

Characterization of the allergen Der f 7 from house dust mite extracts by species-specific and crossreactive monoclonal antibodies

H.-D. SHEN*†, W.-L. LIN*, L.-C. TSAI*†, M. F. TAM‡, K.-Y. CHUA§, H.-L. CHEN*, K.-H. HSIEH¶, C.-S. LI** and W. R. THOMAS††

*Department of Medical Research, Veterans General Hospital-Taipei, †Centre for Immunology, National Yang-Ming University, ‡Institute of Molecular Biology, Academia Sinica, §Graduate Institute of Immunology, College of Medicine, National Taiwan University, ¶Chang Gung Children's Hospital, Tao-Yuan, **Division of Environmental Health Sciences, Institute of Public Health, College of Medicine, National Taiwan University, Taiwan, Republic of China, and ††Institute for Child Health Research, Perth, Western Australia, Australia

Summary

Background The group 7 mite allergens react with IgE in 50% of sera from allergic patients.

Objective To determine the molecular and antigenic characteristics and heterogeneity of Der f 7 in mite extracts.

Methods Monoclonal antibodies (MoAbs) produced from mice immunized with recombinant Der f 7 were examined for crossreactivity to Der p 7 and then used for immunoblotting of 1 and 2-D gel electrophoresis. Deglycosylation was studied with N-glycosidase-F and N-terminal sequencing by Edman degradation. The epitopes of the monoclonal antibodies were compared by cross-inhibitory immunoassays.

Results Immunoblotting of *D. farinae* extracts with all the anti Der f 7 MoAbs showed major reactivities at 31, 30 and 25 kDa. The strongest immunostaining was at 25 kDa which contrasted with Der p 7 where the 31 and 30 kDa bands were strongest. The relative strength of staining however varied between extracts. The 31 and 30 kDa components were glycosylation products of the 25 kDa form which had the N-terminal sequence predicted from cDNA analysis. Two MoAbs stained an 18 kDa band consistent with a degradation product. The 2-D gels showed that different components with pIs from 5.6–6.4. Both species-specific and Der p 7 crossreactive MoAbs were produced and a two-site ELISA assay for detecting group 7 allergen was developed with MoAbs recognizing different epitopes.

Conclusions Der f 7 has been defined by its natural N-terminal sequence and MoAbs. It apparently exists as different glycosylation and degradation products in mite extracts, the relative abundance of which differs with different preparations. A two-site ELISA to measure the allergen was developed.

Keywords: mite allergen, Der f 7, monoclonal antibody, glycosylation, IgE binding, two-site ELISA

Clinical and Experimental Allergy, Vol. 27, pp. 824–832. Submitted 22 August 1996; revised 21 November 1996; accepted 24 December 1996.

Introduction

The group 7 allergens of house dust mites (Der p 7 and Der f 7) have been characterized by cDNA cloning [1,2]. The

Correspondence: Dr W. R. Thomas, Division of Molecular Biology, TVW Telethon Institute for Child Health Research, PO Box 855, West Perth WA 6872, Australia.

cDNA clone of Der p 7 encoding a 215 residue protein, was isolated by plaque radio-immunoassay using serum containing mite-specific IgE antibodies [1]. The cDNA encoding Der f 7 was amplified using polymerase chain reaction (PCR) and cloned into *E. coli*. It encoded a 213 residue protein [2]. Both cDNAs were predicted to contain a 17 amino acid residue leader sequence and the deduced amino acid sequences had 86% identity [1,2].

Both Der p 7 and Der f 7 have been expressed as glutathione S-transferase (GST) fusion proteins [1,2] and the IgE-binding activity of the fusion proteins compared with that of the GST-Der p 2 fusion protein [3]. The results showed that 88%, 46% and 46% of the 41 sera from asthmatics were IgE-positive for Der p 2, Der p 7 and Der f 7, respectively [2,4]. However, although the frequency of IgE to Der p 7 was lower than to Der p 2, 58% of the Der p 7-positive samples bound IgE to Der p 7 as high or higher than the binding to Der p 2 [4]. The group 7 allergens may therefore be as significant as the major group 2 allergen in half the mite allergic patients. Similar observations have been made with skin tests [1].

The group 7 allergens are also of interest because of their molecular heterogeneity and how this affects the estimation of the number of significant allergens determined by Western blotting. MoAbs to Der p 7 [4,5] have previously been shown by 2-D immunoblot to react with mite components of 31, 30 and 26 kDa with pIs from 5.7–6.0 [5]. The 31 and 30 kDa components were the predominant immunostaining species and these reduced to a band of 27 kDa after N-deglycosylation [5]. The MoAb-binding results in this study show even further heterogeneity of the group 7 allergens in *D. farinae* extracts, namely in the number of bands immunostaining in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the predominant molecular species and variation between mite extracts. The major immunostaining species of Der f 7 was 25 kDa, in comparison to 31/30 kDa for natural Der p 7, and this had the N-terminal sequence predicted by the analyses of cDNA. The MoAbs differed in their species specificity and could also be used in a two-site ELISA for detecting antigen.

