



MAPPING OF THE ANTIGENIC AND ALLERGENIC EPITOPES OF *Lol p* VB USING GENE FRAGMENTATION

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(First received 3 May 1994; accepted in revised form 24 August 1994)

Abstract—The recombinant proteins of *Lol p* VA and *Lol p* VB expressed in *E. coli* reacted with IgE antibodies from sera of allergic patients and mAbs FMC A7 and PpV1. Cross-absorption analyses using these recombinant proteins showed that *Lol p* VA and *Lol p* VB possess both similar and unique IgE binding determinants. Gene fragmentation was utilized to localize the antigenic and allergenic determinants of *Lol p* VB. When full-length cDNA of *Lol p* VB was digested into three fragments and expressed as the fusions from the glutathione transferase of pGEX vectors, fragments Met₁–Val₁₉₆ and Asp₁₉₇–Val₃₃₉ bound IgE while fragment Met₁–Pro₉₆ did not. The data suggest that there are at least two IgE binding determinants in *Lol p* VB. In addition, only fragment Met₁–Val₁₉₆ reacted with mAb PpV1. The localization of these determinants was further resolved using random fragment expression libraries. The mAb PpV1 determinant was near the N-terminal region of *Lol p* VB molecule. The IgE binding determinants were distributed in the central region: region I (amino acids 111–195) and II (199–254). These IgE binding determinants are conserved in *Lol p* VA.

Key words: allergy, expression, isoforms, polymorphism, IgE.

INTRODUCTION

Lol p V is one of two major allergens in the pollen of rye-grass and more than 90% of patients allergic to grass pollen have IgE recognizing this allergen (Singh *et al.*, 1991; Smith, 1993). Together with *Lol p* I, these allergens could inhibit nearly all IgE binding to rye-grass pollen extract (Bond *et al.*, 1993).

Lol p V exists in multiple isoforms with M_r ranging from 28 to 32 K (Singh *et al.*, 1991). cDNAs encoding two of the *Lol p* V isoforms have been isolated by IgE plaque radioimmunoassay using human allergic serum (Singh *et al.*, 1991; Ong *et al.*, 1993). The isoforms encoded by these cDNAs have been named *Lol p* VA and *Lol p* VB (Singh *et al.*, 1991; Ong *et al.*, 1993) and show 80% similarity between their deduced amino acid sequences. The calculated M_r of the mature protein of *Lol p* VA and *Lol p* VB are 27 and 31 K, respectively. These genes provide a potential source for producing large quantities of clonally purified allergens for immunologic and clinical experiments. The recombinant allergens retain considerable IgE binding reactivity that is comparable to their natural counterparts (Singh *et al.*, 1991; Ong *et al.*, 1993). The high degree of allergenicity of these recombinant

proteins (reP) suggest that the production of peptides expressed from cDNA fragments and synthetic peptides would permit epitope analysis. In this study, we report that *Lol p* VA and *Lol p* VB isoforms possessed similar and unique IgE binding determinants. The antigenic and allergenic determinants of *Lol p* VB were localized using random fragment expression libraries.

MATERIALS AND METHODS

cDNA probes

cDNA clone 12R coding for *Lol p* VA (Singh *et al.*, 1991). cDNA clone 19R coding for *Lol p* VB (Ong *et al.*, 1993). The fragments of clone 19R used as DNA probes were as follows: probe 1, *Eco*RI–*Hin*CII fragment, bases – 18 to 588; probe 2, *Hin*CII–*Eco*RI fragment, bases 589 to 1265; probe 3, *Eco*RI–*Pvu*II fragment, bases – 18 to 274; and probe 4, *Dde*I–*Eco*RI fragment, bases 793 to 1265.

Antibodies

Anti-glutathione-S-transferase (GST) polyclonal antisera raised in rabbit were obtained from Dr M. Wright, Walter and Eliza Hall Institute, Parkville, Victoria, Australia. Monoclonal antibody (mAb) PpV1 was a gift from Dr F. Matthiesen, ALK Laboratories, Denmark (Matthiesen *et al.*, 1990). The preparation and characterization of mAb FMC A7 has been described elsewhere (Smart *et al.*, 1983).

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Abbreviations: GST, glutathione-S-transferase; IgE, immunoglobulin E; mAb, monoclonal antibody; reFP, recombinant fusion protein; reP, recombinant protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Gel electrophoresis and immunoblotting

SDS-PAGE was carried out according to Laemmli (1970). Immediately after electrophoresis, the separated proteins were either Coomassie stained or transferred at 4°C to nitrocellulose (BA 0.45 µm, Schleicher and Schuell, Dassel, Germany) according to Towbin *et al.* (1979). Immunodetection methods used to detect proteins on Western blots were as described (Ong *et al.*, 1990).

