

Isolation of cDNA Coding for the Major Mite Allergen *Der p II* by IgE Plaque Immunoassay¹

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Abstract. A lambda gt11 library made with cDNA from the house dust mite *Dermatophagoides pteronyssinus* was screened with human allergic serum by IgE plaque radioimmunoassay. This resulted in the isolation of clones coding for the major allergen *Der p II*. The cDNA coded for a 129-residue protein of 14,131 daltons with no N-glycosylation sites. No sequence homology with other proteins was evident. The *Der p II* expressed in *Escherichia coli* reacted with IgE in 14 of 17 sera from mite-allergic patients giving clonal evidence for its designation as a major allergen. This, along with previous work, has resulted in the cloning of the two major mite allergens.

Introduction

Allergens present in house dust mite extracts have been examined by a variety of techniques. The first major allergen clearly characterised from *Dermatophagoides pteronyssinus* is now known as *Der p I*. Purified *Der p I* has been shown by skin test and radioimmunoassay to react with IgE in about 90% of patients allergic to mites [1-3]. Monoclonal antibodies have now been used to clearly define *Der p I* as well as related allergens from other mite species, *Dermatophagoides farinae* and *Dermatophagoides microceras* [4, 5]. More recently a cDNA clone has been used to provide the amino acid sequence for *Der p I* which is homologous to a number of cysteine proteases, including papain and actinidin [6, 7].

The use of cross-radioimmuno-electrophoresis to detect IgE-binding compounds in mite extracts confirmed the importance of *Der p I* and revealed another major allergen, *Der p II* (formerly dpX) [5, 8], which reacted with IgE in almost 80% of allergic sera. Apart from *Der p I* and *Der p II*, these studies indicated the existence of another 4 components which reacted with serum from up to 20% of mite allergic

patients [8, 9]. Like *Der p I*, *Der p II* and its *D. farinae* counterpart have now been more clearly defined with monoclonal antibodies [10]. We now describe the use of allergic serum to probe lambda gt11 expression libraries by IgE plaque immunoassay. This has resulted in the isolation of a cDNA clone coding for *Der p II*. The allergen as produced by this clone reacts frequently with IgE from mite-allergic sera giving clonal evidence of its importance. The IgE plaque immunoassay has potential for isolating further allergens.

Materials and Methods

IgE Plaque Radioimmunoassay

A lambda gt11 cDNA library containing 5×10^5 recombinants was prepared from adult *D. pteronyssinus* supplied by the Commonwealth Serum Laboratories, Parkville, Australia [6]. For immunoscreening, an overnight culture of *Escherichia coli* Y1090 [11] was diluted 1/50 in L broth and incubated at 37°C to an OD₆₅₀ of 0.6. The bacteria were pelleted and resuspended in 400 µl for every 50 ml of broth. For 14.5-cm Petri dishes, 300 µl of Y1090 were incubated with 10^4 pfu phage for 30 min at room temperature and then plated on LB agar in 9 ml of 0.7% agar overlay and incubated for 3 h at 42°C (when plaques usually became visible). At this time a nitrocellulose filter, which had been saturated with 10 mM isopropyl β-D-thiogalactoside and dried, was placed on top of the lawn. The incubation continued overnight at 37°C. The filter was then removed and washed in 0.01 M Tris-hydrochloride, 0.15 M NaCl, 0.05% Tween 20 v/v, pH 8, (TNT) buffer with gentle rocking for 20 min. The filter was then incubated with serum for 2 h at room temperature with rocking and then washed three times for 30-min

¹ This is publication No. 326 of the Clinical Immunology Research Unit of the Princess Margaret Children's Medical Research Foundation.



Fig. 1. High density IgE immunoscreening. Plaques were plated at 10^4 per 14.5-cm dish and screened for IgE reactive clones. To perform this lifts on nitrocellulose filters were reacted with allergic human serum and ^{125}I -anti-IgE and developed after 48-hour autoradiography. The arrow shows a dot signifying an IgE-reactive clone.

