Mutants of the major ryegrass pollen allergen, Lol p 5, with reduced IgE-binding capacity: candidates for grass pollen-specific immunotherapy

Ines Swoboda[1], Nicole De Weerd[1], Prem L. Bhalla[1], Verena Niederberger[2], W. R. Sperr[3], Peter Valent[3], Helga Kahlert[4], Helmut Fiebig[4], Petra Verdino[5], Walter Keller[5], Christof Ebner[6], Susanne Spitzauer[7], Rudolf Valenta[6] and Mohan B. Singh[1]

[1] Plant Molecular Biology and Biotechnology Laboratory, Institute of Land and Food Resources, The University of Melbourne, Parkville, Australia
[2] Department of Otorhinolaryngology, Vienna General Hospital, University of Vienna, Vienna, Austria
[3] Department of Internal Medicine I, Division of Hematology and Hemostaseology, Vienna General Hospital, University of Vienna, Vienna, Austria
[4] Allergopharma Joachim Ganzer KG, Reinbek bei Hamburg, Germany
[5] Division of Structural Biology, Institute for Chemistry, Graz, Austria
[6] Department of Pathophysiology, Vienna General Hospital, University of Vienna, Vienna, Austria
[7] Inst. of Medical and Clinical Chemistry, Vienna General Hospital, University of Vienna, Vienna, Austria

More than 400 million individuals are sensitized to grass pollen allergens. Group 5 allergens represent the most potent grass pollen allergens recognized by more than 80 % of grass pollen allergic patients. The aim of our study was to reduce the allergenic activity of group 5 allergens for specific immunotherapy of grass pollen allergy. Based on B- and T-cell epitope mapping studies and on sequence comparison of group 5 allergens from different grasses, point mutations were introduced by site-directed mutagenesis in highly conserved sequence domains of Lol p 5, the group 5 allergen from ryegrass. We obtained Lol p 5 mutants with low IgE-binding capacity and reduced allergenic activity as determined by basophil histamine release and by skin prick testing in allergic patients. Circular dichroism analysis showed that these mutants exhibited an overall structural fold similar to the recombinant Lol p 5 wild-type allergen. In addition, Lol p 5 mutants retained the ability to induce proliferation of group 5 allergen-specific T cell lines and clones. Our results demonstrate that a few point mutations in the Lol p 5 sequence yield mutants with reduced allergenic activity that represent potential vaccine candidates for immunotherapy of grass pollen allergy.

Received	7/9/01
Accepted	15/10/01

Key words: Pollen allergen / Allergen engineering / Grass / Hypoallergenic / Immunotherapy

1 Introduction

Type I allergic diseases, such as allergic rhinitis or hayfever, conjunctivitis, allergic asthma and allergic dermatitis, represent a major health problem in industrialized countries, affecting more than 25 % of the world's population [1]. Allergic patients are characterized by their genetically determined tendency to produce IgE antibody responses to otherwise harmless environmental antigens (allergens) in pollen, food, house dust mites, animal dander, fungal spores and insect venoms. Cross-linking of effector cell-bound IgE antibodies by intact allergens containing several binding sites for IgE antibodies leads to the release of biologically active mediators (histamine, leukotrienes) and thus to the immediate symptoms of type I allergy.

Whereas the symptoms of allergic disease (allergic rhinoconjuncitivitis, asthma) can be controlled by pharmacotherapy, specific immunotherapy is the only causative approach towards the treatment of type I allergy [2]. Specific immunotherapy is based on the administration of increasing amounts of the disease-eliciting allergens in the form of allergen-containing extracts. Since its introduction in 1911, the efficacy of specific immunotherapy has been documented by numerous controlled clinical trials but the underlying immunological mechanisms are still a matter of debate [3–5]. Moreover, allergen extracts used for immunotherapy consist of allergenic and non-

[I 22365]

I. S. and N. de W. are joint first authors of this paper.

0014-2980/02/0101-270$17.50+.50/0

allergenic components and may induce severe anaphylactic side effects upon therapeutic administration. With the introduction of recombinant DNA technology into the field of allergen characterization it has become possible to produce recombinant allergens for diagnosis of type I allergy and to reduce the allergenic activity of allergens by recombinant technology or peptide chemistry [6–10].

