

Characterization of the house dust mite allergen Der p 7 by monoclonal antibodies

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Summary

The house dust mite allergen Der p 7, which was defined by cDNA cloning, has been shown to react with about 50% of allergic sera and corresponds to or is antigenically related to at least three different sized components in mite extracts. To characterize these entities, monoclonal antibodies (MoAbs) were generated by immunizing BALB/c mice affinity-purified Der p 7-GST (glutathione S-transferase) fusion protein. MoAbs WH9 and WH22 showed positive reactivity to recombinant Der p 7 negative reactivity to GST and the Der p 5-GST fusion protein in ELISA and immunoblotting. The specificity of both MoAbs was confirmed by inhibition of the ELISA activity by recombinant Der p 7 but not by the recombinant Der p 5. Immunoblot analysis demonstrated that both MoAbs showed reactivities to components with molecular weights (mol. wt.) of 31, 30 and 26 kDa reactive to both MoAbs. At least six major forms with different pI or size were indicated by 2-D gel analysis. In addition to characterization of Der p 7, both MoAbs may also be considered for use in the standardization of Der p 7 in mite extracts.

Clinical and Experimental Allergy, Vol. 25, pp. 416–422. Submitted 24 May 1994; revised 16 October 1994; accepted 1 December 1994.

Introduction

House dust mites have been identified as an important causative agent of bronchial asthma [1]. Data obtained from immunoblotting has shown that more than 30 mite bands react with IgE antibodies and most reactivity is associated with six or seven components [2–4]. For *Dermatophagoides pteronyssinus* seven allergens (Der p 1–7) have been characterized with known cDNA sequences or N-terminal amino acid sequences [5]. Der p 1–4 and 6 have been identified as a cysteine protease, possibly lysozyme, trypsin, amylase and chymotrypsin, respectively [6–10]. No sequence homologues have been found for Der p 5 and Der p 7 in searches of the data banks [11,12]. Absorption studies using affinity-purified rabbit antibodies and allergic serum samples showed that mite components with molecular weight (mol. wt.) of

about 29, 27 and 24 kDa correspond to Der p 7 [12]. Skin tests showed reactivity of Der p 7 in 16/30 (53%) allergic patients and 0/10 of controls [12]. To further characterize Der p 7 molecular antibodies (MoAbs) against this newly identified allergen were generated in the present study. The results obtained provide evidence that mite components with mol. wts. of about 29 and 27 kDa represent different glycosylation products of Der p 7 although further heterogeneity remained.

Materials and methods

Preparation of mite extracts

The house dust mite *D. pteronyssinus* was obtained from CSL Ltd (Parkville, Australia). They were extracted by homogenizing the lyophilized mites resuspended in phosphate-buffered saline (PBS) containing aprotinin (0.1 TIU/ml; Sigma Chemicals Co, St Louis, MO, USA) and phenylmethylsulphonyl fluoride (PMSF,

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1 mM; Sigma). After centrifugation at 10 000 *g* for 30 min, the supernatant was dialysed against 0.05 mol/l of ammonium bicarbonate, pH 8.0. The supernatant was then freeze-dried in aliquots and stored at 4°C.

Preparation of recombinant Der p 7 and Der p 5

Constructs expressing Der p 7 and Der p 5 as fusion proteins from the pGEX vectors were prepared as described [12,13]. The polypeptides fused to glutathione-S-transferase (GST) encoded by the Sj26 gene of the pGEX were affinity purified from glutathione agarose [14].

Development of molecular antibodies

MoAbs against Der p 7 were prepared essentially as described previously [15]. Spleen cells obtained from BALB/c mice immunized with the recombinant Der p 7 were fused with murine plasmacytoma NS-1 cells in the presence of polyethylene glycol (mol. wt. 1500 Da; Merck, Hohenbrunn, Germany). Antibody-producing hybrid cells were screened by the enzyme-linked immunosorbent assay (ELISA) using recombinant GST-Der p 7 and GST alone. Hybridomas producing Der p 7-specific MoAbs were subcloned at least twice by limiting dilutions and then further expanded.