Cross-absorption analysis

Individuals' serum of four grass pollen allergic patients were pre-absorbed with a lysate of *E. coli* transformed with pGEX or pGEX constructs expressing fusions with clone 12R or 19R at 20% (w/v) overnight with shaking. The sera used were diluted 1:10 with PBS and 0.5% BSA.

Slot blot filters were prepared by applying 20 µg/slot of various lysates of *E. coli* transformed with pGEX or pGEX constructs expressing fusions with clone 12R or 19R on nitrocellulose membrane (BA 0.45 µm, Schleicher and Schuell) using the Hybri-Slot manifold (BRL, Life Technology Inc., U.S.A.). Filters were air-dried and washed with PBS for 5 min, followed by overnight incubation with absorbed (inhibited) sera at room temperature with shaking. After serum incubation, the filters were washed and then incubated with ¹²⁵I-rabbit anti-human as described (Ong *et al.*, 1990). The relative reduction in IgE activity, which is proportional to the amount of IgE activity blocked by allergen, is expressed as a percentage of inhibition, calculated as follows: % Inhibition = (A - B) / A × 100 where A = counts without pre-absorption, B = count with pre-absorption.

Construction of random fragment libraries

Two size ranges of the random fragment libraries were constructed according to the method of Greene *et al.* (1990). Library A contained random fragments of 72–118 bp (range 24–40 amino acid residues). Library B contained random fragments of 118–310 bp (range 40–100 amino acid residues). At least 50,000 recombinant plaques from each random fragment library were probed with pooled allergic sera and mAb PpV1.

Immunological screening of random fragment libraries

Immunological screening of the cDNA library was done as described by Ong *et al.* (1993).

DNA screening of purified clones

Purified λ-gt 11 clones were plated out and grown at 37°C overnight. One plaque from each clone was picked and immersed in 20 µl SMC (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM MgSO₄, 0.1% gelatin) for 2 hr. Then, 2 µl of the phage solution were dotted onto a lawn of *E. coli* Y1090 and grown for 4 hr. DNA filters were prepared as described by Sambrook *et al.* (1989). Pre-hybridization and hybridization of DNA filters was performed as described by Ong *et al.* (1993).

DNA sequencing

Lambda DNA of the isolated clones was prepared using Promega Magic Lambda Purification System (Promega, Madison, WI, U.S.A.). The λ-gt 11 DNA was sequenced according to Promega fmol sequencing system (Promega).

Expression of recombinant protein

The cDNA inserts of clones 12R and 19R were excised from purified phages and subcloned into *Eco*RI site of pGEX or pTrc 99 expression vectors. The cDNA inserts of clones 12R and 19R contained the 5' non-coding DNA segment of 39 and 18 bp, respectively. The expression of the recombinant proteins was carried out according to the procedure outlined by Smith and Johnson (1988) and Amann *et al.* (1988), respectively.

RESULTS

Expression of Lol p V cDNAs in *E. coli*

Two expression vectors, pTrc 99A and pGEX-1, were used. The pTrc 99A expression vector contains a strong IPTG-inducible hybrid trp/lac promoter adjacent to the cDNA insertion site and the resulting recombinant products do not have a fusion partner (Amann *et al.*, 1988). The pGEX expression vector is designed to direct the synthesis of the cDNA insert fused with the C-terminal of Sj 26, a 26-kDa GST of the parasitic helminth *Schistosoma japonica* (Smith and Johnson, 1988). The cDNAs of both clone 12R (encoding Lol p VA) and clone 19R (encoding Lol p VB) were ligated in-frame into the pGEX vector (pGEX-12R and pGEX-19R). The cDNA clone 19R was inserted in the correct reading frame of pTrc 99A vector (pTrc-19R). The expressed products were monitored by Western blotting using either human pooled sera, mAb FMC A7 or mAb PpV1. Figure 1 shows that the rePs, pGEX-19R, pGEX-12R and pTrc-19R are recognized by IgE antibodies from an allergic serum pool. The reP pGEX-12R was recognized by mAb FMC A7 but not mAb PpV1 (Fig. 1). The rePs pGEX-19R and pTrc-19R were recognized by mAb PpV1 but not mAb FMC A7 (Fig. 1). These results indicate that the rePs retain the antigenic and allergenic properties of the natural counterparts.

In pGEX-12R, there was an abundance of proteins recognized by both IgE from an allergic serum pool and mAb FMC A7 at M_r 30 K. The size is within the calculated size range determined for the deduced amino acids of cDNA insert of clone 12R. This suggests that the 30 K proteins are the recombinant products minus the fusion partner, 26-kDa GST.

