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Molecular polymorphisms of house dust mite allergens

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ABSTRACT

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Previous studies from this laboratory have described the primary amino acid sequences of the group I and group II allergens from *Dermatophagoides pteronyssinus* and *D. farinae*. This report concentrates on polymorphisms of allergens within a species. Firstly, four cDNA clones of *Der f*II produced by polymerase chain reaction have been sequenced and are compared to the sequences published previously by ourselves and others. Although the sequences come from different sources, Australia and Japan, the overriding conclusion is one of similarity, with only two possible non-conservative changes in the six sequences. The nucleotides were also very conserved including the 3' untranslated regions, although some non-coding differences could be found which may provide a genetic marker. Experiments are reported to help define the group III *D. pteronyssinus* allergens. Previous studies have characterised the group III of *D. farinae* as a Mr 29-kDa molecule which can be defined by monoclonal antibodies. A Mr 17-kDa molecule of *D. pteronyssinus* has been reported with an almost identical N-terminal sequence. Here it is described that *Der f*III isolated from different preparations of spent mite media by affinity chromatography have predominantly Mr 32-, 28- and 21-kDa forms which vary in degree from batch to batch. 83% of adults and 38% of children react with the preparation by radioimmune dot-blot. The difference between the children and adults is statistically significant and reactivity can be to at least the 32- and 28-kDa form. Antisera produced in mice against the *Der f*III react to *D. pteronyssinus* mite extract by Western blotting primarily to a 32-kDa moiety, but also 28- and 21-kDa forms in some extracts.

INTRODUCTION

House dust mites are a group of several species linked together because they release products into the domestic environment which cause allergic disease.

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The products we need to consider are from *Dermatophagoides pteronyssinus*, *D. farinae*, *D. microceras* and *Euroglyphus maynei*. In some areas or even countries one species can be overwhelmingly predominant, whereas in other locations species can be mixed even within individual households (Chapman et al., 1987). In an ideal situation it would be desirable to measure the immune response in terms of IgE and T cell function to each of these allergens. This could then be related to the level of allergen in the environment and to possible formulations for immunotherapy, which would need to take inter- and intraspecies polymorphisms into consideration.

At this stage it is clear that a large percentage of IgE and T cell activity is directed to the group I and group II allergens. For instance van der Zee et al. (1988) found half of the patients they examined had over 60% of their IgE to these components and often this could reach 80%. A vexed question, however, is still the role of other components even when they are minor IgE-binding determinants. The work of Tovey and Baldo (1987) has shown that Western blotting with house dust mite extracts and high concentrations of serum can reveal reactivity to a large number of electrophoretic bands with 10–12 not being uncommon in highly reactive adults. The significance of these reactivities needs to be determined and a starting point would be to determine the degree and affinity of the IgE reactions and the ability of the components to stimulate T cell responses. It is also important to investigate whether or not many of the components which react are related proteins, degradation products or secondary modifications. The grass pollen allergens for example exist as several related entities (Thomas, 1991), with their group I allergens showing homology with group II and III and with several related sequences and electrophoretic types found for group V components.

Thus, despite recent progress, there is much which can be done to characterise the allergens and the immune response to the allergens and much to be gained in the understanding of mite allergy. In this paper we will report ongoing research into the major group II allergens to describe and discuss the importance of polymorphisms with the *D. farinae* species and to describe investigations on the isolation of group III allergens or group III-related proteins from *D. pteronyssinus*. The group III allergens were isolated from *D. farinae* as a 29-kDa protein or glycoprotein by Heymann et al. (1989) and further defined by monoclonal antibodies and the N terminal sequence. A similar sequence was reported for *D. pteronyssinus* by Stewart et al. (1989) who recognised the sequence homology with serum proteases. The protein, however, was 17 kDa, and this and observations to be discussed suggest there may be several related entities in this group.

