

IgE binding studies with large peptides expressed from *Der p* II cDNA constructs

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Summary

The major mite allergen *Der p* II shows marked resistance to denaturation and is expressed from cDNA in bacteria with almost all of its IgE binding activity. Despite these properties, the IgE binding activity appears to be dependent on maintaining the complete primary structure. Random fragment libraries of cDNA, able to code for up to 93 of the 129 amino acid residue protein, did not express IgE binding peptides. Large overlapping peptides 1–69, 69–129 and 42–117 expressed as the fusions from the glutathione transferase of pGEX vectors only had binding activity with IgE in 15 out of 57 sera, and this was typically weak. Sera from children with atopic dermatitis bound IgE in seven out of eight cases but this was also weak compared with their strong reactivity to intact recombinant *Der p* II. The inability of such large peptides to form IgE binding structures suggests that the antigenic determinants of *Der p* II are highly conformational and restricted.

Clinical and Experimental Allergy, Vol. 21, pp. 161–166. Submitted 6 June 1990; revised 9 August 1990; accepted 28 September 1990.

Introduction

Dermatophagoides pteronyssinus and *D. farinae* are two closely related dust mite species present in house dust. The allergens from these mites have been shown to be important risk factors associated with allergic diseases such as asthma, perennial rhinitis and eczema [1]. Two groups of major allergens, each of which reacts with about 80% of allergic sera, have been identified and defined [2,3]. The 25-kD group I allergens (*Der p* I and *Der f* I) are faecal antigens [4] and a cDNA clone for *Der p* I has been shown to share a high sequence homology with a group of thiol proteases [5]. A cDNA clone encoding *Der p* II has also been isolated by IgE plaque radioimmunoassay using a human allergic serum [6]. The inferred amino acid sequence showed that it consists of 129 amino acid residues including six cysteine residues with a calculated molecular weight of 14·131 kD. The cDNA can be expressed in *Escherichia coli* as a fusion protein yielding a highly allergenic molecule [7] such that absorption of allergic sera with the recombinant molecule removes nearly all the IgE binding for the native *Der p* II.

The data available on the B cell epitopes of *Der p* II show that it is extremely resistant to denaturation and can, for example, resist boiling for 10 min [8]. Perhaps more interestingly, it has been shown that its reactivity with IgE is markedly increased after incubation with detergents [9]. However, antigenicity is abolished by reduction and alkylation [8], although it cannot be determined whether this operates by modification of the residues itself or by affecting the conformation. The high degree of antigenicity found in the recombinant molecule suggests that defined sequences may confer antigenicity and that the production of peptides from cDNA fragments would permit epitope analysis. Here we present evidence that the epitopes of *Der p* II are highly dependent on the intact sequence of the molecule. This has been obtained by determining the IgE reactivity of peptides expressed from random fragments of *Der p* II in lambda gt11 libraries and by the antigenicity of large overlapping peptides expressed by pGEX vectors.

Materials and methods

Sera

Fifty-seven human allergic sera were collected from individuals sensitive to house dust mite with skin and

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RAST tests positive for *D. pteronyssinus*. The clinical symptoms were determined from the patients' medical records. All 34 children sera were obtained from patients attending the allergy clinics at Princess Margaret Hospital for Children, Western Australia. Thirteen adult sera were kindly provided by Dr Richard O'Brien, Melbourne, Victoria, Australia, and the rest were obtained from patients in Western Australia. Adult sera were from rhinitis and asthma patients; the children had rhinitis (two), atopic dermatitis (eight) and asthma only (24). A hyper IgE serum (a gift from Dr N. R. Lynch, Institute of Biomedicine, Caracas, Venezuela) containing 100 000 IU/ml of IgE was used as a control, as it has been shown that this serum does not contain anti-*Der p* II reactivity. All sera were absorbed with an equal volume of *E. coli* lysate overnight at 4°C. Non-fat milk powder and sodium azide were added to 5% and 0.02%, respectively. All allergic sera were used at 1/2 dilution. Hyper IgE serum was used at 1000 IU/ml.

Preparation of native *Der p* II

Native *Der p* II was purified from *D. pteronyssinus* spent growth medium using monoclonal affinity chromatography as described [2]. The monoclonal anti-*Der p* II was kindly provided by Dr R. Aalberse, University of Amsterdam, The Netherlands and M. D. Chapman, Charlottesville, Virginia.

