at 20 mg/mL in phosphate-buffered saline (PBS) containing aprotinin (0.1 TIU/mL; Sigma Chemical Co., St Louis, MO, USA) and phenylmethylsulfonyl fluoride (PMSF, 1 mM; Sigma). After centrifugation at 10 000 *g* for 30 min, the supernatant was dialysed against 50 mM ammonium bicarbonate, pH 8.0 and freeze-dried in aliquots for storage at 4°C.

Preparation of recombinant Der p 7 and Der p 2

Der p 7 and Der p 2 were produced as fusion proteins from the pGEX vectors [1,3]. The polypeptides fused to glutathione-S-transferase (GST) at their N-termini were affinity purified from glutathione agarose [4].

Inhibition of IgE-binding to natural Der p 7 by rDer p 7

The difference between IgE-binding of recombinant and natural Der p 7 was analysed by competitive solid phase radioimmunoassay (RIA). Firstly, the monoclonal antibody (MoAb) WH9 [5] against Der p 7 was coated onto the wells of flexible polyvinyl microtitre plate (Costar Corp., Cambridge, Mass. USA) by the addition of 50 µL of MoAb WH9 (20 µg/mL) per well and incubated at 37°C for 2 h. The wells were blocked with 1% skim milk at 37°C for 1 h then 50 µL of a whole mite extract of *D. pteronyssinus* (200 µg/mL) was added into each well which were incubated at 4°C for 16 h. After washing

with PBS containing 0.05% Tween 20 (PBST), serum samples containing IgE antibodies against Der p 7 which had been mixed individually with different amounts of rDer p 7 or PBST alone were added to the wells and incubated at 37°C for 4 h. Further washing was performed and ¹²⁵I anti-human IgE monoclonal antibodies (2.1.5 available from Silenius Laboratories, Mt Hawthorn, Victoria) were added into each well and incubated at room temperature for 1.5 h. After washing with PBST, the radioactivity of each well was counted in a gamma counter (LKB, Turku, Finland). Each experimental determination was carried out in duplicate and the mean cpm of duplicate wells was calculated and used. The % inhibition of IgE-binding was calculated as:

$$\% \text{ inhibition} = \frac{\text{cpm of serum mixed with PBST} - \text{cpm of serum mixed with rDer p 7}}{\text{cpm of serum mixed with PBST}} \times 100$$

IgE radioimmunoassay (RIA)

The presence of Der p 7 and Der p 2-specific IgE antibodies in sera of 41 children known to be allergic to mite extracts by previous skin-test was analysed by RIA using procedures similar to that described above. The sera were the same as used previously [2] and were from children attending the allergic clinic of the National Taiwan University Hospital, Taipei. All were known to react to mite extract, which was established through a skin test. Serum samples tested were added individually into wells of flexible microtitre plates (Costar Corp.) coated with rDer p 7, rDer p 2 or the fusion partner GST at 0.05 µg/well. After incubation overnight at 4°C, the presence of specific IgE antibodies was detected by incubation with ¹²⁵I-labelled monoclonal anti-human IgE antibodies. The radioactivity of each well was then counted in a gamma counter. An average of two readings from two separate determinations was calculated. The result of RIA was expressed as count index which was calculated by dividing the average reading of individual test serum by the average reading of the cord serum sample included in the same assay.

Development of MoAbs

MoAbs against Der p 7 and Der f 7 were prepared from BALB/c mice immunized with rDer p 7 or rDer f 7 [5]. Antibody-producing hybrid cells were screened by the enzyme-linked immunosorbent assay (ELISA) and SDS-PAGE-immunoblotting using rDer p 7, rDer f 7 and GST alone. The isotype of the MoAbs was determined

by ELISA with class-specific antisera (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