Cross-reactivity between recombinant Lol p VA and Lol p VB

To examine if unique IgE binding determinants exist between the two isoforms of Lol p V, rePs pGEX-12R and pGEX-19R were used in a cross-absorption analysis. IgE antibodies from sera of four grass pollen allergic patients were individually pre-absorbed with each of the rePs pGEX-12R and pGEX-19R. The pre-absorbed serum

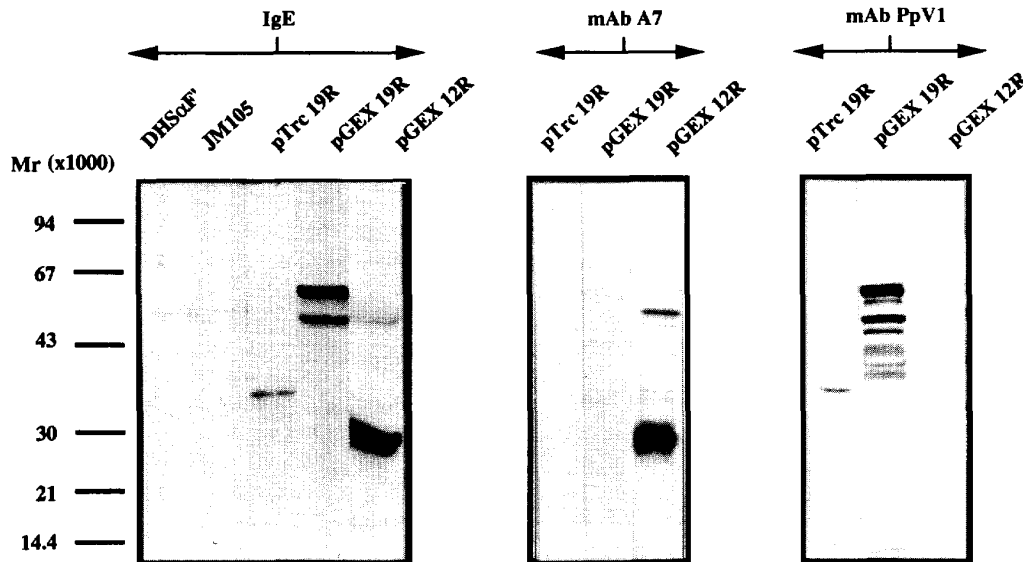


Fig. 1. Immunoblot analysis of recombinant *Lol p V* proteins. SDS-PAGE immunoblot sections were generated with the following antigens: (1) DHS α F' negative control bacterial lysate; (2) JM105 negative control bacterial lysate; (3) bacterial lysate containing pTrc 19R; (4) bacterial lysate containing pGEX 19R; (5) bacterial lysate containing pGEX 12R. Each lane was loaded with 40 μ g of the extract. These blots were probed with pooled allergic human sera at 1:10 dilutions, mAb FMC A7 and mAb PpV1.

was then tested by slot blot radioimmunoassay to assess changes in reactivity to the rePs pGEX-12R and pGEX-19R. In the patients' sera tested, reP pGEX-12R could remove all IgE reactivity to itself, but not reP pGEX-19R. Similarly, absorption of patients' sera with reP pGEX-19R abrogated all reactivity to itself, but not reP pGEX-12R (data not shown).

Tables 1 and 2 show the percentage of cross-absorption using rePs pGEX-12R and pGEX-19R as inhibitors. The capacity of reP pGEX-12R used to deplete binding of IgE from allergic patients' sera to reP pGEX-19R varied between the four sera used. The level of depletion ranged between 3 and 78%. Similarly, the ability of reP pGEX-19R to deplete binding of IgE from allergic patients' sera to reP pGEX-12R varied between the four sera used. The level of depletion ranged between 31 and 60%. These results indicate that unique IgE

binding determinant/s exist between the two *Lol p V* isoforms.

Epitope mapping of *Lol p VB* using random fragment libraries

As a preliminary step to map the antigenic and allergenic determinants of *Lol p VB*, full-length cDNA of clone 19R was digested into three fragments and subcloned into pGEX 1-3 expression vectors. Figure 2 shows that the rePs expressed by fragment Met₁-Val₁₉₆ and Asp₁₉₇-Val₃₃₉ were reactive with IgE antibodies from an allergic serum pool whereas only fragment Met₁-Val₁₉₆ gave a positive signal when tested with mAb PpV1. Fragment Met₁-Pro₉₁ was non-reactive when tested with both IgE antibodies from an allergic serum pool and mAb PpV1. These results indicate that *Lol p VB* contains at least two different IgE binding determinants.