IgE antibody radioimmunoassay

Dot blot filters were prepared by applying 2–50 µg/spot of the various recombinant peptides on nitrocellulose membrane (Trans-Blot, Bio-Rad Laboratories, Richmond, VA) using the Bio-Dot apparatus (Bio-Rad). Filters were air-dried and washed in TNT buffer (100 mM Tris HCl, 0.15 M NaCl with 0.05% Tween 20, pH 7.4) for 5 min, followed by overnight serum incubation at 4°C with rocking. After serum incubation, filters were washed in TNT buffer for 5 min and then incubated with ¹²⁵I-monoclonal anti-IgE (2 × 10⁶ c.p.m./ml; 10⁸ c.p.m./µg) for 2 hr at room temperature with rocking. Filters were then given three 15-min washes in TNT. Autoradiography was carried out at –70°C with intensifying screens for 24–48 hr.

Random fragment libraries

The cDNA encoding *Der p* II was isolated from pUC19 pII(C1) [5,6] by gel electrophoresis of the *Eco*RI digested plasmid. Ten micrograms of cDNA were then sonicated on a Branston Sonifyer until a range of fragments from 50–300 bp was observed by agarose gel electrophoresis.

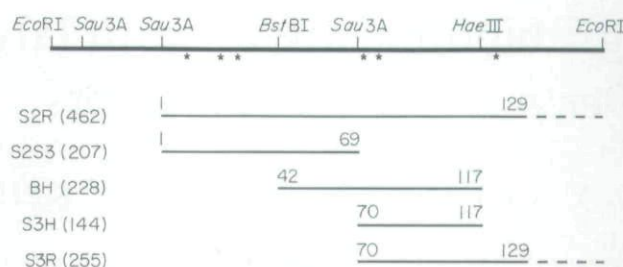


Fig. 1. Restriction map of *Der p* II cDNA used to construct fragments. Numbers in parentheses are base pairs and numbers above the line are amino acid numbers of the 129 residue protein. *Cysteines at positions 8, 21, 27, 73, 78 and 119.

The sonicated DNA was then electrophoresed in 15% polyacrylamide in 0.5 × TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA [10]) and fractions corresponding to fragments in the size ranges 60–118, 118–194 and 194–281 bp were excised. The DNA was then isolated and blunt ends repaired with Klenow enzyme (Boehringer Mannheim, Mannheim, Germany) and ligated to *Eco*RI linkers (New England Biolabs, Beverly, MA). The fragments were then cloned in lambda gt11 for plaque immunoassays [11]. The libraries contained 28 500, 170 000 and 180 000 recombinants for each size range, respectively. This procedure has been described in detail for *Der p* I cDNA [12].

Expression of peptides from restriction endonuclease fragments

Cloning vectors used in this study were pGEX-1 and pGEX-2T [13] which express sequences as a fusion to the carboxy terminus of the glutathione transferase from *Schistosoma japonicum*. The generation of restriction fragments S2-R (462 bp), S2-S3, S2-S3 (207 bp) and S3-R (255 bp) (Fig. 1) comprising amino acid residues 1–129, 1–69 and 69–129, respectively, was carried out by partial digestion of *Der p* II cDNA insert with *Sau*3AI. Four micrograms of *Der p* II cDNA insert (a 597-bp *Eco*RI fragment) were digested with *Sau*3AI and the linear DNA molecules were gel-isolated on a 1.4% low-melting-point agarose gel. DNA fragments were recovered by phenol extraction. The gel purified DNA fragments were then cloned into *Bam*HI–*Eco*RI-digested pGEX-1 vector. Fragment S3-H (residues 69–117) was gel-purified from a double digestion of the *Der p* II cDNA with *Sau*3AI and *Hae*III after blunt-ending cloned in *Sma*I-digested pGEX-1 vector. B-H fragment (residues 42–117) was generated by *Bst*IIb (New England Biolabs) and *Hae*III digestion of *Der p* II cDNA. The ends of the fragment were filled in with Klenow and ligated into *Sma*I-digested pGEX-2T. The various constructs were transformed into

Table 1. Fusion peptides produced from pGEX *Der p II* fragments

cDNA fragments	Residue no.	Estimated size of fusion protein produced (kD)
S ₂ -R	1-129	41
S ₂ -S ₃	1-69	35
B-H	42-117	36
S ₃ -H	69-117	32
S ₃ -R	69-129	34

Fragments were created by restriction enzyme digests using *Sau* 3AI sites S₂ and S₃ (see [7]); *Eco*RI, R; *Hae*III, H; and *Bst*IIb.