Cross-inhibition ELISA

MoAbs from ascitic fluid were purified by precipitation with ammonium sulphate at a final concentration of 40% saturation, dialysed against 50 mM ammonium bicarbonate and lyophilized. Biotinylation of MoAbs was then performed according to the Cold Spring Harbor Laboratory [6]. Briefly, 40 μ L of N-hydroxysuccinimide biotin (Sigma) at 10 mg/mL in dimethyl sulfoxide was mixed with 1 mL of MoAb at 2 mg/mL in 0.1 M sodium borate buffer pH 8.8. The mixture was then incubated at room temperature for 4 h. After the addition of 32 μ L of 1 M NH_4Cl , the mixture was incubated at room temperature for 10 min and then dialysed against PBS to remove uncoupled biotin. The biotinylated MoAb was titrated by ELISA using plates coated with rDer p 7 at 0.05 μ g/well to find a suitable dilution factor to be used in the inhibition. Wells of microtitre plates coated with rDer p 7 were then incubated with unmodified MoAbs at a concentration of 10 μ g/mL or with PBST alone at 37°C for 1 h. After washing with PBST, diluted biotinylated MoAbs were added to each well and incubated at 37°C for another hour. Wells were washed again with PBST and 50 μ L of a solution containing peroxidase-conjugated streptavidin (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was added for 1 h at 37°C. Following the washing

procedure, 50 μ L of a substrate solution (Bio-Rad, peroxidase substrate kit 172-1064, containing 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] and hydrogen peroxide) was added to each well and incubated at 37°C for 30 min. The colour change was measured by the absorbance at 415 nm on a Titertek Multiskan (Flow Laboratories, McLean, Virginia, USA). An average of two readings from two separate determinations was calculated. The percent inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{A415 without inhibiting MoAb} - \text{A415 with inhibiting MoAb}}{\text{A415 without inhibiting MoAb}} \times 100$$

Inhibitory RIA

The ability of MoAbs to inhibit the reactivity between IgE and rDer p 7 was analysed by RIA. The wells of microtitre plates coated with rDer p 7 at 0.05 μ g/well were incubated with different amounts of various MoAbs at 37°C for 1 h. After washing with PBST, serum samples from asthmatic children known to contain IgE antibodies against Der p 7 were added into each well. After incubation overnight at 4°C, the presence of specific IgE antibodies was detected by incubation with ^{125}I -labelled monoclonal anti-human IgE antibodies. The radioactivity of each well was then counted in a gamma counter. An average of two readings from two separate determinations was calculated. The

Table 1. Inhibition of IgE-binding to solid-phase natural Der p 7 by recombinant Der p 7

Inhibitors (μ g/mL)	% Inhibition			
	Serum 1 (%)	Serum 2 (%)	Serum 3 (%)	Serum 4 (%)
rDer p 7				
100	91	73	91	73
10	88	66	86	71
1.0	80	66	67	53
0.1	27	38	13	-3
rDer p 2				
100	-3	8	-3	-2
10	-4	7	3	-2
1.0	-9	2	-1	1
0.1	18	7	2	1

The IgE binding of serum without inhibitor was 1184, 242, 1280 and 526 cpm for serum samples 1, 2, 3 and 4 respectively.