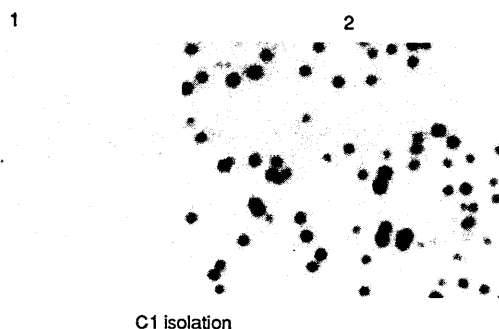


Fig. 2. Plaque purification by immunoscreening. A section of a filter of high density IgE immunoscreen (10,000 pfu/plate; left) with a positive plaque and a primary purification (approximately 1,000 pfu/plate) after picking IgE-reactive plaques (right).

periods with TNT. The serum used was first diluted 50:50 with an *E. coli* extract [11], incubated overnight then clarified by centrifugation (3,000 g 10 min). Non-fat milk and sodium azide were added to 5 and 0.02%, respectively. To develop the IgE reactivity the filter was rocked in a solution of ^{125}I -labelled anti-IgE for 2 h at room temperature followed by three 30-min washes with TNT. The anti-IgE was a mouse monoclonal 2.1.5, a gift from Dr. K.J. Turner (also available from Silenus Laboratories Pty. Ltd., Hawthorn, Victoria) and was used at 30 ng/ml coupled with 10^5 dpm/ng ^{125}I in TNT [12]. It was labelled by the chloramine T method. The filter was autoradiographed with intensifying screens, usually for 48 h at -70°C .

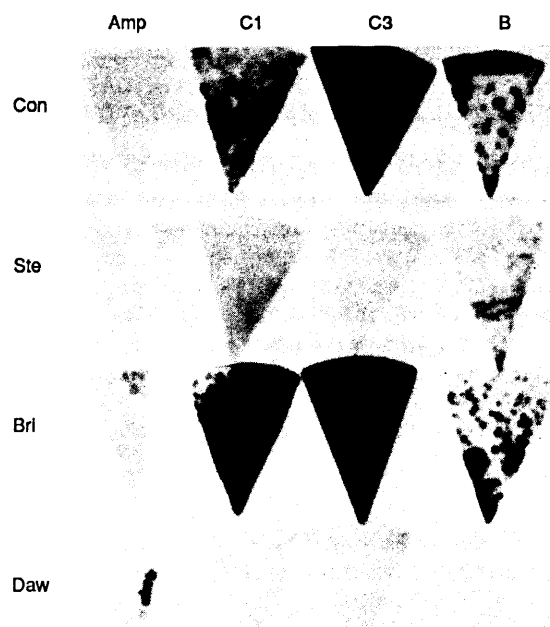


Fig. 3. Reactivity of serum from allergic patients with cDNA clones lambda gt11 pII (C1), (C2) and (B). Plaques were plated at high density (10,000 pfu/filter) and nitrocellulose lifts taken. Segments were used for IgE immunoassay with different mite-allergic sera. The assay shows that serum from Con and Bri reacts strongly with clones C1, C3 and B. Serum from Ste had weaker reactivity while Daw serum showed no reaction. The column headed Amp shows reactivity with lifts from plates plated with the vector control lambda gt11 Amp [24].

DNA Analysis

DNA from lambda gt11 clones of interest was isolated by a rapid PEG precipitation method as described previously [7] and digested with *Eco* R1 enzyme. The insert was subcloned into pUC9 plasmid by standard procedures. DNA probe was radiolabelled with ^{32}P by nick translation. Hybridisation of phage lifts was carried out at 42°C in 50% formamide, $6\times$ SSC, $1\times$ Denhardt's solution, 0.1 mg/ml of poly U, 0.1 mg/ml of poly C and 0.4 mg/ml of denatured salmon sperm DNA for 16 h. Washings were performed in $2\times$ SSC and 0.1% SDS at 65°C . cDNA sequencing was performed by the dideoxynucleotide chain termination method [13, 14] after subcloning into the M13-derived vectors, mp18 and mp19.