Table 2. Frequency of IgE reactivity of allergic sera with recombinant *Der p II* peptides and native *Der p II*

<i>Der p II</i> peptides comprising residues	1-129	1-69	69-117	69-129
Adult sera (<i>n</i> =23)	23/23	2/23	2/23	2/23
Children's sera (<i>n</i> =34)	32/34	13/34	5/34	13/34

Results show the fraction of sera with positive dot-blots to peptides.

E. coli strain TG1. Transformants were screened by colony DNA hybridization using ³²P-labelled DNA probes. *Sau*3AI, *Bam*HI, *Eco*RI and *Sma*I were purchased from Toyobo, Osaka, Japan. All DNA manipulations, and DNA hybridization transformation experiments were carried out by standard methods described [13].

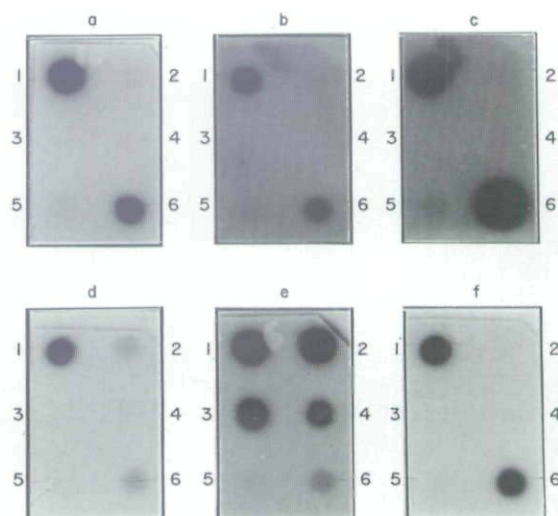
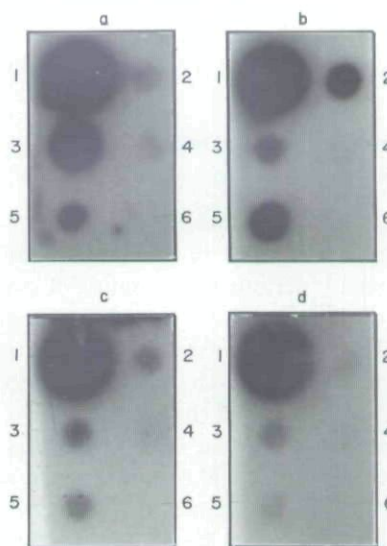
Protein analysis

Bacterial lysates from the *E. coli* transformants harbouring the various recombinant constructs were prepared and fusion proteins were purified by glutathione agarose affinity chromatography according to methods described [13]. Protein concentrations were determined spectrophotometrically. An E₁^{1%}_{cm} 280 nm of 5 was used based on previous comparison of OD₂₈₀ and Folin's estimations. Samples were also analysed on Coomassie blue 250 stained SDS-PAGE [14] gels.

Results

Screening of *Der p II* cDNA random fragment libraries with human allergic serum

The cDNA clone encoding *Der p II* was originally isolated from the clone lambda gt11 pII(C1) which reacted with

**Fig. 2.** Reactivity of sera to *Der p II* fusion peptides. Ten micrograms of whole *Der p II* peptide 1-129 (1); 1-69 (2); 69-129 (3); 69-117 (4); GEX vector control (5); and 2 µg native *Der p II* (6) were reacted with sera from allergic patients and IgE reactivity determined by ¹²⁵I and IgE. Panels a-c are examples of children, and panels d-f of adults. As summarized in Table 1, reactivity to peptide fragments was usually absent or weak. The reactivity in panel e is one of the few sera with reactivity to peptides.**Fig. 3.** Reactivity of allergen sera from patients with atopic dermatitis to *Der p II* fusion peptides. Ten micrograms of recombinant *Der p II* (1-129) (1); 1-69 (2); 69-129 (3); 69-117 (4); native *Der p II* (2 µg) (5); and GEX control (6) were reacted with different sera as shown for four examples in panels a-d.