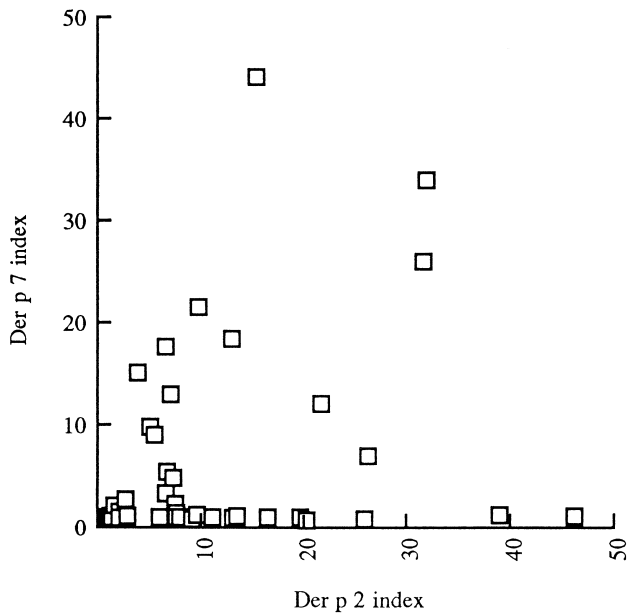


Fig. 1. The distribution of specific IgE count indices to recombinant Der p 2 and Der p 7 by 41 sera from patients allergic to house dust mite and one from a patient allergic to pollen only. The cord serum binding (index of 1) was 39 cpm for rDer p 7 and 45 for rDer p 2. The pollen allergic only had indices of 0.9 and 1.1 for Der p 2 and Der p 7.

% inhibition was calculated as:

$$\% \text{ inhibition} = \frac{\text{cpm without inhibiting MoAb} - \text{cpm with inhibiting MoAb}}{\text{cpm without MoAb}} \times 100$$

Results

IgE-binding by recombinant and natural Der p 7

The ability of rDer p 7 to inhibit IgE-binding to natural Der p 7 was analysed by solid-phase competitive RIA using four serum samples. A dose-dependent inhibition of IgE-binding to natural Der p 7 was observed for all samples (Table 1). Among these four samples, nos. 1, 3 and 4 were pools of sera from three different asthmatic patients. The IgE binding of sample nos. 1 and 3 (Table 1) were inhibited to 91% with 100 µg/mL of rDer p 7. Samples 2 and 4 showed 73% inhibition with this concentration of rDer p 7 while no inhibition was observed with rDer p 2 (Table 1). As a further control it was shown that 100 µg/mL of rDer p 7 produced only 1% inhibition of IgE binding to rDer p 2.

Table 2. Immunological characteristics of MoAbs against Der p 7

MoAbs	ELISA Activity (A415)		Immunoglobulin isotype
	rDer p 7	rDer f 7	
WH9*	2.099	1.766	IgG2b, k
WH14	1.965	1.134	IgG1, k
WH22*	2.229	2.063	IgG1, k
WH27	2.114	1.645	IgG2b, k
WP8	2.155	1.769	IgG1, k
HD19+	2.201	2.059	IgG1, k
P40#	0.142	0.129	IgG1, k

*MoAbs against Der p 7 generated by us before [5].

+MoAb reacted with Der p 7 obtained by immunization of mice with rDer f 7.

#P40 represents a MoAb against the 68 kD allergen of *Penicillium notatum*, which was used as a control.

IgE antibodies against Der p 2 and Der p 7 in asthmatic sera

IgE antibodies to rDer p 7 and rDer p 2 in sera of 41 asthmatic children were compared by RIA. The results of the RIA are expressed as a count index as described in the Materials and methods. The average cord serum binding was 39 cpm for rDer p 7 and 45 for rDer p 2. Serum samples with specific count indices equal to or greater than 1.5 (which is equivalent to the mean ± 3 sd of the cord serum binding to rDer p 7) were considered as positive. The results in Fig. 1 show that except for nos. 27, 28, 33, 34 and 39, all the sera reacted to Der p 2 with IgE count indices greater than 1.5. Der p 7-specific IgE indices greater than or equal to 1.5 were found in 19 (46%) of the 41 patients all of whom were also positive to Der p 2. Surprisingly however, 11 (58%) of the 19 sera tested showed Der p 7-specific count indices which were greater than or equal to the indices of Der p 2 (Fig. 1). With the exception of two low sera these indices are quite high. The control serum, serum no. 42, was from a patient allergic to pollen only. All sera showed no significant binding to recombinant GST.