Materials and methods

Preparation of recombinant Der f 7 and Der p 7 (rDer p 7 and rDer f 7)

Recombinant Der f 7 and Der p 7 were produced as fusion proteins from the pGEX vectors [1,2]. The polypeptides were fused to glutathione-S-transferase (GST) at the predicted N-termini of the mature proteins and were affinity purified with glutathione agarose [6].

Mite extracts of D. farinae

D. farinae from Allergon AB (Engelholm, Sweden) 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 phenylmethyl-sulfonyl 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. In addition, the extract of *D. farinae* obtained from Greer Laboratories (Lenoir, NC, USA) was also included in the present study.

Development of MoAbs

MoAbs against Der f 7 were prepared from BALB/c mice immunized with rDer f 7 [4]. Antibody-producing hybrid cells were screened by the enzyme-linked immunosorbent assay (ELISA) [5] and SDS-PAGE-immunoblotting using rDer f 7 and GST alone. Hybridomas producing MoAbs against Der f 7 were subcloned at least twice by limiting dilutions and then further expanded. The isotype of the MoAbs was determined by ELISA with class-specific antisera (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

SDS-PAGE and 2D gel electrophoresis

The SDS-PAGE and 2D gel electrophoresis were performed essentially as described [5]. For SDS-PAGE, the sample to be analysed was mixed with an equal volume of double-concentration sample buffer (Tris buffer containing 2% (w/v) sodium dodecylsulfate (SDS), 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.00125% (w/v) Bromophenol Blue, pH 6.8), boiled for 5 min and electrophoretically separated on a 4% stacking and 12.5% separating SDS-polyacrylamide gel. For 2D gel electrophoresis, isoelectric focusing in the first dimension was carried out for 14 000 Vh in a 3.5% polyacrylamide, containing 0.2% N, N'-methylenebisacrylamide, 8.8 M urea, 2% carrier ampholytes (pH 3–10), and 1.9% of Nonidet P-40. The gels were cast in glass tubes. Pre-focusing was carried out for 1 h at 200 V. Approximately 300 µg of the whole mite extract mixed with an equal volume of the sample buffer containing 0.05 M CHES (cyclohexylamino ethane sulfonic acid), pH 9.5, 2% (w/v) SDS, 1% (w/v) dithiothreitol and 10% (v/v) glycerol was loaded onto each tube. At the end of the isoelectric focusing step, the gels were extruded from the glass tubes and equilibrated in a Tris buffer, pH 6.8, containing glycerol (10%), SDS (2.1%) and Bromophenol Blue. The second dimension of electrophoresis was carried out on slab gels consisting of 13% polyacrylamide. Low molecular weight

standards (Pharmacia, Uppsala, Sweden) were used to estimate the molecular weight of the protein components. Carbamylated creatine kinase obtained from BDH (Poole, UK) was used as the charge standard for calculating the isoelectric point of the resolved proteins.

Immunoblotting

Components of the mite extracts or recombinant proteins recognized by MoAbs produced were analysed by gel electrophoresis and immunoblotting [5]. Briefly, mite components or recombinant proteins separated by SDS-PAGE or by the 2D gel electrophoresis were transferred electrophoretically onto polyvinylidene difluoride membrane (PVDF; 0.45 µm, Millipore, Bedford, MA, USA). After blocking with 1% skimmed milk, the blot was incubated with supernatant from cultured hybridoma cells at room temperature for 1 h. After washing, all the blots were incubated with affinity-purified horseradish peroxidase-labelled goat anti-mouse IgG (H + L) (Bio-Rad) for 1 h at room temperature. After washing, the blot was developed in a substrate solution of acetate buffer (50 mM, pH 5.0) containing 1 mM 3-amino-9-ethyl-carbazole (Sigma) and 0.015% (v/v) hydrogen peroxide for about 5 min. The reaction was stopped by washing the blots with water. The deposition of red stain which demonstrated antigen-antibody binding was recorded by photography. For a control, the supernatant of cultured hybridoma cells containing MoAb P40 against the 68 kDa allergen of *Penicillium notatum* was used.

Deglycosylation

Enzymatic deglycosylation of mite components was performed according to the protocol recommended by the manufacturer (Boehringer Mannheim, Germany). The sample (30 µg of the mite extract) was boiled in 10 µL of 1% SDS for 2 min. After the addition of 40 µL of a sodium phosphate buffer, 20 mM, pH 7.2 containing 10 mM of sodium azide, 50 mM of EDTA and 0.5% (v/v) of Nonidet P-40, the sample was boiled for another 2 min. After cooling, 1.2 units of N-glycosidase F (Boehringer Mannheim) were added and the sample was then incubated at 37°C for 16 h. As a control, the same amount of sample was treated and incubated as described without adding enzyme. The enzyme-treated and non-treated samples were analysed by SDS-PAGE and immunoblotting with MoAbs.