Results

Isolation of IgE cDNA Mite Clones by Plaque Radioimmuno Assay

Serum from an allergic patient (A.M.) was chosen because it contained IgE reacting to a large number of mite components as judged by Western blotting. After screening 4 high density filters by plaque immunoas-

Table 1. Nucleotide and inferred amino acid sequence of lambda gt11 pII (C1)

CACAAATTCTTCTTTCTTCCTTACTACTGATCATTAACTGAAAACAAAACCAAACAAACCAT																63
-17 -10 TCAAA ATG ATG TAC AAA ATT TTG TGT CTT TCA TTG TTG GTC GCA GCC GTT Met Met Tyr Lys Ile Leu Cys Leu Ser Leu Leu Val Ala Ala Val																113
-1 1 10 GCT CGT GAT CAA GTC GAT GTC AAA GAT TGT GCC AAT CAT GAA ATC AAA Ala Arg Asp Gln Val Asp Val Lys Asp Cys Ala Asn His Glu Ile Lys																161
20 30 AAA GTT TTG GTA CCA GGA TGC CAT GGT TCA GAA CCA TGT ATC ATT CAT Lys Val Leu Val Pro Gly Cys His Gly Ser Glu Pro Cys Ile Ile His																209
40 CGT GGT AAA CCA TTC CAA TTG GAA GCC GTT TTC GAA GCC AAC CAA AAC Arg Gly Lys Pro Phe Gln Leu Glu Ala Val Phe Glu Ala Asn Gln Asn																257
50 60 ACA AAA ACG GTC AAA ATT GAA ATC AAA GCC TCA ATC GAT GGT TTA GAA Tho Lys Thr Ala Lys Ile Glu Ile Lys Ala Ser Ile Asp Gly Leu Glu																305
70 GTT GAT GTT CCC GGT ATC GAT CCA AAT GCA TGC CAT TAC ATG AAA TGC Val Asp Val Pro Gly Ile Asp Pro Asn Ala Cys His Tyr Met Lys Cys																353
80 90 CCA TTG GTT AAA GGA CAA CAA TAT GAT ATT AAA TAT ACA TGG AAT GTT Pro Leu Val Lys Gly Gln Gln Tyr Asp Ile Lys Tyr Thr Trp Asn Val																401
100 10 CCG AAA ATT GCA CCA AAA TCT GAA AAT GTT GTC GTC ACT GTT AAA GTT Pro Lys Ile Ala Pro Lys Ser Glu Asn Val Val Val Thr Val Lys Val																449
120 ATG GGT GAT GAT GGT GTT TTG GCC TGT GCT ATT GCT ACT CAT GCT AAA Met Gly Asp Asp Gly Val Leu Ala Cys Ala Ile Ala Thr His Ala Lys																497
129 ATC CGC GAT <u>TAAATCAAACAAAATTTATTGATTTTGTAAATCACAAATGATTGATTTTCTT</u> Ile Arg Asp																557
TCCAAAAAAAAAATAAATAAAATTTTGGAATTC																581

Numbers to the right are nucleotide positions and numbers above the sequence are amino acid residues. Positive amino acid residues begin with the known N-terminal sequence of *Derp* II. The first 40 residues which correspond to this sequence were published in Chapman et al. [17]. The amino acid sequence -1 through -17 resembles a leader sequence with a highly hydrophobic core. The stop signal and polyadenylation site are underlined.

say with a monoclonal anti-IgE (10,000 pfu/filter) 4 plaques were found to give positive reactions by autoradiography (fig. 1). These reactions were small on high density screening, but after plaque purification and seeding at low density the reactivity became pronounced (fig. 2). Preliminary studies performed after purification showed that these plaques did not react with non-allergic serum (see below). To test for possible sequence relatedness between these plaques, a DNA insert from one clone (C1) was isolated and tested with the other clones by Benton hybridisation [15]. All four clones isolated by IgE immunoassay hybridised, but C1 did not hybridise to a phage clone containing cDNA coding for *Der p* I. To test IgE reactivity further, a series of allergic and some non-allergic sera were used for plaque immunoassay against three of the clones, C1, C3 and B, as well as a vector control for gt11 (fig. 3). All non-allergic sera tested showed no reactivity. Of 17 mite-allergic sera, 14 showed reactivity to the clones, but not the gt11 vector control. Sera either reacted to all recombinant clones or to none (fig. 3) consistent with the hybridisation evidence that they contained related cDNA. The possibility that IgE was non-specifically binding to the clones was unlikely because the sera used had similar IgE levels as measured by PRIST assays. The high frequency of reactivity with different allergic sera suggested that this cDNA may code for an important allergen so one clone was investigated further.