MoAbs against Der p 7

In addition to MoAbs WH9 and WH22 which were previously generated [5], four more MoAbs which showed positive ELISA reactivity against Der p 7 were isolated (Table 2). MoAb HD19 was produced by immunization with Der f 7, the others from mice immunized with Der p 7. The isotype of MoAbs WH9 and

Table 3. Inhibition of biotinylated MoAbs to solid-phase rDer p 7 by unlabelled MoAbs (% inhibition)

Inhibitors (10 µg/mL)	Biotinylated MoAbs					
	WH9 (%)	WH22 (%)	WH27 (%)	WH14 (%)	WP8 (%)	HD19 (%)
WH9	61	68	61	-4	11	8
WH22	51	55	49	3	-5	9
WH27	65	65	64	-2	8	2
WH14	0	-8	-4	76	58	50
WP8	8	9	11	78	62	61
HD19	10	5	9	85	68	77
P40	-1	4	-5	3	8	6

WH27 is IgG2b with k light chain, and the isotype of other MoAbs is IgG1, k (Table 2). All five MoAbs obtained by immunization with Der p 7 also showed positive ELISA reactivity to Der f 7 (Table 2). They also all showed positive SDS-PAGE-immunoblotting reactivity to both rDer p 7 and rDer f 7 and negative ELISA and immunoblotting reactivity to recombinant GST (data not shown). All six MoAbs reacted with bands of 31, 30 and 26 kDa in mite extracts as previously reported for WH9 and WH22 [5].

Mapping of epitopes recognized by MoAbs

The relationship between epitopes on Der p 7 recognized by the MoAbs was analysed by inhibitory ELISA. The MoAbs were purified, biotinylated and titrated against rDer p 7 to find a concentration which gave absorbance values between 1.0 and 2.0 which were on the responsive region of the titration curve. Unmodified MoAbs were then used to inhibit binding. The results in Table 3 show the percent inhibition of individual MoAb to six different biotinylated MoAbs. When 10 µg/mL of MoAb was used, the homologous antibodies showed at least 55–77% inhibition compared with the control P40 MoAb which showed 8%. Two groups with different patterns of inhibition were noted. One consisting of WH9, WH22 and WH27 inhibited themselves and had little effect on binding by WH14, WP8 and HD19, and MoAbs WH14, WP8 and HD19 showed a reciprocal pattern. Therefore at least two different epitope patterns have been defined.

Analysis of epitopes recognized by human IgE and MoAbs

To analyse the relationship between epitopes recognized by human IgE and mouse MoAbs, five of the six MoAbs were tested for their ability to inhibit the reactivity

between IgE antibody and Der p 7. The results in Fig. 2 show inhibition experiments performed with IgE reactivity in individual sera from two patients and one pooled serum sample. The MoAbs WH9, WH22, WP8 and HD19 produced a significant dose-dependent inhibition of all samples while only a borderline inhibitory activity up to 17%, was observed for MoAb WH14. Inhibition was not observed when the control MoAb P40 was used. Thus, although WH14, WP8 and HD19 inhibited each other in ELISA, the ability of WH14 to inhibit IgE reactivity was clearly distinct. Similarly, WH9 and WH22 although both inhibitory had a different pattern with the sera from different patients.

Discussion

Der p 7 was originally isolated by its IgE-binding activity in a recombinant house dust mite expression library [1]. It appeared important because it caused skin reactions or bound IgE in the sera of about 50% of allergic patients and was interesting because Western blotting showed it was present in house dust mite extracts in at least three molecular weight forms (31, 30 and 26 kDa). At least some of the heterogeneity is due to glycosylation [5]. In order to estimate the importance of Der p 7 as an antigen, the IgE binding activity of rDer p 7 has been compared here to the natural Der p 7 and to the major house dust mite allergen Der p 2. The results showed that rDer p 7 inhibits 73–91% of IgE-binding to solid-phase natural Der p 7. This indicates that rDer p 7 expressed as a GST fusion retained much of the IgE reactivity of its natural counterpart and is suitable to estimate the importance of the allergen which has not been isolated from house dust mite extracts in practical quantities. Although not 100%, the degree of inhibition is similar to