N-terminal amino acid sequence analysis

Components in the extract of *D. farinae* prepared by using mites obtained from Allergon AB were separated by 2D gel electrophoresis and transferred electrophoretically onto a PVDF membrane. The membrane-bound proteins were visualized by Coomassie blue staining. The 25 kDa component reacted with MoAb against Der f 7 was then excised from the membrane for amino acid sequence analysis. Automated cycles of Edman degradation were performed with an ABI Procise Model 491 sequencer (Applied Biosystems, Inc., Foster City, CA, USA) according to the manufacturer's instruction.

Cross-inhibition ELISA

MoAbs from ascitic fluid were purified by precipitation with 40% saturated ammonium sulphate, dialysed against 50 mM ammonium bicarbonate and lyophilized. MoAbs were biotinylated according to published protocol [7]. Briefly, 40 microlitre 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 adding 32 µL of 1 M NH₄Cl, the mixture was incubated at room temperature for 10 min and then dialysed against PBS (phosphate buffered saline) to remove uncoupled biotins. The biotinylated MoAb was titrated by ELISA using plates coated with rDer f 7 at 0.05 µg/well to determine the dilution factor to be used in the inhibition assay. Wells of microtitre plates (Costar, Cambridge, MA, USA) coated with rDer f 7 were then incubated with unmodified MoAbs at a concentration of 10 µg/mL in PBST (PBS containing 0.05% [v/v] Tween 20) containing 1% skim milk or with PBST-1% milk 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 µL of a solution containing peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was added and incubated for 1 h at 37°C. Following the washing procedure, 50 µL 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 per cent 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 f 7 was analysed by RIA. The wells of microtitre plates coated with rDer f 7 at 0.05 µg/well were incubated with different amounts of various MoAbs or with PBST containing 1% skim milk alone at 37°C for 1 h. After washing with PBST, serum samples from asthmatic children known to contain IgE antibodies against Der f 7 were added into each well. Incubation was carried out overnight at 4°C. The presence of specific IgE antibodies was detected by incubation with ¹²⁵I-labelled monoclonal anti-human IgE antibodies (2.1.5 available from Silenius Laboratories). The radioactivity of each well was then counted in a gamma counter. An average of two readings from two separate determinations was calculated. The % inhibition was calculated as:

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

Two-site ELISA assay

The content of the group 7 allergens in mite extracts was determined by a two-site ELISA assay using a pair of crossreacting MoAbs against different epitopes of the group 7 mite allergens. Fifty microlitre of purified MoAb WH9 dissolved in carbonate buffer, pH 9.6 at a concentration of 2 µg/mL was coated onto wells of polyvinyl microtitre plates (Costar). After incubation at 4°C for 16 h, the wells were blocked with 1% skimmed milk. Fifty microlitre of recombinant Der p 7 and Der f 7 in different concentrations as well as commercial mite extracts in serial dilutions were added separately into the wells and incubated at 37°C for 1 h. After washing with PBST, 50 µL of diluted biotinylated MoAb HD19 was added to each well and incubated at 37°C for another hour. The wells were washed with PBST again and 50 µL of a solution containing peroxidase-conjugated streptavidin followed by 50 µL of a substrate solution were added to each well as described above in the cross-inhibition ELISA. The colour change was measured by the absorbance at 415 nm on a Titertek Multiskan (Flow Laboratories). An average of two readings from two separate determinations was calculated. The content of the group 7 allergens in mite extracts were calculated according to the standard response curves using recombinant Der p 7 or Der f 7 performed in parallel with each assay. Recombinant Der p 2 [3] and a commercial extract of *P. notatum* were also included in the sandwich ELISA for the background control. The determination of Der p 1 in the extract of *D. pteronyssinus* was performed according to the method described previously [8].

Table 1. Immunological characteristics of MoAbs against Der f 7

MoAbs	ELISA activity (A415)		Immunoglobulin isotype
	rDer p 7	rDer f 7	
HD2	0.089	1.482	IgG2a,k
HD4	0.119	1.571	IgG2b,k
HD10	0.098	1.951	IgG2a,k
HD12	0.089	1.951	IgG2a,k
HD19*	2.201	2.059	IgG1,k
P40+	0.142	0.129	IgG1,k

* MoAb against Der f 7 generated by us before [4].

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

Results

Preparation of MoAbs against Der f 7

In addition to MoAb HD19 that was previously generated by immunization of BALB/c mice with rDer f 7 [4], four more MoAbs that showed positive ELISA reactivity against Der f 7 were isolated (Table 1). The isotype of MoAbs HD2, HD10 and HD12 is IgG2a with κ light chain, and the isotype of MoAb HD4 is IgG2b, κ (Table 1). Although MoAb HD19 reacted with both Der p 7 and Der f 7, all four MoAbs obtained in the present study showed ELISA reactivity to rDer f 7 only (Table 1).