MATERIALS AND METHODS

Mite allergen preparations

Spent mite media was a gift from the Commonwealth Serum Laboratories, Melbourne, Australia and was stored at 4°C. It was then suspended in 0.1 M

Tris pH 7.4 and left overnight at 4°C with stirring and clarified by centrifugation at 10 000 *g* for 30 min. Mite extracts were prepared from dried mites purchased from Commonwealth Serum Laboratories as described (Stewart and Thomas, 1987) and stored as freeze-dried aliquots. For Western blotting extracts were suspended at 20 mg/ml in ammonium bicarbonate.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE gels were performed according to Laemmli (1970) with a standard 8–18% gradient for 10–12-cm gels or 16% for mini Protein II gel assemblies (Bio-Rad, Richmond, Ca). For house dust mite extracts, samples were loaded at 1 mg protein/track and purified proteins were typically electrophoresed at 20–50 μ g/track. For Western blotting proteins were transferred to nitrocellulose by the method of Burnette (1981). Filters were washed in 10 mM Tris-HCl with 0.05% Tween 20 pH 7.4 and then incubated with anti-serum in 5% skimmed milk. Incubation with rabbit and mouse antisera were for 1 h at room temperature and for human IgE overnight at 4°C. Filters were then washed and antibody reactivity developed with ^{125} I anti IgE or ^{125} I protein A and autoradiography, as described in Chua et al. (1990b).

Radioimmune dot-blots

To assay reactivity of allergens with IgE, 10 μ g of purified protein was applied to a nitrocellulose filter with a Bio-Rad transblot apparatus. Filters were washed and assayed as described above and by Chua et al. (1990b) for Western blotting.

Affinity chromatography

The anti *Der f* III used in these studies was supplied by Drs. Heymann, Chapman and Platts-Mills (Charlottesville, Virginia). An ammonium-sulphate-precipitated fraction was conjugated to sepharose 4B by standard protocols, e.g. Chua et al. (1990b), and used to isolate *Der f* III from *D. farinae* spent mite medium. For a 5-ml column, 200 ml of medium was passed over at 5–10 ml/h before washing and the *Der f* III was eluted with water titrated to pH 11 with ammonium hydroxide.

RESULTS

Polymorphism of group II allergens

The importance of the group II allergens has been described by many authors. In our studies with a cDNA clone for instance, it has been shown that 82/84 sera tested had strong anti *Der p* II reactivity by radioimmune dot-blot (Chua et al., 1991). Now cDNA clones and sequences are available for both *Der p* II and *Der f* II (Chua et al., 1990a; Yuuki 1990; Trudinger et al., 1991),

	10	20	30	40	50	60	
pFL1	DQVDVKDCANNEIKKVMVPGCHGSEPCIIHRGKPF	TLEALFDANQNTKAKIEIKASLDGLE					
pFL2	N.....	I.I.....					
MT 3	N.....	I.T.....					
MT 5 (1-92)	S.....	I.I.....					
MT18 (1-84)	N.....	I.I.....					
MT16 (1-70)	N.....	T.I.....					
	70	80	90	100	110	120	130
pFL1	IDVPGIDTNACHFMKCPLVKGQQYDIKYTWNVPKIAPKSENVVVTVKLIGDNGVLACAIATHAKIRD						
pFL2	A.....	V.....					
MT 3	A.....	V.....					
MT 5	I.....						
MT18							
MT16							

Fig. 1. Alignment of translated *Der f*II cDNA sequences obtained from Yuuki et al. (1990) (pFL1 and pFL2) and sequences of products of *Der f*II cDNA generated by polymerase chain reaction (sequences MT 3, 5, 16 and 18). The pFL1 sequence has been used as a prototype and alterations in other sequences shown. The *Der f*II sequence reported by Trudinger et al. (1991) was a composite of clone 3 (70–129) and 16 (1–70). Only partial sequences have been determined for MTS, 16 and 18.

the latter being from two independent laboratories. Subsequent to the publication of Trudinger et al. (1991), we have analysed the sequence of further PCR-generated clones. The alignment in Fig. 1 shows the translated amino acids in comparison with the two sequences reported by Yuuki and colleagues. For comparison we have used the pFL1 sequence reported by these authors as a prototype. The first feature to note is that they are very similar, especially considering their different origins, Australia and Japan. All sequences differ from that of pFL1 by only one amino acid residue and only five residues namely 11, 52, 54, 88 and 111 showing polymorphisms. Residue 88 shows valine/alanine interchanges and residue 111 had valine/isoleucine substitutions. At position 11, one variation was found with a polar asparagine/serine change. Two clones showed isoleucine/threonine substitutions, one at 52 and the other at 54. Although not revealed by the amino acid alignments in Fig. 1, the nucleotide sequences were also very similar with a maximum disparity of only 12 out of the 387 coding bases found.