Sequence Analysis of Lambda gt11 pII (C1)

The DNA from clone lambda gt11 pII (C1) was prepared and after *EcoR* I digestion a 0.6-kb insert was isolated. This was subcloned into mp18 and mp19 for DNA sequence analysis. The sequence (table 1) was 597 bp long. There are two potential translation initiation ATG triplets at bases 69–71 and 72–74. The initiation site was tentatively placed at nucleotides that fulfilled criteria for Kozak consensus sequence for translation initiation site [16]. The open reading frame ends with a termination codon TAA at nucleotides 509–511. The 3' untranslated region does not contain a poly A tail, but a polyadenylation signal (AAT AAA) is present.

The inferred amino acid sequence from nucleotides 126–512 showed that the clone coded for the allergen *Der p* II in that amino acid residues numbered 1–40 are the same as the published sequence for the N-terminal of *Der p* II [17]. The same sequence was obtained by Stewart et al. [18] for the allergen they de-

Table 2. N-terminal amino acid homology of *Der p* II and *Der f* II

<i>Der p</i> II	DQVDVKDCANHEIKKVLVPGCHGSEPCIIHRGKPF
	* * *
<i>Der f</i> II	DQVDVKD?ANNEIKKVMVDG?HGSDP?IIHRGKPF

30 of 34 of the N-terminal amino acids are homologous. * Non-homologous residues. Data for *Der f* II are from Chapman et al. [17]. Data for *Der p* II are from the cDNA sequence (fig. 5) and *Dpt* 22 by Stewart et al. [18] which is identical to that published by Chapman et al. [17], except that the previously unknown residues have been identified as cysteine (C).

signed *Dpt* 22, but is now recognised as *Der p* II. The *Der p* II amino acid residue sequence shows high homology with the N-terminal sequence of *Der f* II available for comparison from Chapman et al. [17] (table 2). A search of available protein sequence data bases using the Match algorithm [19] revealed no significant homology with other known amino acid sequences.

Starting from the N-terminal Asp of mature *Der p* II [17], the open reading frame codes for a protein of 129 amino acids with a calculated molecular weight of 14,131 D, which is consistent with the M_r of 15 k calculated from SDS-PAGE [10] for *Der p* II and *Der f* II. There is no N-glycosylation site in the sequence. The 17 amino acids preceding the N-terminal of mature *Der p* II (residues –1 through –17 in table 1) have a positively charged lysine near the N-terminus, with the remainder being very hydrophobic. This resembles a typical leader sequence [20].

The reading frame of the *Der p* II and its leader sequence is out of the phase with the *lac Z* gene of lambda gt11. The other two reading frames also contain several stop signals. This is in keeping with the finding (data not shown) by SDS-PAGE that lysates of bacteria expressing this clone do not have high molecular weight fusion protein. However, a low molecular weight band (approximately 15 k in size) which reacts with human IgE can be found by Western blotting.

Discussion

Cloning of cDNA coding for allergens can assist several aspects of research into house dust mite al-

lergy. It is also possible that it will lead to the production of new diagnostic and therapeutic agents at least for subpopulations of allergic patients. The ability to produce large quantities of clonally purified, standardised allergens would help quantitate allergic responses and permit otherwise impractical studies. For instance, the concentration of allergen required to study T-cell responses to purified allergens has inhibited studies in this area. In addition to producing large quantities of allergen, the cloning provides sequence data which can be used to help determine the T- and B-cell epitopes of allergens. Eventually this may lead to genetically engineered allergoids or help explain genetic susceptibility to allergic disease.