SDS-PAGE-immunoblotting

The specificity of MoAbs obtained has also been confirmed by SDS-PAGE immunoblotting. The results in Fig. 1 show that all five MoAbs (HD2, 4, 10, 12 and 19) reacted with rDer f 7 (panel A-II, strips a-e) and only MoAb HD19 showed immunoblotting reactivity to rDer p 7 (panel B-II, strip e). ELISA and immunoblotting results, as well as direct tests with the GST protein alone (not shown), have shown that these MoAbs do not react with the GST part of the fusion protein. The monoclonal antibodies HD10 and 12 in track A(c) and (d) gave reactions to several low Mr species of rDer f 7, presumably degradation products.

The results in Fig. 2 show that all five MoAbs mentioned above and another five MoAbs (WH9, 14, 22, 27 and WP8) generated previously by immunization with rDer p 7 [4] reacted predominantly with components of *D. farinae*

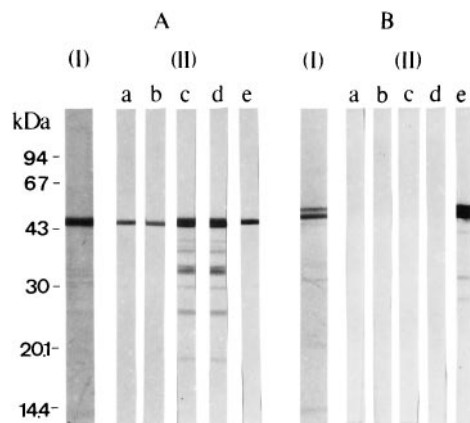


Fig. 1. SDS-PAGE-immunoblot reactivity of MoAbs HD2, 4, 10, 12 and 19 to rDer f 7 and rDer p 7. Panels A-I and B-I show the Coomassie blue-stained protein profile of rDer f 7 and rDer p 7 on PVDF membranes, respectively. Strips a-e in panels A-II and B-II show immunoblotting reactivity of MoAbs HD2 (a), HD4 (b), HD10 (c), HD12 (d) and HD19 (e) to rDer f 7 and rDer p 7, respectively.

(obtained from Allergon AB) with molecular masses of about 31, 30 and 25 kDa. The immunoblotting reactivity of these antibodies to the 25 kDa component was stronger than that to the 31 and 30 kDa components. In addition, MoAbs HD10 and HD12 immunostained to an 18 kDa component. The results are presented in Fig. 2, panel B, strips i and j.

2D-immunoblotting

Components of *D. farinae* (obtained from Allergon AB) reacted with MoAbs against rDer f 7 were characterised further by 2D-immunoblotting. The electrophoretogram of the *D. farinae* extract is presented in Fig. 3A. More than 100

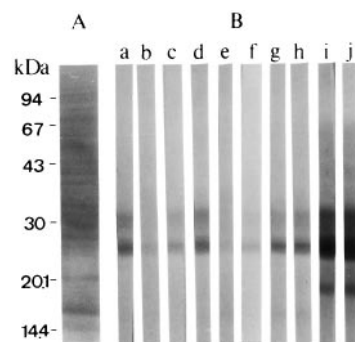


Fig. 2. SDS-PAGE-immunoblot reactivity of 10 different MoAbs to components of *D. farinae*. Panel A shows the Coomassie blue-stained protein profile of *D. farinae* (obtained from Allergon AB) on the PVDF blot. Strips a-j of panel B show the immunoblot reactivity of MoAbs WH9 (a), WH14 (b), WH22 (c), WH27 (d), WP8 (e), HD19 (f), HD2 (g), HD4 (h), HD10 (i) and HD12 (j) to components of *D. farinae*.

protein spots were detected by silver-staining. After immunoblotting, components with molecular mass of about 25.5, 25 and 24.5 kDa and pI values from about 5.6–6.4 were shown to be reactive to MoAb HD12 (Fig. 3B). The arrows in Fig. 3 indicate protein spots in panel A that have enzymatic colour reaction in immunoblotting as shown in panel B.

Deglycosylation

Enzymatic deglycosylation was undertaken to test for the presence of N-linked carbohydrates on Der f 7 which should be present as predicted from the cDNA sequence [2]. Panels A and B in Fig. 4 show the deglycosylation results of *D. farinae* extracts prepared from mites obtained from