To refine the *Lol p VB* linear allergenic and/or antigenic determinants, two random fragment expression libraries were constructed. No plaque was reactive when library A

Table 1. Quantitative analysis of cross-reactivity between recombinant *Lol p VA* and *Lol p VB* proteins

Patient	sr	rl	ma	aa
pGEX 12R	100%	100%	100%	100%
pGEX 19R	56%	3%	40%	78%

Slot blots were generated with the following antigens: (1) DHS α F' negative control bacterial lysate (20 μ g/slot); (2) bacterial lysate containing pGEX 12R (20 μ g/slot); (3) bacterial lysate containing pGEX 19R (20 μ g/slot); and (4) rye-grass pollen extract (7 μ g/slot). The blots were probed with sera pre-absorbed with recombinant *Lol p VA* of four individual patients and the autoradiograph was measured using gamma counter. Percentage of inhibition was calculated as described in Materials and Methods.

Table 2. Quantitative analysis of cross-reactivity between recombinant *Lol p VB* and *Lol p VA* proteins

Patient	sr	rl	ma	aa
pGEX 19R	100%	100%	100%	100%
pGEX 12R	48%	59%	31%	60%

Slot blots were generated as detailed in Table 1. The blots were probed with sera pre-absorbed with recombinant *Lol p VB* of four individual patients and the autoradiograph was measured using a gamma counter. Percentage inhibition was as described in Materials and Methods.