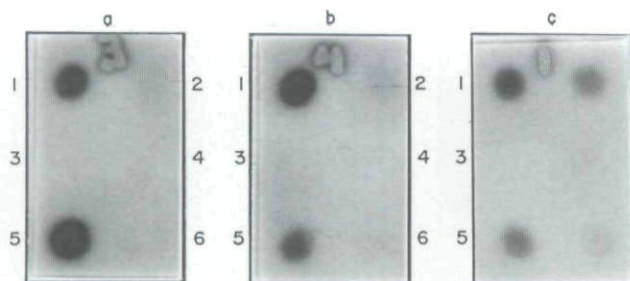


Fig. 4. Reactivity of allergen sera to overlapping peptides. Ten micrograms of recombinant *Der p II* (1–129) (1); 42–117 (2); 1–69 (3); 69–129 (4); native *Der p II* (2 µg) (5); and GEX control (6) were reacted with different sera as shown for three examples by panels a–c.

IgE by plaque immunoassay. To determine whether fragments of the cDNA could express antigenic peptides, random fragments libraries in the size ranges 60–118, 118–194 and 194–281 bp were constructed in lambda gt11 and tested for reactivity by IgE plaque immunoassay. Clones in these libraries could be expected to express peptides of 20–40, 40–65 and 65–95 amino acid residues, respectively, compared with the 129 residues of native *Der p II*. A total of 110 000 plaques from these libraries were screened by IgE radioimmunoassay using two sera with high reactivity to *Der p II*. No positive plaques were obtained. However, when lambda gt11(C1) phage which expressed intact *Der p II* was included as a positive control in the screening by mixing with a random fragment library, plaques with strong IgE reactivity similar to that described by Chua *et al.* [6] were obtained showing the efficacy of the screening procedure.

Expression of Der p II cDNA fragments generated by restriction endonuclease digestions in pGEX

The technique of random fragmentation and plaque immunoassay with lambda gt11 recombinants was adopted because it permits the screening of large numbers of peptides of varying sizes, from different parts of the molecule. The inability to detect IgE reactivity in the libraries therefore suggests that peptides, even in the size range of 65–95 residues did show antigenicity. There are, however, some uncontrolled variables in this procedure such as the stability and yield of peptides produced by the plaques. Several large fragments of the cDNA (Fig. 1) were therefore produced by restriction endonuclease digestion and cloned in the pGEX vectors so peptides could be produced as fusions with the glutathione transferase. These could be then isolated by glutathione agarose affinity chromatography and checked by SDS-PAGE so assays could be performed with defined

quantities. The sizes of the fusion proteins produced by the various constructs are summarized in Table 1. Soluble protein could be isolated from all constructs in sufficient quantity for immunoassay. The SDS-PAGE profiles showed that over 75% of the protein had the expected molecular weight.

IgE binding studies

Allergic sera from 34 children (< 12 yr) and 23 adults were used in the dot-blot assay to compare the IgE binding capacity of the various peptides expressed from the enzyme restricted cDNA fragments. Initially, peptides from fragment encoding residues 1–69, 69–129, 69–117 as well as the full-length molecules 1–129 were examined. All sera reactive with the native *Der p II* by IgE dot-blot reacted with recombinant *Der p II*, i.e. 32 out of 34 children's sera and all 23 adult sera. Only two of the adult sera and 13 of the children's sera had some reactivity to fragments (Table 2). However, even the sera reactive with peptides had weak binding, except for one adult serum with relatively strong binding to the peptide 1–69 and 69–129. Figure 2 shows six examples of dot-blot assays against the fragments; panel e is the adult with strong binding to the fragments. Examination of the records of the children showing IgE reactivity to peptides showed that seven out of 13 of the reactive sera had atopic dermatitis and they comprised seven out of eight of the atopic dermatitis patients. Again, however, the reactivity against the fragments was often weak compared with the intense signal given by the group to the full-length molecule (Fig. 3).

Because nearly all sera showed no or little reactivity to the large peptide fragments, particularly the 'half molecules' 1–69 and 69–129, a fragment B–H expressing the overlapping peptide 42–117 was produced. Twelve out of the 57 sera were selected to test for reactivity. Sera that were negative to 1–69 and 69–129 showed no reactivity or only weak binding (Fig. 4). Weak reactors to 1–69 and 69–129 showed no significant increase in reactivity to 42–117.