For some investigations and applications, it is important to consider the number of allergens which produce the allergic response as well as the contribution of a particular allergen to the total response. As outlined in the introduction, studies to date have shown that 80–90% of patients have IgE to both *Der p* I and *Der p* II. Another 4 components are recognised by 10–20% of sera while other allergens cause sporadic reactions. The allergen *Der p* I is remarkable in that it often binds 60% of IgE antibody in mite allergic serum [1, 2, 21] although recent studies have shown that this may not have the same degree of biological significance in terms of the ability to produce skin reactions [21]. Thus while there is a certain degree of complexity, it is feasible that the allergens responsible for much of the response for many people may be cloned. The availability of cloned allergens will also help elucidate the significance of responses to particular allergens. It also should be noted that little attention has been paid to analysing the allergens recognised by different clinically defined groups. One study in this direction has indicated a fairly homogeneous response of eczematous patients dominated by reactivity to *Der p* II [22].

To obtain clones expressing allergens from our lambda gt11 expression libraries, an IgE plaque immunoassay was used. Serum was diluted 1/2 in a bacterial lysate before use. An ¹²⁵I-labelled monoclonal anti-IgE was used to develop reactions and presumably because of the low concentration of IgE in serum (1 µg/ml) low backgrounds were achieved. Of 4 plaques picked from screening 40,000 recombinant pfu, all were related as judged by DNA hybridisation. They also all gave similar reactivity to any particular serum indicating they were the same allergen. Because only a relatively small number of plaques were

screened, in a library made from mRNA isolated from a whole organism, it is feasible that other allergens will be isolated by further screening for IgE reactivity.

The nucleotide and inferred amino acid sequence of the cDNA coding for this allergen showed that the first 40 residues were identical to the known sequence of *Der p* II published by Chapman et al. [17]. This had very high homology with the N terminal of the related protein from *D. farinae*, *Der f* II. This combined with the calculated molecular weight of 14,131 daltons which is similar to previous estimates [10] almost certainly identifies the cDNA as coding for *Der p* II. Apart from this sequence data, the fact that 14 of 17 sera from people allergic to mites reacted with the clone showed that it was an important allergen and because of its high frequency of reactivity could probably only be *Der p* I or *Der p* II.

The sequence presented here for *Der p* II will be useful for defining the allergen, comparing it to similar allergens from other mites and for epitope analysis. Our previous sequence of a *Der p* I cDNA clone allowed us to identify this allergen as a cysteine protease related to papain and actinidin. Because the X-ray crystallographic structure of these two proteins has been determined, this homology has provided a structural basis for epitope analysis. Like papain [23], however, the antigenicity of *Der p* I appears highly dependent on the tertiary structure, so while recombinant molecules react with rabbit anti-*Der p* I, reactivity to IgE in allergic serum is poor. The *Der p* II clones in contrast are highly reactive with allergic sera, although more analysis is required to determine the degree of antigenicity and conservation of epitopes. As shown by the sequence analysis and our SDS-PAGE studies the recombinant allergen is not expressed as a fusion. This does not account for its allergenicity since further constructs made from this clone (manuscript in preparation) have produced fusions which are equally allergenic. The expression of eukaryotic DNA from lambda gt11 as a non-fusion has been recorded before, but the mechanism has not been fully explored [24, 25].

In conclusion, the success of using IgE plaque immunoscreening to isolate allergens from gt11 libraries has been demonstrated and the primary structure of *Der p* II obtained. This technique will lead to a better characterisation of allergens, and provide material for studies such as T-cell responses. Given the strong reactivity of *Der p* II, the use of this technique to iso-

late other cDNA clones expressing allergens can be anticipated. The approach, however, must be taken with some caution, however, since the major allergen *Der p* I could not be detected by this assay [6].

Acknowledgements

This work was supported by the Children's Medical Research Foundation, the National Health and Medical Research Council of Australia, the Australian National Biotechnology Programme Research Grants Scheme and the Asthma Foundation of Western Australia.

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Received: April 26, 1989

Accepted: October 23, 1989

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