Group III-related allergens

The group III allergen *Der f*III has been defined by Heymann et al. (1989) as a 29-kDa molecule which reacts with the monoclonal antibody 5A12 and others. To investigate the equivalent allergen in *D. pteronyssinus* a monoclonal antibody to *Der f*III, a gift from Drs. Heymann, Chapman and Platts-Mills, was tested for reactivity to a *D. pteronyssinus* extract by Western blotting. No reactivity could be detected despite binding to a 30-kDa component in a *D. farinae* extract so a strategy to produce a polyclonal anti *Der f*III with

Der p III cross reactivity was adopted. The monoclonal antibody was coupled to Sepharose and used to affinity purify *Der f* III from *D. farinae* spent mite medium. In our first isolation three bands which stained almost equally with Coomassie blue were obtained at Mr 32, 28 and 21 kDa (Fig. 2 tracks 2–4). Some very minor bands at 60 kDa and between 28 and 20 kDa were also detectable although they are not visible in the figure. These are perhaps worth noting because similar bands do not appear after our purification procedures with monoclonal anti *Der p* I and *Der p* II antibodies. Other purifications with the monoclonal antibody and different batches of spent mite media did not, however, produce the three equally staining bands. This is shown for another isolation in Fig. 2 with little material visible at 30 kDa. Another variation is again shown in Fig. 3 where the 32-kDa band was very predominant and the 29- and 21-kDa moieties only visible with close inspection. On Western blotting with two allergic sera using the first preparation described in Fig. 2, IgE antibodies were detected to the 32- and 28-kDa components (not shown). The frequency of sera containing IgE reacting with the *Der f* III was also investigated by radioimmune dot-blot using this preparation. It was found that 15/18 allergic adults reacted strongly (83%) compared to 7/18 children

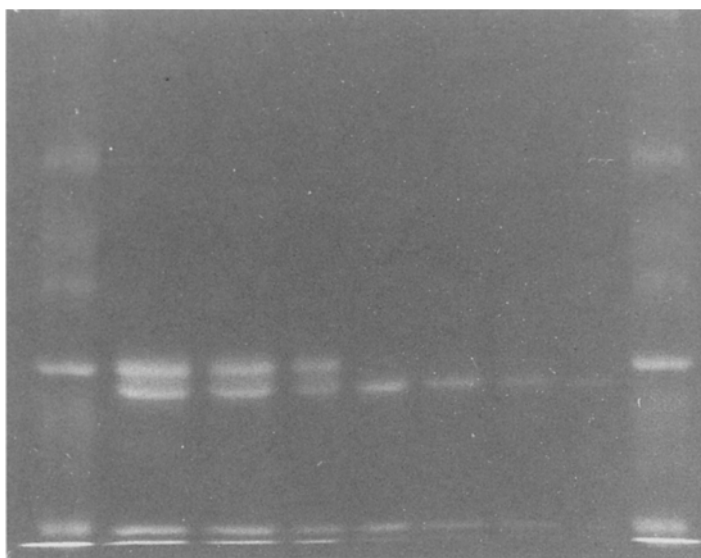


Fig. 2. SDS (13%) polyacrylamide gel electrophoresis of *Der f* III preparations isolated from spent mite media with an anti *Der f* III monoclonal antibody (1). Tracks 2–4 and 5–7 are doubling dilutions of two different preparations. The bands of Mr of 32 kDa and 28 kDa were predominant in the first preparation and the 28-kDa band predominant in the second. A weaker staining band at about 22 kDa is seen in the first preparation. A 14–15-kDa band is quite strong in both preparations (track 2). The molecular weight markers of 97, 66, 43, 31, 21.5 and 14.5 kDa are in tracks 1 and 8.