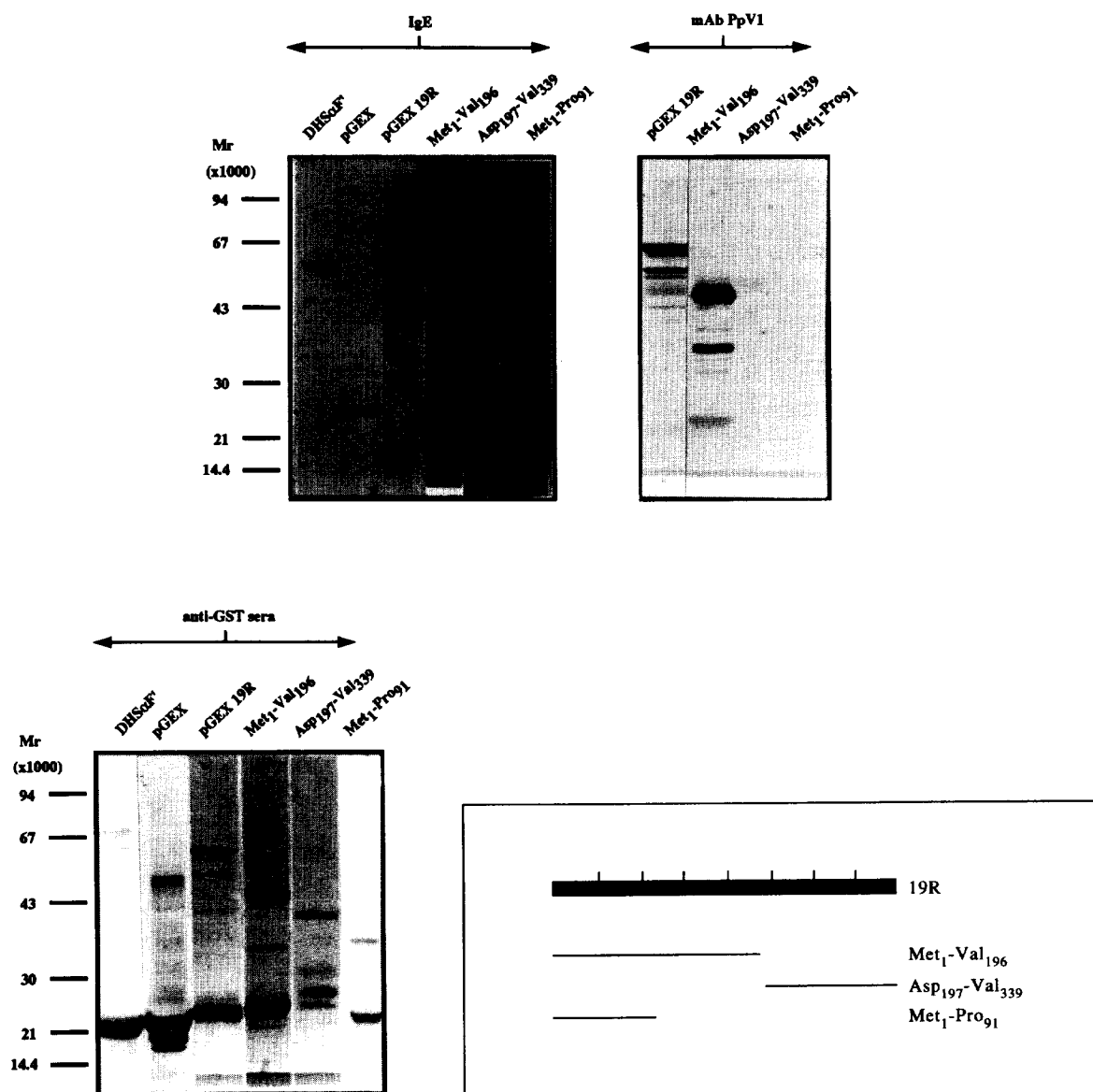


Fig. 2. Mapping of IgE and mAb-reacting epitopes by immunoblotting of fusion proteins encoded by full-length clone 19R and truncated fragments expressed in pGEX expression vectors. The truncated fragments are as illustrated schematically above. The position of size markers are shown on the left. In all cases, 40 μ g of bacteria lysate protein was loaded per lane. Western blots were probed with IgE antibodies from pooled allergic sera, mAb PpV1 and anti-GST sera. The controls were protein lysates of bacteria cells transformed with non-recombinant plasmid (pGEX) and protein lysates of bacteria cells without plasmid (DHS α F).

was probed with pooled allergic sera and mAb PpV1. In contrast, 114 IgE reactive clones and 32 mAb PpV1 reactive clones were obtained from library B. Out of these 32 mAb reactive clones, 15 also gave positive signals with pooled allergic sera. All the IgE reactive clones were retested with pooled allergic sera. Eighty-five clones (75%) gave a positive signal on retesting and were purified. The signal intensities of IgE reactive clones varied. Nineteen of the mAb and mAb-IgE reacting clones gave a positive signal on retesting with mAb PpV1.

To align these different random fragment clones to the

full-length clone of 19R, plaque DNA-lifts from all the clones were obtained for hybridization. Fifty-four IgE binding clones hybridized to probe 1 (–18 to 588 bases) whereas 16 IgE binding clones hybridized to probe 2 (589 to 1265 bases). There were 15 IgE binding clones that hybridized to both probe 1 and 2. None of the IgE binding clones hybridized to probe 3 (–18 to 274 bases) nor to probe 4 (793 to 1265 bases). Ten of these IgE binding clones were selected for sequencing. All the mAb and mAb-IgE reacting clones hybridized to probe 1. Eight of these clones were selected for sequencing.

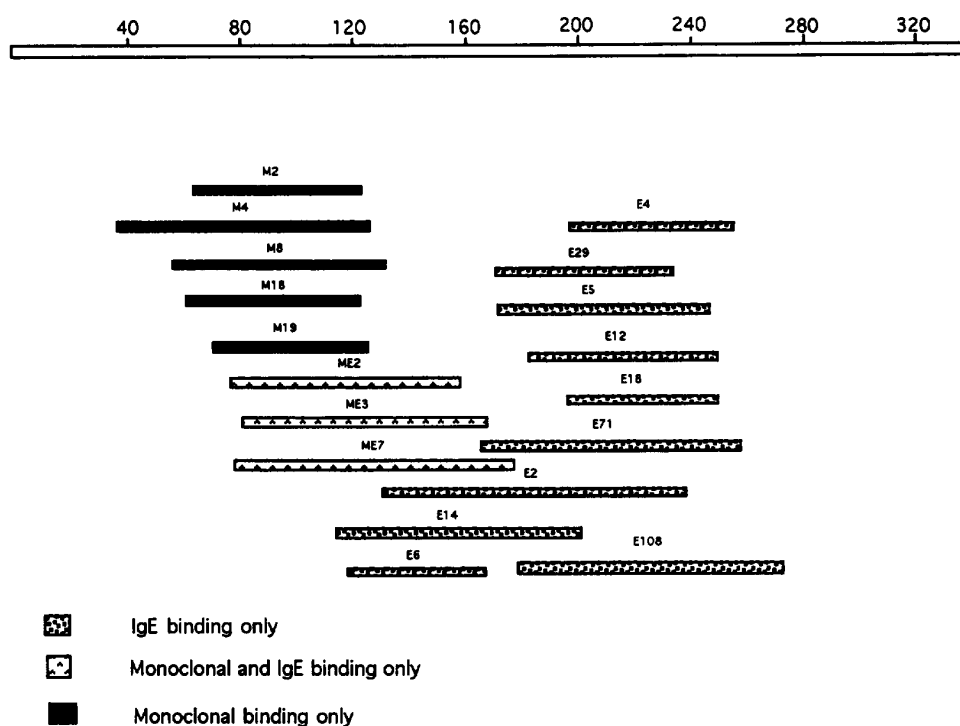


Fig. 3. Map showing the location of the 18 random fragment clones. The open bar represents the full-length coding region of clone 19R with the number of amino acid residues on top.