ELISA

The ELISA was performed as described [15]. The antigens used were coated separately onto wells of polyvinyl microtitre plates (Costar, Cambridge, MA, USA) by the addition of 50 µl of a 0.5 µg/ml solution of recombinant proteins in carbonate buffer, pH 9.6. After blocking with 1% skimmed milk, culture supernatant solutions of hybridoma cells were added to each well and incubated for 1 h at 37°C. For inhibitory ELISA, different concentrations of recombinant proteins were added into the culture supernatant and incubated as described above. After washing with PBS containing 0.05% Tween 20 (PBST, peroxidase-conjugated goat anti-mouse IgG (whole molecule, Bio-Rad, Richmond, VA, USA) was added to each well and incubated for 1 h at 37°C. Wells were washed again with PBST and 50 µl of substrate solution (Bio-Rad, peroxidase substrate kit 172-1064, containing 2,2'-di-[3-ethylbenzthiazoline-6-sulfonic acid] and hydrogen peroxide) was added to each well for 30 min at 37°C. A colour change was detected by measurement of the absorbance at 415 nm on a Titertek Multiskan (Flow Laboratories, McLean, VA, USA). Culture supernatant solutions of hybridoma cells containing MoAbs against fungal proteins which have been

generated by us were used as negative control. An average of two readings from two separate determinations was calculated. Possible ELISA activity in the screening was attributed to supernatant which showed absorbance greater than 1.2 times the absorbance obtained with the negative control supernatant. The present inhibition was calculated as follows:

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

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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis-SDS-PAGE.

2-D gel electrophoresis

The 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 containing glycerol (10%), SDS (2.1%), and Bromophenol Blue, pH 6.8. The second dimension of electrophoresis was carried out on slab gels consisting of 13% polyacrylamide. Low mol. wt. standards (Pharmacia, Uppsala, Sweden) were used to determine the molecular weight of the protein components. Carbamylated creatinine kinase obtained from BDH (Poole, UK) was used as the charge standard for calculation of isoelectric point of the resolved proteins.

Immunoblotting

The components of recombinant proteins or components of the whole mite extracts recognized by MoAbs produced were analysed by gel electrophoresis and immunoblotting [15,16]. Briefly, recombinant proteins or components of the mite extracts were separated by SDS-PAGE or by the

Table 1. Immunological characteristics of MoAbs against Der p 7

MoAbs	ELISA activity (A415)	
	Der p 7	Whole mite extract
WH9	2.223 (46.3*)	0.161 (3.1)
WH22	2.2395 (49.9)	0.266 (5.2)
P40	0.048 (1)	0.051 (1)

*Data in parentheses represent the index value calculated by dividing the absorbance of MoAb tested by the absorbance of control MoAb P40 which was against a fungal protein.

two-dimensional (2D) gel electrophoresis and transferred electrophoretically onto polyvinylidene difluoride membrane (0.45 μ m, Millipore, Bedford, MA, USA). After blocking with 1% skimmed milk, the blot was incubated with culture supernatant solutions of hybridoma cells. For absorption studies, supernatant solutions which have been incubated overnight at 4°C

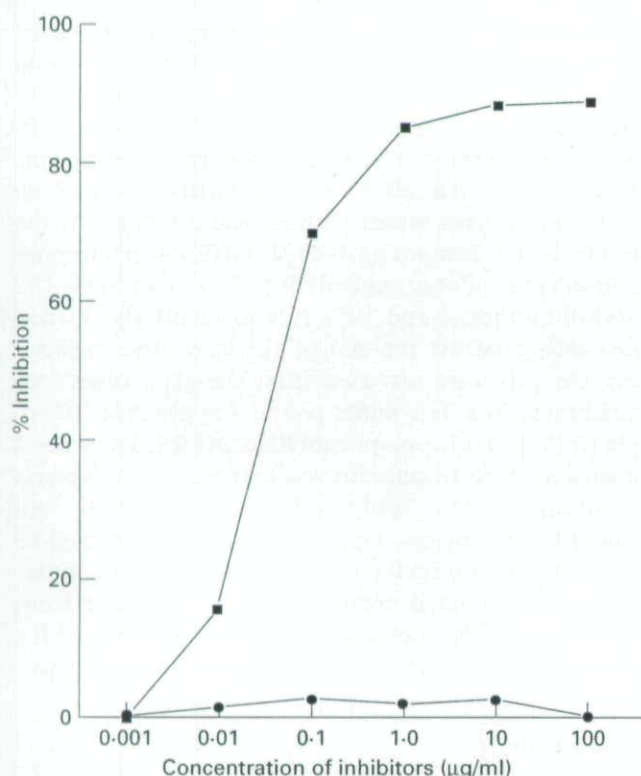


Fig. 1. Dose-dependent inhibition of ELISA activity of MoAb WH9 to recombinant Der p 7 by Der p 7-GST fusion protein but not by the Der p 5-GST fusion protein. The % inhibition was calculated as described in the Materials and methods. ■, r Der p 7. ●, r Der p 5.