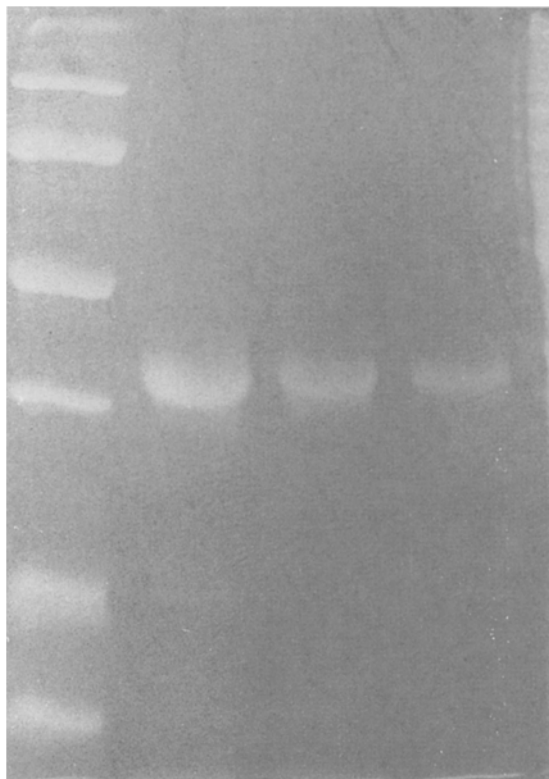


Fig. 3. SDS (13%) polyacrylamide gel electrophoresis of a *Der f*III preparation isolated from spent mite medium (2). The isolations, performed by affinity chromatography in the same way as for Fig. 2, show a major band at Mr 32 kDa and a minor at 21 kDa (track 1). Track 1 contains the molecular weight markers of 97, 66, 43, 31, 21.5 and 14.5 kDa and tracks 2–4 dilutions of the *Der f*III preparation.

(38%). Examples of the reactivities are shown in Fig. 4. This is statistically significant ($\chi^2 P < 0.01$) but no differences in the intensity of the autoradiographs of children and adults was noticed.

The *Der f*III isolated and described in Fig. 2 tracks 2–4 was used to immunise rabbits and mice in an attempt to produce a polyclonal antiserum cross reactive with *Der p* III. In addition the *Der f*III was used to construct an affinity column to isolate anti group III specificities from a rabbit immunised with whole *D. pteronyssinus* extract. The mouse antiserum and the affinity-purified antibodies both showed strong reactivity to a 32-kDa band when examined by Western blotting against a *D. pteronyssinus* mite extract with lesser reactivities to a 28- and 21-kDa band. Figure 5 shows a Western blot with a very predominant 32-kDa reactivity to a mouse anti *Der f*III. The rabbit anti-

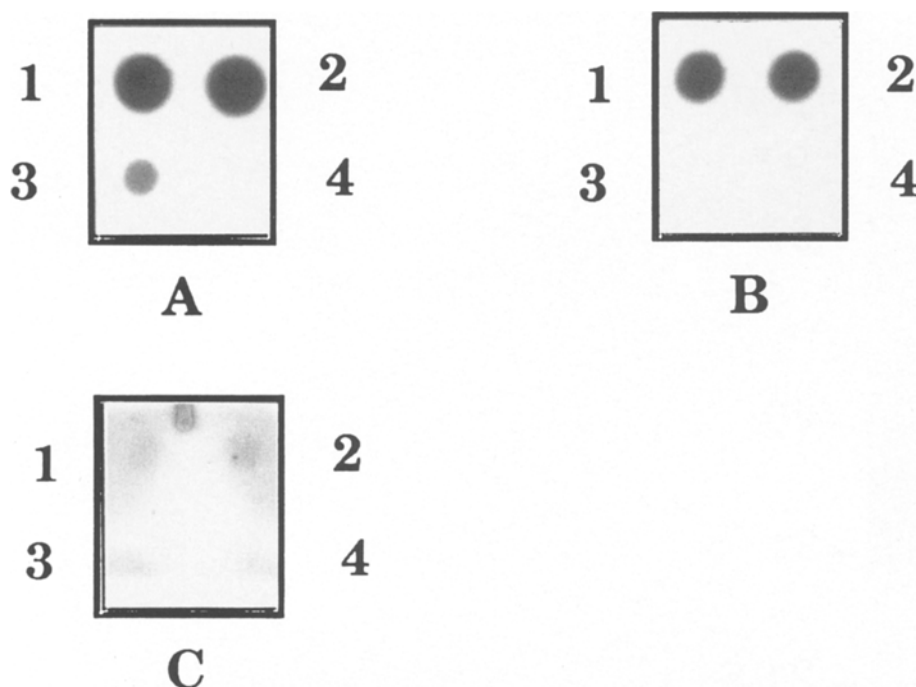


Fig. 4. Radioimmune dot-blot to detect anti *Der f*III IgE antibody. Panel A and B show reactivities with sera from allergic patients and C with a hyper IgE control serum used at 1000 IU of IgE per ml. Positions 1 and 2 are reactivities to recombinant *Der p* II (positive controls), position 3 is *Der f*III and position 4 is a glutathione transferase protein used as a negative control. The patient in panel A was chosen to illustrate a positive reaction and panel B a negative.

serum had the same 32-kDa specificity, but with several other reactivities corresponding to both higher and lower molecular weight bands.