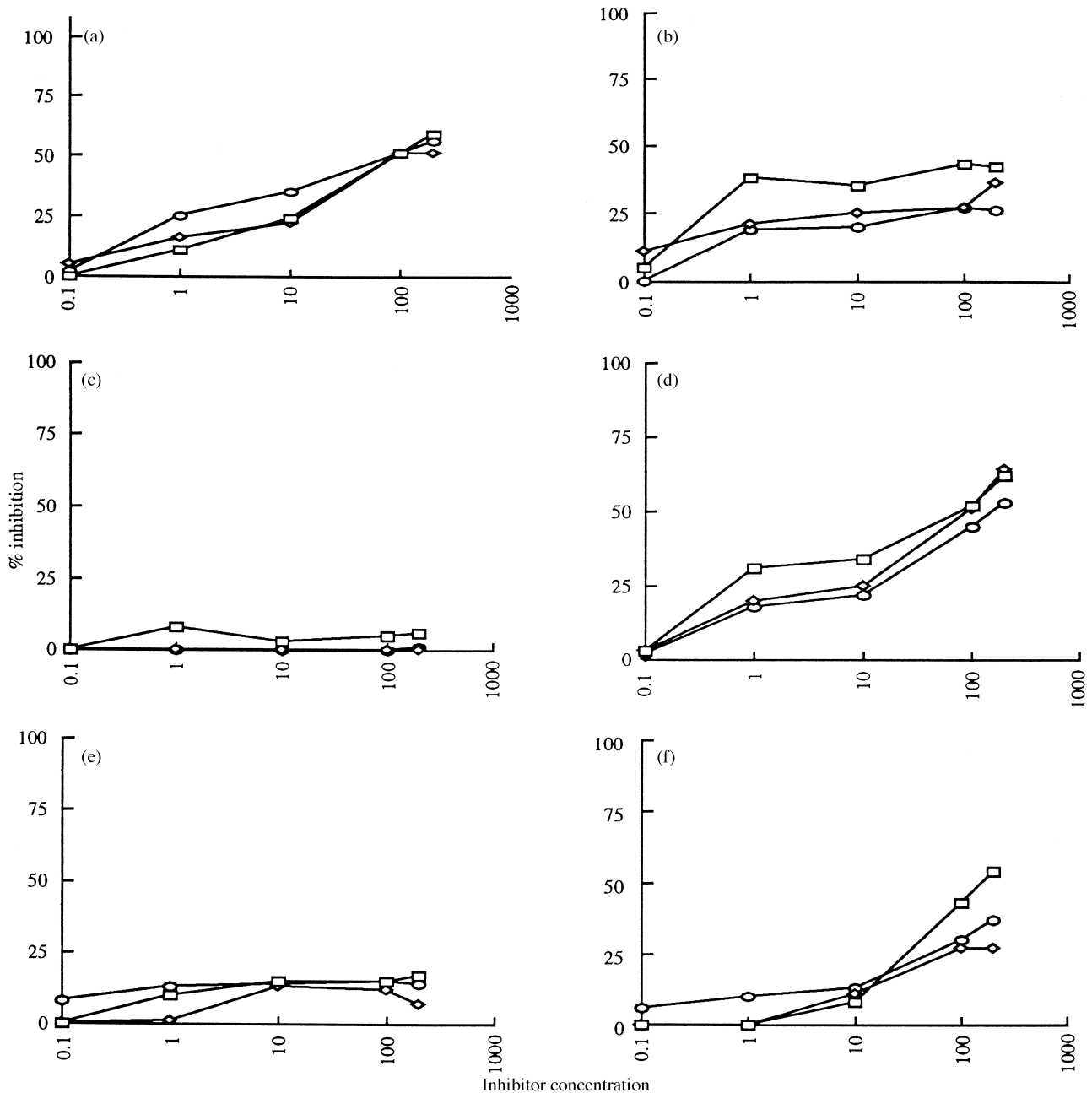


Fig. 2. Inhibition of IgE binding to solid-phase rDer p 7 by different monoclonal antibodies. The inhibitor concentration is from 0.1 to 200 $\mu\text{g/mL}$. The standard deviation for the triplicates for each point is an average of 2.5, 2.8, 2.8, 3.6, 4.6 and 4.3 for monoclonal antibodies, (a) HD19, (b) WH22, (c) P40, (d) WH9, (e) WH14, (f) WP8 respectively. The IgE binding of serum samples, $\square$, 1, $\diamond$, 2 and $\circ$, 3 was 722, 385 and 1035 cpm respectively.