Figure 3 shows the position of the sequenced fragments with respect to the full-length clone 19R coding sequence. The results indicate that IgE binding determinants were distributed at the centre of *Lol p* VB molecule while mAb PpV1 binding determinant was near the N-terminal.

IgE binding studies

Six of these random fragment clones were selected for testing the recombinant fusion proteins (reFPs) with 18 allergic patients' sera. The method used was as described by Valenta *et al.* (1992) and was designed to indicate the

Table 3. Specificity of IgE antibodies from human sera to random fragment recombinant proteins

Patients	12R	19R	M8	ME7	E14	E2	E5	E4
1	+	+	+	+	—	+	+	+
2	+	+	+	+	+	+	+	+
3	+	+	—	—	—	—	—	—
4	+	+	—	+	+	+	+	+
5	—	—	—	—	—	—	—	—
6	+	+	+	+	+	+	+	+
7	—	—	—	—	—	—	—	—
8	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+
10	+	+	—	+	+	+	—	—
11	—	—	—	—	—	—	—	—
12	+	+	—	+	+	+	—	—
13	+	+	—	+	+	+	—	—
14	+	+	—	+	+	+	—	—
15	—	—	—	—	—	—	—	—
16	+	+	—	+	+	+	—	—
17	+	+	—	+	+	+	+	+
18	+	+	—	+	+	+	—	—

Recombinant proteins were expressed from λ -gt 11 phages onto nitrocellulose using 10 mM isopropyl- β -thiogalactosidase as described by Ong *et al.* (1993). + shows there was binding of IgE antibodies to reFP. — shows there was no binding of IgE antibodies to reFP.

presence or absence of IgE binding to the recombinant protein. These sera contained IgE reactive with natural *Lol p V* on Western blots (data not shown). Seventy-eight percent (14/18) of patients have IgE directed against reFP 12R and reFP 19R (Table 3). Thirteen of these patients reacted positively with reFP ME7 and E2 while twelve of them have IgE recognizing reFPs E14. Seven of these patients have IgE reactive with reFPs E4 and E5, and five have IgE reactive with reFP M8. The IgE antibodies from the serum of one patient recognized reFPs 12R and 19R but had no binding to any of the random fragment reFPs.

The pattern of binding of these individual patients' sera to the random fragment reFPs varied (Table 3). There were four patients that possessed IgE directed against all the six reFPs while two patients had IgE reactive with five reFPs except M8. There were six patients that possessed IgE directed against reFPs ME7, E14 and E2 while one patient had IgE reactive to reFPs M8, ME7, E2, E4 and E5. Most of the patients' IgE bound strongly to reFPs ME7, E14 and E2. These results show that IgE antibodies from different individuals bound to different determinants. IgE antibodies from most allergic sera showed binding to determinants between amino acids 76–237 (between ME7 and E2). These results indicate that there is one main IgE binding determinant among these sampled populations.

IgE was affinity purified from reFPs E14 and E4. E14 and E4 correspond to amino acids 111–195 and 199–254 of clone 19R, respectively. The affinity purified IgE from reFPs E14 and E4 bound to reFP 12R (data not shown). These results suggest that these determinants encoded by random fragment E14 and E4 are present in reFP 12R. The affinity purified IgE from reFP E14 did not react with reFP E4. Similarly, the affinity purified IgE from reFP E4 did not react with reFP E14. Both of these affinity purified IgE bound to reFP E2.

DISCUSSION

In this report, we have shown that two isoforms of a major rye-grass pollen allergen, *Lol p VA* and *Lol p VB* possess both similar and unique allergenic determinants. There are two dominant IgE binding determinants in *Lol p VB*: region I (located between amino acids 111 to 195, fragment E14) and region II (located between amino acids 199 to 254, fragment E4).