DISCUSSION

The complete primary amino acid sequences of the group I and II allergens of *D. pteronyssinus* and *D. farinae* are known. Some information, although limited, is also available for *Der m* I and *Eur m* I (Hill et al., 1991). Given the evidence outlined in the introduction that antibodies to these components often constitute most of the IgE specificities in many allergic patients, their role in the allergic process is probably considerable, although the extent has still to be determined. These sequences open the avenue for investigating the use of peptides for modulating T cell function and to mediate immunotherapy by regulating IgE production or modifying lymphokines which control effector cells such as eosinophils (O'Hehir et al., 1991) and it can be antici-

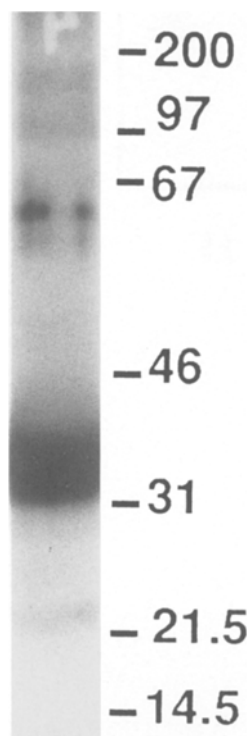


Fig. 5. Western immunoblot of *D. pteronyssinus* extract reacting with mouse anti *Der f* III. The extract (1 mg/tract) was electrophoresed on a 8–16% SDS-PAGE gel and after transfer to nitrocellulose was blotted with mouse anti *Der f* III at a 1/10 dilution then developed with ^{125}I protein A. This extract shows the major reactivity at 32 kDa.

pated that in the near future considerable information on the T cell epitopes recognised by allergic patients will be generated. One critical question will be to determine the number of peptides which should be in a cocktail to modulate the immune response of at least most allergic individuals. This will depend on the diversity of major histocompatibility restriction elements, the T cell receptors and the allergens. As well as providing information to produce immunoregulatory peptides the cDNA will also permit the production of suf-

ficient allergen to examine T cell function during diagnosis and treatment. The value of this has not yet been explored, but the advent of simplified, PCR-based, multiple lymphokine assays (Ehlers and Smith, 1991) along with synthetic allergens should greatly assist investigations.

A comparison of the sequence of a *Der p* I and *Der f* I molecule has been published (Dilworth et al., 1991). Although obviously related with 82% amino acid homology there are many differences and non-conservative substitutions which would largely effect the T cell epitopes. Both similarities and difference in the B cell epitopes have been documented, but no information is available for T cells. In the light of the sequence data this would seem important. Some information on T cell cross reactivity exists for the group II allergens and, surprisingly most have shown species specificity, although still only a limited number of clones have been examined (O'Hehir et al., 1987). In another study with polyclonal stimulation, T cells from allergic patients in Melbourne, Australia tended to make proliferative responses equally to *Der p* II and *Der f* II with the exception of two patients with a bias to *Der p* II (O'Brien et al., 1991). Despite the fact that most house dust mites in Australia are *D. pteronyssinus* there are other populations which could have caused sensitisation (Colloff et al., 1991) so experiments on a clonal level with limit dilution analysis are required. The group II allergens have very similar amino acid sequences showing 88% homology between *Der p* II and *Der f* II (Trudinger et al., 1991). Moreover, many of the changes are conservative with only a few substitutions, to residues 11 histidine/asparagine, 19 proline/aspartate, 52 isoleucine/threonine, 70 proline/threonine and 114 aspartate/asparagine, which could be expected to greatly affect T cell epitopes. At the moment there are no published data on group I sequence polymorphisms within a species. This when it becomes available will be of interest because of the degree of interspecies sequence divergence. The data available for the group II allergens reported here, by Yuuki et al. (1990) and by Trudinger et al. (1991) show that such variation is minor. All six sequences available for comparison differ, but each by only one amino acid residue. The alterations at residue 88 and 111 are conservative substitutions of small aliphatic groups, for example valine/isoleucine changes. The differences at 52 and 54 do involve nonconservative isoleucine/threonine changes and the one asparagine/serine shift at position 11, although a polar substitution, could have functional significance.