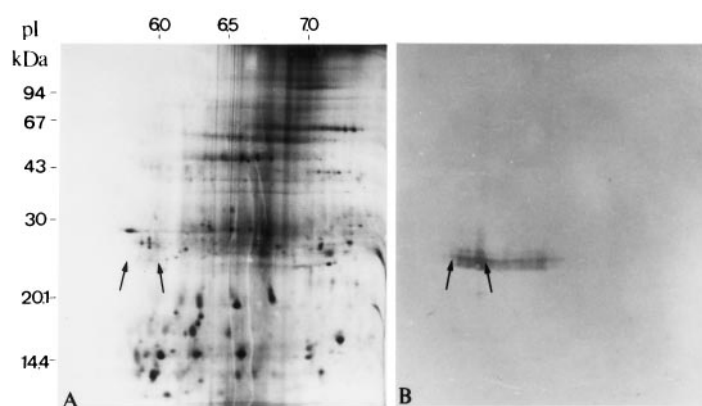


Fig. 3. 2D-immunoblot analysis of components of *D. farinae* reacted with MoAb HD12. Panel A demonstrates the silver-stained 2D protein profile of an extract of *D. farinae* prepared by using mites obtained from Allergon AB. Panel B shows the immunoblot reactivity of MoAb HD12 to components of *D. farinae* separated by two-dimensional gel electrophoresis. The arrows indicate protein spots in panel A which showed enzymatic colour reaction in immunoblotting as shown in panel B.

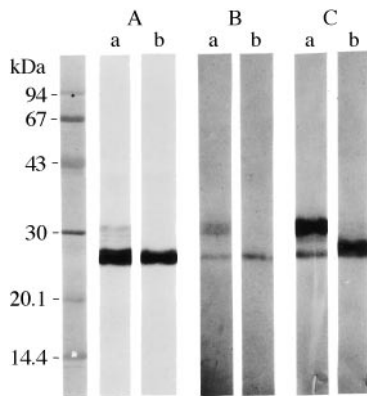


Fig. 4. SDS-PAGE-immunoblotting reactivity of MoAbs HD12 (panels A and B) and WH9 (panel C) to components of *D. farinae* (panels A and B) and *D. pteronyssinus* (panel C) without (strips a) and with the treatment of N-glycosidase F (strips b). The extract used in panel A was prepared using *D. farinae* obtained from Allergon AB. The extract of *D. farinae* used in panel B was obtained from Greer Laboratories.

Allergon AB and Greer Laboratories, Inc., respectively. In the controls without treatment with N-glycosidase F the extracts from both companies had components with molecular mass of about 31, 30 and 25 kDa which were reactive to HD12 (strips a of panels A and B). The Allergon AB extracts showed additional bands at 29 and 26 kDa (Fig. 4, A-a). The Allergon extract in panel A had almost all of its immunoblotting reactivity to MoAb HD12 in the 25 kDa component whereas the reactivities against the 31, 30 and 25 kDa components in the *D. farinae* extract obtained from Greer Laboratories were similar (panel B, strip a). The reactivity of *D. pteronyssinus* extracts was, as found previously, predominantly to the high molecular weight bands. After treatment with N-glycosidase F, the 25 kDa component in both extracts was shown to be still reactive to the MoAb HD12 in immunoblotting as shown in Fig. 4, strips b of panels A and B. As a control, an extract of *D. pteronyssinus* prepared by using mites obtained from CSL Ltd (Parkville, Australia) was deglycosylated and reacted with MoAb WH9. The results are presented in panel C of Fig. 4.

N-terminal amino acid sequence analysis

The 25 kDa component of *D. farinae*, with pI values from about 5.6–6.1, was subjected to Edman degradation. The sequence of the first 25 amino acid residues was determined to be DPIHYDKITEEINKAIDDAIAAIEQ. The experimental result is identical to that deduced from the cDNA sequence of Der f 7 [2], and shows that we correctly assigned the length of the leader sequence and the first amino acid residue of the mature protein.

Table 2. Inhibition of biotinylated MoAbs to solid-phase rDer f 7 by unlabeled MoAbs (% inhibition)

Inhibitors (10 µg/mL)	Biotinylated MoAbs				
	HD2 (%)	HD4 (%)	HD10 (%)	HD12 (%)	HD19 (%)
HD2	55	73	0	–5	90
HD4	56	72	0	–8	90
HD10	6	4	62	78	4
HD12	1	–2	58	79	1
HD19	52	48	–1	–9	81
P40*	1	–3	–5	2	–2

* A MoAb against the 68 kDa allergen of *Penicillium notatum*.

Mapping of epitopes recognized by MoAbs

The relationship between the epitopes on Der f 7 recognized by the MoAbs was analysed by inhibitory ELISA. The MoAbs were purified, biotinylated and titrated against rDer f 7 to determine the concentration required to give an absorbance values between 1.0 and 2.0 which are in the responsive region of the dose–response titration curve. Unmodified MoAbs were then used to inhibit binding. The results in Table 2 show the per cent inhibition of individual MoAb to five different biotinylated MoAbs. When 10 µg/mL of MoAb was used, the homologous antibodies showed 55–81% inhibition. In control experiments the anti *Penicillium notatum* MoAb P40 showed 2% inhibition. Two groups with different patterns of inhibition were noted. MoAbs HD2, HD4 and HD19 were self-inhibitory but had little effect on the binding of HD10 and HD12. MoAbs HD10 and HD12 showed a reciprocal pattern. Since HD2 and HD4 are Der f 7-specific, the epitope(s) recognized by both MoAbs should be different from that recognized by the MoAb HD19 which crossreacted with Der p 7.