that reported for homologous inhibition of Der p 1 and Der p 2 [7,8]

The frequency of patients with IgE antibody recognizing Der p 7 was like previous studies about 50%, considerably less than the 88% recognition of Der p 2. As can be anticipated from these figures, all of the patients who recognized Der p 7 also recognized Der

p 2. Importantly, however, the majority of patients who recognized Der p 7 had binding indices (and absolute levels) greater than Der p 2. For the most part, although not always, the patients who recognized both Der p 2 and Der p 7 were higher responders for Der p 2 so the levels of binding to Der p 7 was often large. For example, of the binding indices greater than 10 in Fig. 1, 14 were to Der p

2 and 9 to Der p 7. The frequencing of binding of Der p 2 and Der p 7 is similar to the skin test data [1] and unpublished observations have shown the weal-and-erythema reaction to Der p 7 can be greater than that to Der p 2. Since Der p 2, used as a reference here, is along with Der p 1 [9], a dominant allergen contributing over half of the anti-mite IgE in over 50% of sera [9,10] the Der p 7 may be quite important. This differs considerably to the Der p 6 chymotrypsin allergen. Although this allergen has a frequency of reactivity only slightly less than Der p 7, the degree of binding compared to Der p 1 and Der p 2 was consistently small [11]. It should be noted that as for Der p 7 the IgE binding activity of the natural Der p 2 and rDer p 2 is very similar [3].

The *D. farinae* equivalent Der f 7 has now been cloned and shown to have 86% sequence homology with Der p 7 [2]. It also reacts with a similar number of sera although to a lesser degree [2] presumably due to the preponderance of *D. pteronyssinus* in northern Taiwan where this study was performed. Here we show that MoAbs did, however, react well with Der p 7 and Der f 7 and in this case is different to the group 1 allergens where most MoAbs are species-specific [12].

Cross-inhibition studies with the MoAbs showed these fell into two groups with WH9, WH22 and WH27 giving cross-inhibition and WH14, WP8 and HD19 having a reciprocal pattern. When these MoAbs were used to inhibit the IgE reactivity to Der p 7 in a small panel of three sera further complexity was observed. The MoAb WH14 was distinctive in that it was poor at inhibiting IgE-binding while WP8 and HD19 which was in the same cross-inhibiting group gave significant inhibition although WP8 was less effective at inhibiting responses in two out of three sera. Although WH14 did not inhibit any sera it was just as active in inhibiting ELISA to itself and the other MoAbs. WH9 and WH22 which were in the same cross-inhibitory group gave slightly different patterns of inhibition of IgE with different patients with WH22 being less inhibitory for two sera. Thus, the IgE response to Der p 7 is both large in many patients and as heterogeneous in antibody specificities as responses to the major allergens [8,12]. The same heterogeneity has been observed by studying IgE-binding to recombinant fusion peptides for Der p 1 [13].

The monoclonal antibodies described here with different binding specifications will have further application in double-site binding assays for determining Der p 7 concentrations and standardising extracts and for preparing the natural allergen. The fact that all the monoclonals show the reactivity with the same three molecular weight species on SDS-PAGE confirms they are different forms of the same allergen.

In conclusion, although the allergen Der p 7 has only recently been recognized and reacts with 50% of allergic sera, the IgE response to this allergen is often large and more important than the major allergen Der p 2 used as a reference in these studies. The sequence information and MoAbs will be useful in further evaluating responses to this important allergen including T cell and lymphokine analyses.

Acknowledgments

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