Some of the changes are, however, found in the cDNA generated by the polymerase chain reaction and may be the result of editing errors during amplification (Reiss et al., 1990) and need corroboration. The N-terminal sequence obtained from *Der f* II protein by Heymann et al. (1989) for instance shows the dominant asparagine residue found in most sequences. From previous analysis only 80% of PCR products of this size should be perfect, so we think the sequence of Yuuki should be taken as the prototype for *Der f* II. What is, however, obvious and a valid interpretation from the data is that

large differences are not abundant although separate studies will be required to see if variation exists in the wider environment. These similarities are probably more significant when it is considered the cultures were from mites in different countries. The CSL *D. farinae* preparation was from mites obtained in Sydney, although they could of course originate from visitors. Although not shown in the results presented here the nucleotide sequences are also highly conserved with only 12 changes between the clones from Japan and Australia (data from Yuuki et al., 1990 and Trudinger et al., 1991). The 3' non-coding region is also highly conserved especially given that significant differences were found here between *Der p* II and *Der f* II (Trudinger et al., 1991). This most noticeable divergence is found in one of the clones of Yuuki; pFL1 has a block of six adenosines following the stop codon compared to a block of nine adenosines in the other clones. This may provide a marker to study clonal divergence in environmental studies. While discussing the 3' sequence it should be pointed out that the 'poly A' sequences shown by Yuuki et al. (1990) at the end of their pFL1 and pFL2 are unlikely to be a polyadenylation site because the sequences for *Der p* II and *Der f* II described by Chua et al. (1990a) and Trudinger et al. (1991) extend beyond this with other non-A nucleotides and because there is no AATAAA-polyadenylation signal. A signal does exist in the *Der p* II and *Der f* II sequences described in our laboratories, but the available sequence only extends a few bases beyond this site and polyadenylation occurs 20–30 bars after the signal. No 5' non-coding sequence is available for *Der f* II.

The data presented on group III allergens introduces some important questions which can in time be answered. Firstly is it a major allergen? From Western blotting a 32/38-kDa doublet shows reactions of a high frequency, at least 70% (Tovey and Baldo, 1987). We report here using affinity-purified *Der f* III and radioimmune dot-blot that 83% of adults and 38% of children reacted to *Der f* III. The question is, therefore, one of degree and affinity. The results of Heymann et al. (1989) who found only 20% reactivity using a method which detects high-affinity interactions suggest most anti group III reactivities are of low affinity. Our studies on the frequency and degree of IgE reactivity were not extended because our population in Australia would have been primarily sensitised to *D. pteronyssinus* and only *Der f* III was available so any detailed analysis would be difficult to interpret. It does, however, differ considerably from those of Heymann and more investigation is required. The importance of minor reactivities, if group IIIs are minor, are at present difficult to evaluate. With respect to immunotherapy the significance will become apparent after effective procedures to ameliorate the allergic responses with purified or synthetic group I and II allergens are implemented. That it is not necessary to remove reactivity to all allergens for clinical improvement had been indicated by avoidance studies where useful improvements in bronchial hyperreactivity have been achieved by mite avoidance alone in patients

sensitive to mites and pollen (Platts-Mills and Chapman, 1987). The purifications with monoclonal anti *Der f* III described here show there are at least three related entities in mite extracts and possibly more. The amount present varied in different extracts, but it is clear that anti group III specificities can react with components at Mr 32, 38 and 21 kDa and possibly others. These may be breakdown products or related molecules such as secondary modifications. Since Stewart et al. (1989) have pointed out that these allergens are, by sequence homology, serine proteases, the presence of zymogens, isoenzymes and related enzymes must be considered. It is also important to note that the *Der p* III sequence obtained by Stewart et al. (1989) was from a 17-kDa molecule compared to the 29-kDa moiety described by Heymann et al. (1989). The work reported here shows clearly that *D. pteronyssinus* like *D. farinae* has a 32-kDa/28-kDa group III-like allergen in addition to lower molecular weight forms. Since they correspond to bands reacting at high frequency with IgE on Western blotting this further study is warranted. Furthermore, the comparison of the childrens versus adults reactivity suggests that despite being very strong responders to group I and group II components, they have less diverse reactivities.

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