with an equal volume of *Escherichia coli* (*E. coli*) lysates containing recombinant Der p 7 or another GST fusion with a *Haemophilus influenzae* protein (D15) were used. After washing procedures, all the blots were incubated with affinity-purified horseradish peroxidase-labeled 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

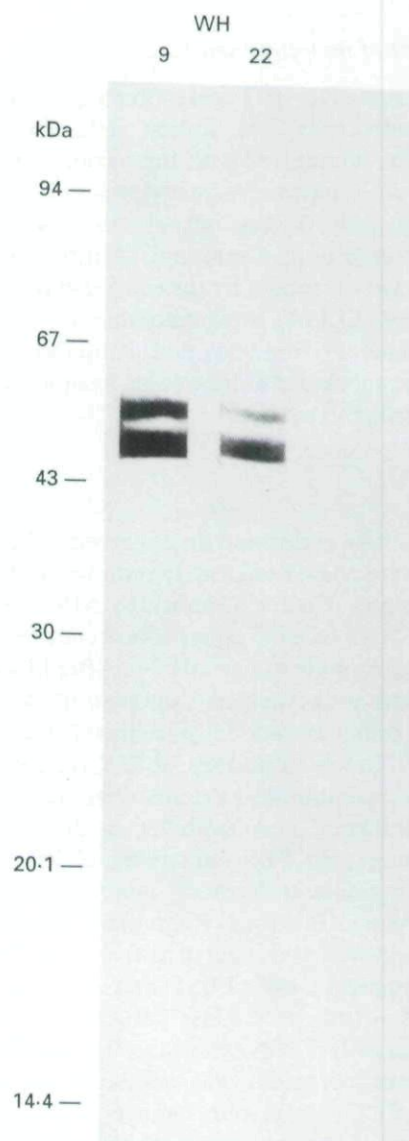


Fig. 2. Immunoblotting activity of MoAbs WH9 and WH22 to recombinant Der p 7.

5.0) containing 1 mM 3-amino-9-ethyl-carbazole (Sigma) and 0.015% (v/v) hydrogen peroxide for about 5 min. After washing with water, the deposition of the red stain which demonstrated antigen-antibody binding was recorded by photography. The same control culture

supernatant solutions used in ELISA were used as negative control in the immunoblotting.

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 109 µl of 1% SDS for 2 min. After the addition of 40 µl of