Expression of recombinant proteins in bacteria

The availability of the cDNA coding for *Lol p VA* and *Lol p VB* makes it possible to produce these allergens in large quantities using recombinant techniques. This provides a source of pure allergens for experimental, diagnostic and therapeutic uses. In addition, as biochemical purification of individual isoforms are tedious, these rePs can provide a source of pure isoform proteins. In previous studies, reFPs of *Lol p VA* and *Lol p VB* have been shown to react with IgE antibodies from sera of allergic patients (Singh *et al.*, 1991; Ong *et al.*, 1993). The lacZ fusion proteins produced using lambda gt 11 vector were insoluble and low in yield, thus not

suitable for cross-absorption analysis. In this study, two expression vectors, pGEX and pTrc 99A, were utilized. The rePs produced reacted with IgE from sera of allergic patients and with either mAb A7 or mAb PpV1.

The rePs produced were relatively soluble and the yield of reP 19R expressed using pTrc vector is lower than pGEX. A low level of production of rePs was obtained using pTrc vector containing 12R cDNA (I. J. Griffith; pers. comm.) and cDNA encoding *Fel d I* chain 1 and chain 2 (Rogers *et al.*, 1993). In contrast, a high level of expression was observed with pTrc containing cDNA encoding *Amb a I* and *Amb a II* (Kuo *et al.*, 1993).

The two isoforms of *Lol p V* share some common IgE binding determinants. This has previously been shown using affinity purified IgE (Ong *et al.*, 1993). The cross-absorption analysis in this study has confirmed this finding. In addition, the analysis revealed that each isoform possesses its own unique IgE binding determinant/s despite the 80% similarity between the amino acid sequences. The percentage of inhibition using the two isoforms varied markedly between the four patients tested. Three of the patients have at least 50% of the IgE antibodies recognizing both reFP *Lol p V*. It is interesting to note that in one patient (rl), the IgE recognizing reFP-*Lol p VA* cross-react with reFP-*Lol p VB* but not *vice versa*. The reason for this observation is not clear and cannot be interpreted using the present data.

The marked variation observed in cross-absorption analysis is in agreement with the direct dot blot analysis using IgE from 30 patients' sera of a sampled population showing variation occurred in the signal of IgE binding intensities (Ong *et al.*, 1993). These differences have also been observed in short ragweed, *Amb a I* isoforms; a minority of patients' IgE reacts with only the recombinant *Amb a I.1* whereas most patients' IgE reacts with the recombinant *Amb a I.1* as well as with the *Amb a I.2* and *Amb a I.3* proteins (Bond *et al.*, 1991). Thus it is of importance to localize the IgE binding determinant/s of *Lol p VB*.

Epitope mapping of Lol p VB

Since recombinant *Lol p VB*, expressed in bacteria, is immunoreactive with IgE antibodies, its linear antigenic and allergenic determinants can be determined using random fragment expression libraries. Positive clones from the random fragment libraries produced by mechanical shearing of cDNA coding for *Lol p VB* were detected, comprising peptides with more than 55 amino acid residues. This observation explains the absence of detectable positive clones in library A (24–40 amino acids). In *Der p II*, random fragment libraries coding for more than 93 of the 129 amino acids were needed for immunoreactivity with IgE antibodies (Chua *et al.*, 1991).

IgE binding determinants of *Lol p VB* are sited at the centre of the molecule. In this region there seems to be two major IgE binding determinants: I (amino acids 111–195) and II (199–254). These conclusions are based on two observations. First, the IgE binding pattern of the 18 patients' sera fall into two groups. Second, affinity purified IgE from clone E14 (region I) does not bind to

reFP E4 (region II) and *vice versa*. Assuming that these IgE binding determinants are linear, region I can be resolved to amino acids 131-156 based on comparison of random fragments E2 and ME2, resulting in a determinant of 25 residues. Similarly, region II can be resolved to amino acids 199-233 based on comparison of random fragments E4 and E29, resulting in a determinant of 34 residues.

Comparison of these IgE binding regions with that of the corresponding regions of *Lol p* VA shows high similarity between the two isoforms. This suggests that these two IgE binding determinants in *Lol p* VB are conserved in *Lol p* VA. This has been experimentally confirmed since affinity purified IgE from fragment E14 (region I) and fragment E4 (region II) bound to *Lol p* VA.