Blocking of IgE binding by MoAbs

The newly described species-specific MoAb HD2 and 4 and HD10 and 12 were examined for their ability to block IgE binding to rDer f 7. The results in Table 3 show blocking experiments with four serum samples. Samples 1 and 4 were from individual patients and 2 and 4 were each pools of three patients. Significant inhibition was achieved by the MoAb in some but not all sera, the highest being 49% for serum 1 and MoAb HD2. The pattern of inhibition was similar for HD2 and HD4 and for HD10 and HD12 in accord with their cross-inhibition groups in Table 2. The blocking was compared with the crossreactive HD19 and although it was cross-inhibitory for HD2 and HD4 binding the pattern for IgE inhibition was different.

Table 3. Blocking of IgE-binding to solid phase rDer f 7 by different MoAbs against the group 7 allergens

Inhibitor	concentration ($\mu\text{g/mL}$)	Serum 1 (%)	Serum 2 (%)	Serum 3 (%)	Serum 4 (%)
HD2	10	46	17	8	36
	1.0	49	10	6	23
	0.1	3	-4	2	19
HD4	10	41	15	11	26
	1.0	35	11	10	21
	0.1	1	-3	-5	13
HD10	10	13	0	15	13
	1.0	4	-8	-5	8
	0.1	3	ND*	-6	14
HD12	10	26	9	2	14
	1.0	8	5	5	15
	0.1	9	ND	-8	ND
HD19	10	38	30	23	19
	1.0	43	6	14	19
	0.1	7	-5	9	9
P40**	10	-2	-10	-7	11
	1.0	-3	-10	-7	5
	0.1	2	-10	-4	10

* Not done.

** A MoAb against the 68 kDa allergen of *Penicillium notatum*.

The standard deviation for the duplicates for each point is an average of 4.0, 4.0, 5.6, 4.0, 4.5 and 3.3 for MoAbs HD2, HD4, HD10, HD12, HD19 and P40, respectively. The IgE binding of serum samples, 1, 2, 3 and 4 was 250 ± 20 , 413 ± 63 , 2239 ± 104 and 456 ± 54 cpm, respectively.

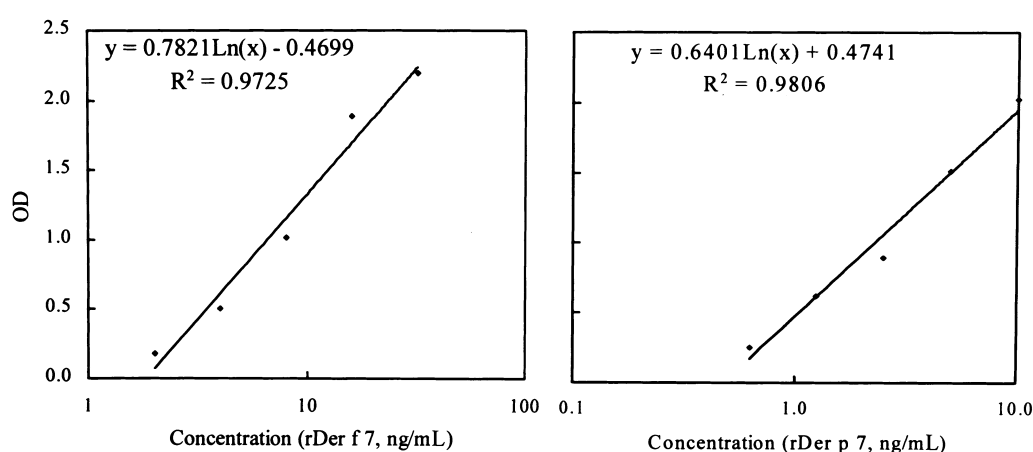


Fig. 5. The standard response curves for a two-site ELISA assay in the determination of the content of Der f 7 (left) and Der p 7 (right) in commercial mite extracts. Plates were coated with MoAb WH9, incubated with doubling dilutions of recombinant Der f 7 and Der p 7, respectively, and then with biotinylated MoAb HD19 as second antibody.

Determination of the content of Der p 7 and Der f 7 in mite extracts

In a sandwich ELISA using capture WH9 and biotinylated HD19, the content of Der f 7 in the extract of *D. farinae* (Greer, GB64, 37B-9UR3) was determined to be 30 ng/mL. The concentrations of Der p 7 and Der p 1 were 720 ng/mL and 40 µg/mL, respectively when the *D. pteronyssinus* extract (Greer, GB70-18B-7UR2) obtained from Greer Lab was analysed. The standard response-curves for the quantitation of Der f 7 and Der p 7 are shown in Fig. 5, respectively. Only background readings were obtained when corresponding concentrations of recombinant Der p 2 and dilutions of an extract of *P. notatum* (Greer, BM19-45B-1A2) were included in the same assay (data not shown). The background readings were subtracted from the readings of the corresponding concentrations of recombinant Der p 7 and Der f 7 and the readings of different dilutions of the test samples before the construction of the standard curves and calculation of the content of the group 7 allergens in mite extracts.