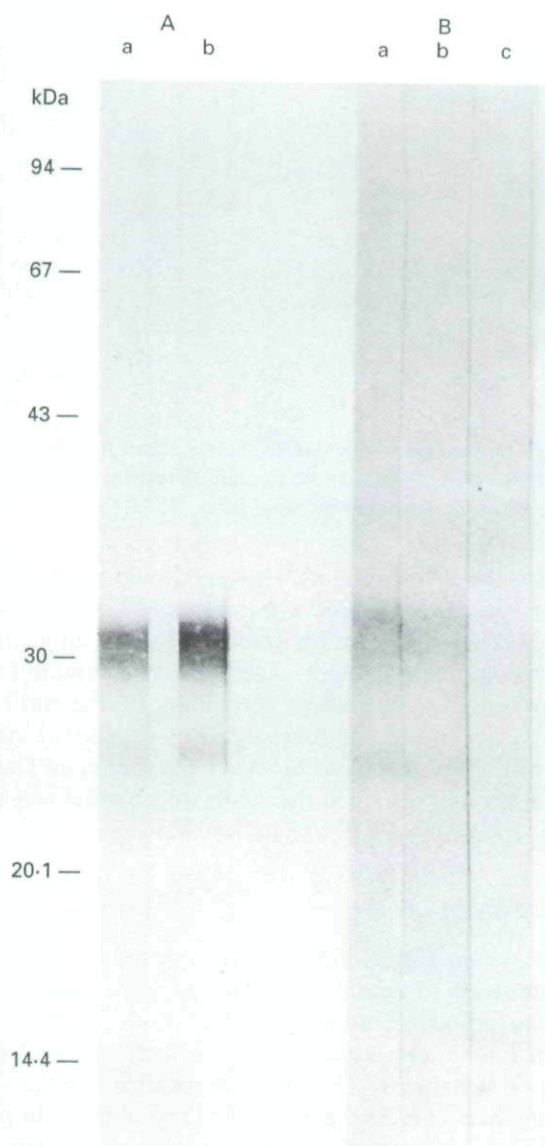


Fig. 3. Immunoblotting activity of MoAbs WH9 (A-a) and WH22 (A-b) to the whole extract of *Dermatophagoides pteronyssinus*. The activity of WH22 to the mite extract (B-a) was absorbed by pre-incubation of the antibody solution with *E. coli* lysate containing recombinant Der p 7 (B-c) but was not inhibited by pre-incubation of WH22 with lysate containing an unrelated GST fusion protein D15 (B-b).

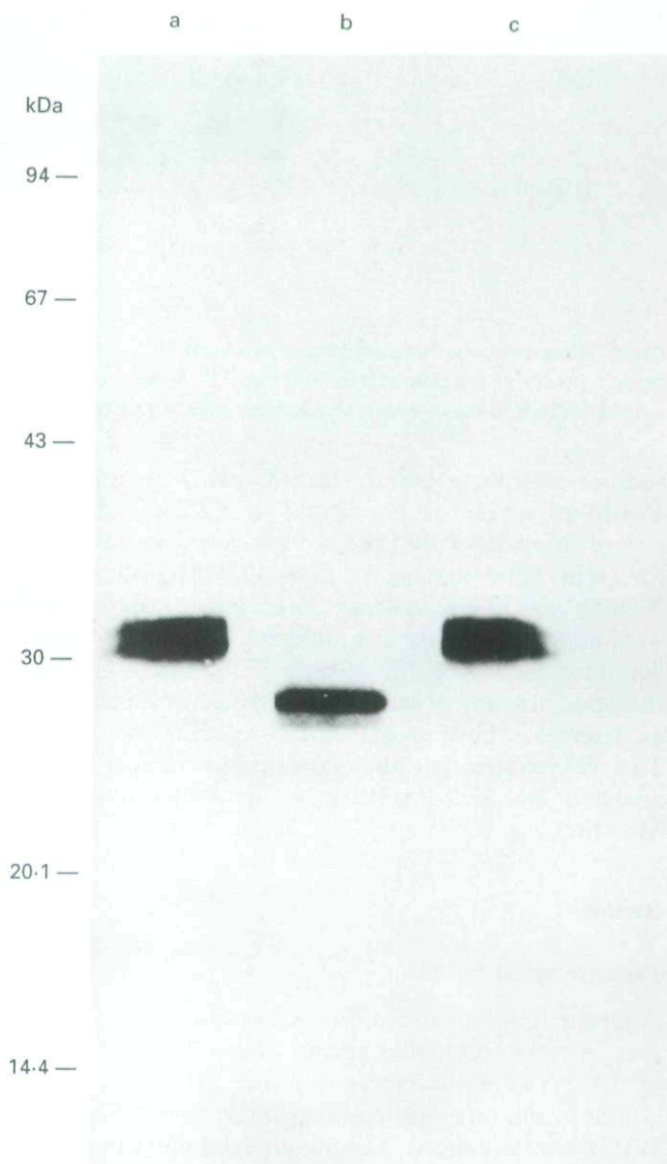


Fig. 4. Immunoblotting reactivities of MoAb WH9 to mite components without (a) and with the treatment of *N*-glycosidase F (b) and *O*-glycosidase (c).