Discussion

The present study using another panel of human sera has demonstrated that there is a good correlation in IgE reactivity between native and recombinant *Der p II*, further confirming our earlier report that the recombinant *Der p II* cDNA when expressed intact in *E. coli* retains most of the IgE binding capacity of the native counterpart [7]. This along with reports of others showing resistance or enhancement of *Der p II* antigenicity with boiling or detergent treatment [8,9] suggested that B cell

epitopes may be expressed by short linear sequences or, alternatively, if a complex structure is required for IgE binding the epitopes may be restricted because it is unlikely that all the conformational structures would be equally resistant to denaturation.

The first approach to examine *Der p* II epitopes was to use random fragment libraries. In this technique a large number of fragments representing a variety of sizes and orientations can be produced by random shearing and screened en masse (10 000/Petri dish) by plaque immunoassay. No IgE-reactive plaques were found in 150 000 plaques containing fragments, although full-length *Der p* II gave intense IgE binding signals in the assays. Three libraries of fragments were examined: library 1 encoded peptides in the range of 20–40 residues; library 2, 40–65 residues; and library 3, 65–95. This indicates that virtually no fragment with less than 95 residues expressed IgE binding activity, even though full-length *Der p* II has only 129 amino acids. Several factors could account for these results, such as the peptides expressed from the fragments being particularly unstable, insoluble or toxic to the bacteria. In order to control for these factors three large overlapping cDNA fragments were cloned to express peptides as fusions with the glutathione transferase of the pGEX vectors which could be isolated by affinity chromatography. Restriction digests were thus used to clone cDNA fragments encoding peptides 1–69, 69–129, and 42–117. Most of the fusion protein from these constructs were shown by SDS-PAGE to be produced with the expected molecular weight showing little breakdown; therefore, the purified soluble peptides could be used for qualitative serology. Only 15 out of 57 allergic sera had some IgE reactivity, and with the exception of one person, they were very weak compared with the reactivity to *Der p* II. The epitopes therefore appear to be very dependent on structures expressed by large peptides or the complete molecule. The finding that the atopic dermatitis patients gave an over-representation of reactivity to the peptides (seven out of eight reacted) is in keeping with their intense reactivity to the whole recombinant and native *Der p* II molecule. It is also in keeping with the observation of Thompson *et al.* [15] showing high IgE binding of eczematous patient to *Der p* II. However, even the reactions of this group to peptides were greatly reduced compared with the whole molecule. Given that most of the peptides consisted of the 27 000 mol. wt. glutathione transferase, very little difference should be expected in their adherence to nitrocellulose. It was, however, noteworthy that inhibition of binding to *Der p* II could not be achieved by peptides similar to that reported for the whole recombinant molecule [7].

At face value these observations are consistent with the finding that most epitopes of globular proteins are

discontinuous [16]. However, most of these studies have been derived from responses of animals to injected antigen. The response of humans to a continuous exposure to low doses of antigen could be subject to a number of regulatory influences and warrants further investigation. Some studies of human IgE epitopes recognized after natural exposure have been performed and these have shown a large degree of reactivity with relatively small peptides. The synthetic peptides of the birch and hazel major allergen [17], the Chironomid haemoglobin [18] and cod fish allergen [19] show very significant binding. The structural basis for the inability of the large peptides of *Der p* II to react may be speculated on. Although there is no sequence homology *Der p* II has a superficial resemblance to lysozyme in that it has the same number of amino acid residues (129) and six cysteines. The C₈, C₂₁, C₂₇ and C₁₁₉ are in similar positions to those in lysozyme, while the C₇₃ and C₇₈ are about 20 residues proximal to their lysozyme counterparts. If the disulphide binding between the 3 N terminal and 3 C terminal cysteines occurs in *Der p* II in a fashion similar to lysozyme, then the domain structure requires interaction between N and C terminal determinants. For mice the major antigenic determinant of lysozyme is formed by an interaction of the N and C terminal peptides [20]. The current peptides from *Der p* II, although large (55% of the molecule), do not allow interaction of residues surrounding of the C terminal and N terminal cysteines. Experiments to further examine the antigenicity will involve the location of the disulphide bonds and attempts to renature putative structures from mixtures of peptides under conditions favouring disulphide bond formation. Alternatively, attempts to isolate antigenic proteolytic fragments could be made.

Acknowledgments

This study was supported by the National Health and Medical Research Council and the Asthma Foundation of Western Australia.

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