Discussion

The cDNA, of Der p 7 and Der f 7 allergens encodes 198 and 196 amino acid polypeptides with a calculated molecular mass of 22 kDa. Here it is shown using a panel of MoAb for immunoblotting that in *D. farinae* extracts Der f 7 exists in 31, 30, 29, 26, 25 and 18 kDa forms. Digestion with N-glycosidase F has indicated the components of molecular mass 31–26 kDa are N-glycosylation products of the 25 kDa component. The sequences of Der p 7 and Der f 7 contain an N-glycosylation site at residues 134–136 and a similar reduction of molecular size with glycosidase treatment has been described for rDer p 7. The most dominant band detected in *D. farinae* was the 25 kDa moiety whereas for *D. pteronyssinus* the 31, 30 kDa bands were the most dominant. However, although the 25 kDa species was more dominant in Der f 7 the two *D. farinae* extracts examined were different. The 25 kDa component was by far the most dominant in one extract while in another 30 and 25 kDa components showed equal staining. Clearly this heterogeneity and variation would present a complicated pattern for analyses attempting to determine the relative importance of different allergens by techniques such as Western blotting and comparing *D. farinae* and *D. pteronyssinus* extracts.

The MoAbs also detected an 18 kDa component of the *D. farinae*. This had quite strong immunostaining but was only detected by the MoAbs 10 and 12. These MoAbs are of the same isotype and are mutually cross-inhibitory so they may well have originated from the same clone. The 18 kDa reactivity could represent a coincidental crossreactivity to

another protein but two findings suggest it is more significant. Firstly, these MoAbs but not the others reacted with a series of low molecular weight bands of recombinant Der f 7 suggesting they have an ability to bind to degradation products. Secondly our absorption studies with rDer p 7 on the IgE binding to *D. pteronyssinus* extracts showed absorption at bands estimated to be at 13 and 11.5 kDa as well as the expected bands corresponding to the larger molecules [1]. It therefore appears that degradation products of the group 7 allergens can persist in extracts, again providing a complication for allergen analyses.

A pertinent comparison can be made with results of O'Neill and Baldo [9]. They found that IgE antibodies affinity purified from a 30–32 kDa region of an SDS-PAGE gel reacted with bands of 32, 30 and 25 kDa as well as smaller 15 kDa bands. Similarly IgE affinity purified with a 25 kDa region reacted back to bands of 32, 30 and 25 kDa. These results were interpreted as crossreactivities between the major group 1 (25 kDa) and group 3 (32/30 kDa) allergens but now the most obvious interpretation of this and the staining of the smaller band is reactivity with the different forms of Der p 7. It also remains to be determined what proportion of the IgE binding bands frequently found in these regions are Der p 7 and not Der p 1 or 3.

In 2D-immunoblotting, Der p 7 showed at least six major forms with pI values ranging from 5.6 to 6.0 [5]. In the present study, Der f 7 showed pI values ranging from 5.6 to 6.4. However, components with pI values from 5.6 to 6.1 have stronger colour reactivity to MoAb HD12 than the other isoforms. They probably represent the major isoforms of Der f 7. Furthermore, possibly due to the presence of small amount of N-glycosylated forms of Der f 7, the reactivities of the 31–29 kDa components were barely detectable in 2D-immunoblotting in the present study. The N-terminal sequence of the 25 kDa protein with pI values of about 5.6–6.1 was determined. The protein has an N-terminal Asp (D), as predicted previously by us from the cDNA sequence [2]. Since the first 15 amino acids of the deduced mature Der p 7 and Der f 7 proteins are identical [2], it is conceivable that Der p 7 has also an N-terminal Asp (D).

Two different reaction patterns to Der f 7 were observed for the MoAbs HD2, 4, 10, 12 and 19 in a cross-inhibition ELISA. The HD10 and 12 cross-inhibit each other but not the other MoAbs while HD2, 4 and 19 had a reciprocal pattern. However, HD2 and four were species-specific while HD19 was crossreactive suggesting different but related epitopes. Lind *et al.* [10] and Chapman [11] have also reported that MoAbs directed against the same or closely positioned site on the antigen surface were found to have different degrees of species specificity. In our studies on the blocking of binding of IgE to rDer f 7 the pattern for the inhibition of different sera correlated with the two

cross-inhibitory groups of species-specific MoAbs HD2 and 4 and HD10 and 12 but the pattern for the crossreactive HD19 was different.