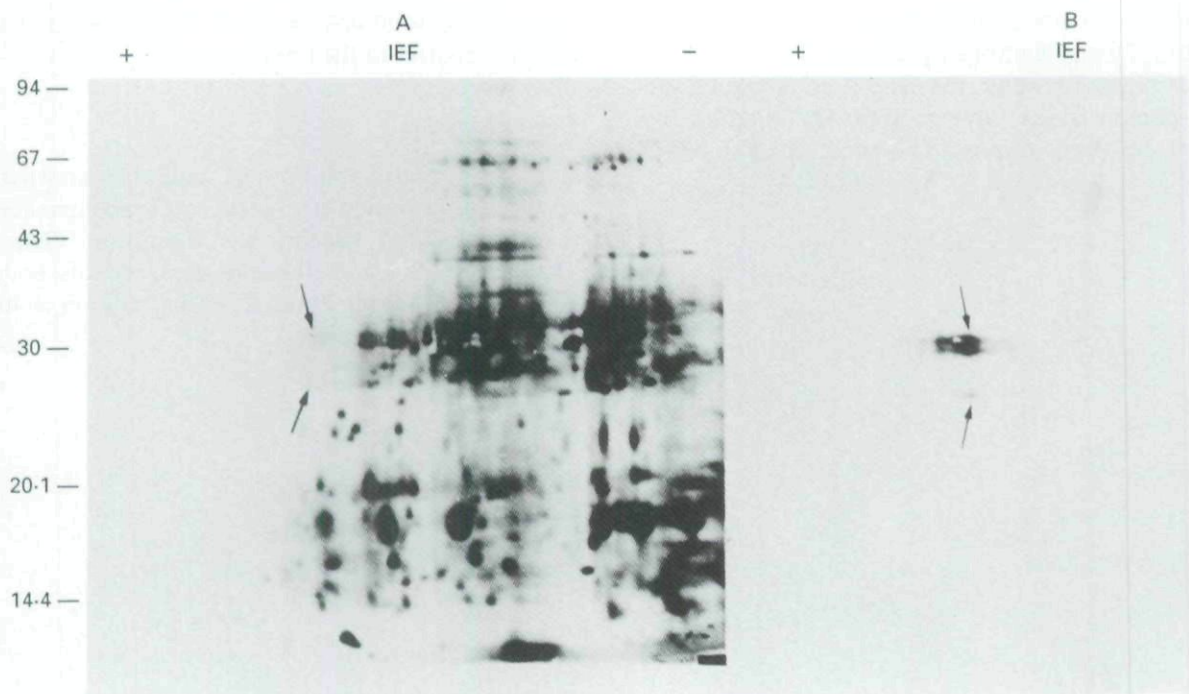


Fig. 5. 2D-immunoblotting activity of MoAb WH22 to the whole extract of *Dermatophagoides pteronyssinus*. Panel A shows the 2D protein profile of the mite extract and panel B shows the reactivity with MoAb WH22. The arrows in panel A indicate protein spots corresponding to those which showed enzyme colour reaction in immunoblotting as shown in panel B.

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 again for 2 min. After cooling, 1.2 units of *N*-glycosidase F or 7.5 mU of *O*-glycosidase (Boehringer, Mannheim) were added separately into different tubes. The sample was then incubated for 16 h at 37°C. For a control, the same amount of sample was treated and incubated as described above except that no enzyme was added. The enzyme-treated and non-treated samples were analysed by SDS-PAGE and immunoblotting with MoAbs.

Results

Preparation of MoAbs

After the fusion of spleen cells with NS-1 cells, hybridomas secreting antibodies against Der p 7 were screened by ELISA. The antibody-producing hybridomas were cloned by limiting dilution. Two stable clones WH9 and WH22 were expanded. The immunoglobulin isotypes of WH9 and WH22 are IgG2b, k and IgG1, k respectively (Table 1). Both MoAbs showed strong ELISA activity to recombinant Der p 7 (Table 1) and negative ELISA activity to control GST and to Der p 5-GST fusion

protein (data not shown). However, only weak ELISA activity of both MoAbs to extracts of *D. pteronyssinus* was detected (Table 1). The results in Fig. 1 demonstrated that recombinant Der p 7 rather than recombinant Der p 5 showed dose-dependent inhibition of the antibody activity in the ELISA. A similar dose-dependent inhibition of ELISA activity of MoAbs to recombinant Der p 7 was also observed when the whole mite extract was used in the inhibitory ELISA (data not shown).

SDS-PAGE immunoblotting

As shown in Fig. 2, both MoAbs reacted strongly with the doublets of recombinant Der p 7. No reaction was detected in immunoblotting when both MoAbs were reacted with the control GST or with Der p 5-GST (data not shown). These results confirm further that both MoAbs are Der p 7 specific. The blotting in panel A of Fig. 3 shows that both MoAbs reacted with the mite components with mol. wts. of about 31, 30 and 26 kDa. The reactivity was not detected after absorption of WH22 with *E. coli* lysates containing recombinant Der p 7 as shown in Fig. 3B-c. For a control, the same bands were still observed after absorption of WH22 with an unrelated pGEX construct (D15) (Fig. 3B-b). A similar result was also observed for WH9 (data not shown).