The lack of binding of IgE from an allergic serum pool to N- or C-terminal peptides of *Lol p* VB is interesting. This was confirmed using the fragment Met₁-Pro₉₁ expressed as a fusion protein, which also did not bind IgE. These results are in contrast with many published observations that the N- and C-termini of proteins are relatively mobile and readily exposed to the binding of antibodies (Berzofsky, 1985). For example, *Lol p* I possesses an IgE binding determinant found at the C-terminal end of the molecule (Esch and Klapper, 1989; Perez *et al.*, 1990; Griffith *et al.*, 1991). The C-terminal end of *Lol p* I is highly hydrophilic (Griffith *et al.*, 1991). *Bet v* I and *Cor a* I possess IgE binding determinants located at their N-termini (Elsayed *et al.*, 1989; Vik *et al.*, 1993). Greene *et al.* (1990) could not demonstrate any antigenic determinant at the N- or C-termini of *Der p* I using a random fragment library and anti-*Der p* I antibodies. An explanation is that the C-terminus of *Der p* I is hydrophobic and buried or inaccessible after folding (Greene *et al.*, 1990). This explanation may apply to *Lol p* VB, as the N- and C-termini of the molecule are highly hydrophobic and contain many alanine residues.

No IgE binding determinant has been detected in *Lol p* VA at the N-terminus using a random fragment library and a series of 12-mer overlapping synthetic peptides and IgE antibodies from 50 patients' sera (C. Suphioglu, pers. comm.). However, two of these patients possessed IgE reacting strongly to the C-terminus of *Lol p* VA using synthetic peptides. The equivalent region of *Lol p* VB has 80% similarity in amino acid sequence with the corresponding region in *Lol p* VA. These results suggest that in *Lol p* VA and *Lol p* VB, the C-terminal IgE binding determinant is unlikely to be dominant.

There are several disadvantages of probing random fragment libraries with pooled human sera: (1) Most allergic patients' sera contain low IgE titre against minor determinants. When human sera were pooled, the titre of these IgE decreased further due to dilution in the pool of serum. Thus the IgE reactive clones detected with pooled serum mainly express major or dominant determinants; (2) In a random fragment library, different sizes of peptides are expressed. These peptides, when probed with human IgE gave different intensities of binding. Generally, clones that gave high intensities of binding were selected that usually contain large inserts. In this

study, few clones were detected with low binding intensities when library A was probed with human IgE. As binding intensities did not differ greatly from background, these clones were not analysed further.

mAb PpV1's (raised against *Phl p* V) binding determinant of clone 19R was determined using random fragment libraries. As the N-terminal fragment Met₁-Pro₉₁ did not react with mAb PpV1, while fragment M2 (Ala₆₄-Asp₁₂₀) did react with this mAb, these data suggest that the antigenic determinant is located between amino acids Ala₉₂-Asp₁₂₀ (28 residues). mAb PpV1 recognizes *Lol p* VB but not *Lol p* VA (Ong *et al.*, 1993). This antigenic binding determinant is therefore "isoform-specific". By comparing the amino acid sequences of *Lol p* VB, *Lol p* VA and *Phl p* VA, the amino acids critical to the function of the determinant can be deduced. This region of *Lol p* VB shows only a low level of similarity to the corresponding region of *Lol p* VA. Peptides corresponding to these amino acid sequence in both *Lol p* VA and *Lol p* VB could be used as immunogens to generate anti-*Lol p* VA and anti-*Lol p* VB specific antisera, respectively. For example, the deduced amino acid sequences of *Amb a* I.1 and *Amb a* II share 66% identity (73% homology) (Rogers *et al.*, 1991). Kuo *et al.* (1993) have successfully used peptides of *Amb a* I.1 and *Amb a* II based on their divergence in sequence, to generate antisera that were specific to each individual allergens. This approach of generating antisera that can differentially bind polymorphic forms of an allergen may be useful in purifying each individual isoform.

CONCLUSIONS

Lol p VA and *Lol p* VB allergens possess both similar and unique allergenic determinants. The allergenic determinants of *Lol p* VB have been determined using random fragment libraries. There are two major allergenic determinants located in the central region of *Lol p* VB: region I (between amino acids 111 and 195) and region II (between amino acids 199 and 254). These regions which contain allergenic determinants are conserved in *Lol p* VA. Thus, comparison between the deduced amino acid sequences of the isoforms help in elucidating allergenic and antigenic determinants. Knowledge of the amino acid sequences of all *Lol p* V isoforms (there are eight isoforms of *Lol p* V; Smith *et al.*, 1994) and the extent of polymorphism will be useful to determine the identity and number of these determinants. In addition, identification of regions containing "isoform specific" determinants could permit the synthesis of short peptides (10-25 amino acids) that could be used as immunogens to raise antisera that are specific, for example, to *Lol p* VB but not *Lol p* VA. This approach of generating antisera that can distinctly bind related isoforms may be useful as other multigene families of allergenic proteins are defined.

Acknowledgements—The authors gratefully thank Drs P. M. Smith, C. Suphioglu and I. J. Griffith for their helpful discussions and the Australian National Health and Medical

Research Council, Asthma Foundation of Victoria, Baker Shaw Trust, for financial support.

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