Most MoAbs produced to the group 1 house dust mite allergens (which have 81% sequence identity [12]) have shown species specificity with respect to their reactivity to Der p 1 and Der f 1 [11]. Species-specific MoAb to the group 2 allergens which have 88% sequence homology [13] were not so forthcoming but have now been described [14,15]. Previous MoAbs produced against rDer p 7 (WH9, 14, 22, 27 and WP8) and rDer f 7 (H19) have crossreacted with the group 7 allergens of both species which show 86% identity. It was therefore significant to produce the four MoAbs which are specific for Der f 7. The MoAbs described here and elsewhere [4,5] had different binding sites as judged by cross-inhibition of allergen-binding so their ability to measure allergen levels by a two site MoAb assay [15–17] was tested. The combination of HD19 and WH9 shown here was accordingly able to produce a titration measuring both Der f 7 and Der p 7. The concentration of the group 7 in house dust mite extracts of about 1 µg/mL was considerably less than Der p 1. It can induce large IgE responses in a significant number of people so it is possible its concentration, perhaps for reasons of stability, is under-represented in the extract compared to natural exposure. Alternatively if the natural exposure is low it raises interesting questions on the effect of the concentration on the induction of sensitization and on acute and chronic inflammatory responses.

Acknowledgements

This work was supported by grants of the National Health Research Institutes (DOH85-HR-508) and the Veterans General Hospital-Taipei of the Republic of China. We would like to thank Mr Chia-hsi Chan for his technical assistance in the two-site ELISA assay.

References

- Shen HD, Chua KY, Lin KL, Hsieh KH, Thomas WR. Molecular cloning of a house dust mite allergen with common antibody binding specificities with multiple components in mite extracts. *Clin Exp Allergy* 1993; 23:934–40.
- Shen HD, Chua KY, Lin WL, Hsieh KH, Thomas WR. Molecular cloning and immunological characterization of the house dust mite allergen Der f 7. *Clin Exp Allergy* 1995; 25:1000–6.
- Chua KY, Dilworth RJ, Thomas WR. Expression of Dermatophagoides pteronyssinus allergen, Der p II, in *Escherichia coli* and the binding studies with human IgE. *Int Arch Allergy Appl Immunol* 1990; 91:124–9.
- Shen HD, Chua KY, Lin WL, Chen HL, Hsieh KH, Thomas WR. IgE and monoclonal antibody binding by the mite allergen Der p 7. *Clin Exp Allergy* 1996; 26:308–15.
- Shen HD, Chua KY, Lin WL, Hsieh KH, Thomas WR. Characterization of the house dust mite allergen Der p 7 by monoclonal antibodies. *Clin Exp Allergy* 1995; 25:416–22.
- Smith DB, Johnson KS. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 1988; 67:31–40.
- Harlow E, Lane D. *Antibodies. A laboratory manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1988.
- Li CS, Wan GH, Hsieh KH, Chua KY, Lin RH. Seasonal variation of house dust mite allergen (Der p I) in a subtropical climate. *J Allergy Clin Immunol* 1994; 94:131–4.
- O'Neill GM, Baldo BA. Intraspecies cross-reactivity of house dust mite allergens. *Electrophoresis* 1993; 14:923–5.
- Lind P, Hansen OC, Horn N. The binding of mouse hybridoma and human IgE antibodies to the major fecal allergen, Der p I, of *Dermatophagoides pteronyssinus*. Relative binding site location and species specificity studied by solid-phase inhibition assays with radiolabeled antigen. *J Immunol* 1988; 140:4256–62.
- Chapman MD, Heymann PW, Platts-Mills TAE. Epitope mapping of two major inhalant allergens, Der p I and Der f I, from mites of the genus *Dermatophagoides*. *J Immunol* 1987; 139:1479–84.
- Dilworth RJ, Chua KY, Thoms WR. Sequence analysis of cDNA coding for a major house dust mite allergen, Der f I. *Clin Exp Allergy* 1991; 21:25–32.
- Trudinger M, Chua KY, Thomas WR. cDNA encoding the major mite allergen Der f II. *Clin Exp Allergy* 1991; 21:33–7.
- Akagawa M, Mori T, Ando T, Okudaira H. Specific measurement of a major mite allergen, Der f II, by an enzyme-linked immunosorbent assay system using monoclonal anti-Der f II antibodies. *Biosci Biotech Biochem* 1992; 56:1725–7.
- Ovsyannikova IG, Vailes LD, Li Y, Heymann PW, Chapman MD. Monoclonal antibodies to group II *Dermatophagoides* spp. allergens: murine immune response, epitope analysis, and development of a two-site ELISA. *J Allergy Clin Immunol* 1994; 94:537–46.
- Chapman MD, Heymann PW, Wilkins SR, Brown MJ, Platts-Mills TAE. Monoclonal immunoassays for major dust mite (*Dermatophagoides*) allergens, Der p I and Der f I, and quantitative analysis of the allergen content of mite and house dust extracts. *J Allergy Clin Immunol* 1987; 80:184–94.
- Luczynska CM, Arruda LK, Platts-Mills TAE, Miller JD, Lopez M, Chapman MD. A two-site monoclonal antibody ELISA for the quantitation of the major *Dermatophagoides* spp. allergens, Der p I and Der f I. *J Immunol Methods* 1989; 118:227–35.