Deglycosylation

Enzymatic deglycosylation was undertaken to test for the presence of *N*-glycosylated carbohydrates on Der p 7 as predicted from the cDNA sequence [12]. Figure 4 shows that after treatment with *N*-glycosidase F, immunoblotting reactivity of WH9 to mite components with mol. wts. of about 27 and 26 kDa was observed (Fig. 4B). However, components with mol. wts. of about 31, 30 and 26 kDa were still shown to be reactive to WH9 in control experiments containing buffer only (Fig. 4A) or with the enzyme O-glycosidase (Fig. 4C). A similar result was observed when MoAb WH22 was used (data not shown).

2D-immunoblotting

The reaction of mite components with MoAbs was analysed further by 2D-immunoblotting. Figure 5A demonstrates the 2D protein profile of the whole extract of *D. pteronyssinus*. More than 100 protein spots were detected in the silver-stained 2D gel. After immunoblotting, components with pI values from about 5.6–6.0 and mol. wts. of about 31, 30 and 26 kDa were shown to be reactive to WH22 (Fig. 5B). A similar result was obtained for WH9 in 2D-immunoblotting (data not shown). The result also showed that there are at least six major isoforms of Der p 7 with pI values from about 5.7–6.0 in the whole mite extract (Fig. 5B). The arrows in Fig. 5A indicate protein spots corresponding to those which showed enzymatic colour reaction in immunoblotting as shown in Fig. 5B.

Discussion

A cDNA clone of Der p 7 has been isolated earlier from a lambda gt11 library of *D. pteronyssinus* cDNA by plaque radio-immunoassay [12]. Sera from 14/38 allergic children reacted strongly with the polypeptide produced by the clone (37%) [12]. Skin tests with Der p 7-GST fusion protein showed reactivity in 16/30 (53%) allergic patients. Absorption studies using recombinant Der p 7 previously performed showed that mite components with mol. wts. of about 29, 27 and 24 kDa were found to have specificities to affinity-purified rabbit antibodies and IgE in allergic sera [12]. To further characterize natural Der p 7, two MoAbs were generated in the present study by immunization of BALB/c mice with Der p 7-GST fusion protein. The specificity of both MoAbs to Der p 7 was demonstrated by their negative ELISA and immunoblotting reactivities to GST and to recombinant Der p 5 as well as by the dose-dependent inhibition of the ELISA by recombinant Der p 7 but not by recombinant Der p 5. In the present study, mite components with mol.

wts. similar to those identified previously [12] by using affinity-purified rabbit and human IgE antibodies were shown to be reactive to MoAbs generated. The slight discrepancy is probably due in part to the use of non-gradient gels in this study.

Since a single *N*-glycosylation site was found in the cDNA sequence of Der p 7 [12], the enzyme *N*-glycosidase F which cleaves *N*-linked oligosaccharides between *N*-acetylglucosamine and the asparagine residue was used in the present study. The results showed that the 31 and 30 kDa reactivities may be different glycosylation products of Der p 7 with a mol. wt. of about 27 kDa. It is interesting that the higher molecular doublet appears to resolve into one band after *N*-glycosidase treatment suggesting glycosylation may be required for the difference in the bands migrating at 31 and 30 kDa. However, since this component and the 26 kDa entity is still considerably higher than the calculated mol. wt. 22 kDa of the polypeptide, there may be a different modification product of Der p 7 or another mite component with a common antibody specificity with Der p 7. The fact that all of molecular sizes react with both MoAbs, however, strongly suggests the former. Since the O-glycosidase gave no change in molecular height it is possible it was not effective on the mite allergen preparation.

Since only weak ELISA reactivity of both MoAbs to the whole mite extract was observed, the possibility of a low content of Der p 7 in mite extract should be considered. Monoclonal antibodies will therefore provide a method for standardization of this newly-identified allergen in mite extract.

Acknowledgment

This work was supported by the grant NSC83-0418-B075-0011BB from the National Science Council of the Republic of China.

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