As a model allergen we used Lol p 5, the group 5 allergen of ryegrass (*Lolium perenne*) pollen [11]. Allergens belonging to group 5 represent the most potent allergenic molecules in grass pollen. They react with IgE antibodies of more than 80 % of the individuals allergic to grass pollen and contain the majority of grass pollen-specific IgE epitopes [12–14]. Based on extensive IgE epitope mapping using overlapping recombinant fragments and synthetic peptides [15, 16] and sequence comparison of group 5 allergens we identified strictly conserved IgE binding sites in group 5 allergens with little overlap with dominant T-cell epitopes [17]. Using site-directed mutagenesis we introduced point mutations into the conserved IgE-bearing sites of Lol p 5 and generated a series of 9 Lol p 5 mutants. Lol p 5 mutants with reduced allergenic activity but retained structural features of the Lol p 5 wildtype allergen could be identified. Their potential usefulness for immunotherapy of grass pollen allergy is discussed.

2 Results

2.1 Exchange of conserved amino acid residues in putative IgE-reactive regions of Lol p 5 by site-directed mutagenesis

On the basis of B- and T-cell epitope mapping studies and of sequence comparison of group 5 allergens from different grasses, we generated nine variants of Lol p 5. In five of them, amino acid residues were exchanged in single domains (mut 1–mut 5), which were then combined in different ways to obtain the triple-mutants mut 6 to mut 9. The sites of amino acid exchanges have been depicted in Fig. 1 A and B.

Previous studies had shown that a synthetic peptide of 12 amino acids, AANAPPADKFKI, corresponding to domain D1, bound IgE from sera of grass pollen-allergic patients and a mouse anti-Lol p 5 monoclonal antibody (A7). A truncated version (APPADKFKI) of this synthetic peptide retained both human IgE and mAb A7 reactivity [16]. Comparison of the truncated sequence with corresponding sequences of Lol p 5 isoforms and homologues indicated that Lys 57 is the only highly conserved amino acid residue. Therefore, this lysine residue was replaced by alanine in mut 1 (Fig. 1 A; Fig. 1 B). Also

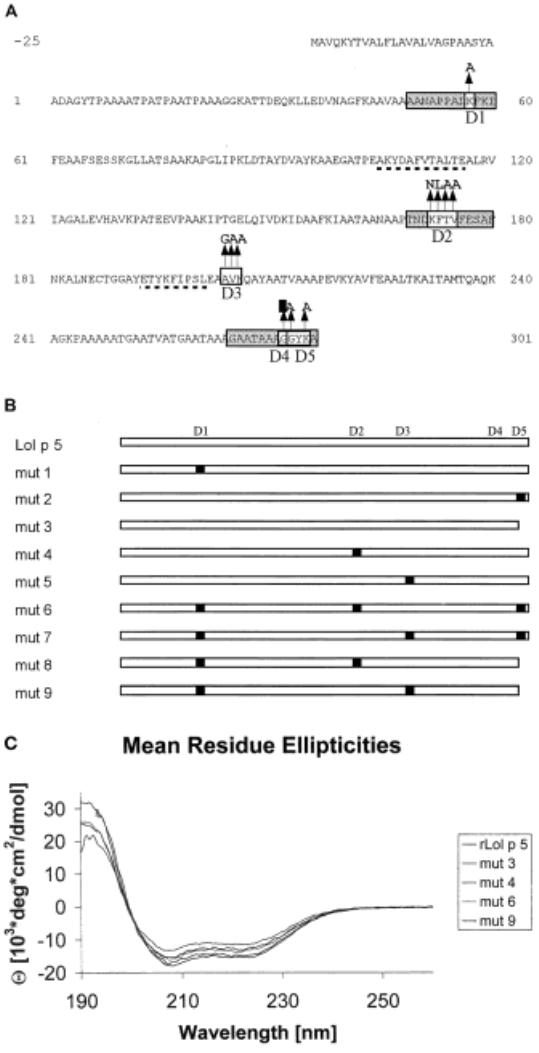


Fig. 1. Description of Lol p 5 mutants. A, amino acid sequence of Lol p 5 showing the domains (D1 to D5) where mutations were introduced. Amino acids of Lol p 5 exchanged are indicated by clear boxes with new sequences given in bold. The sequences corresponding to short IgE-reactive synthetic peptides are shown in gray boxes. T cell epitope peptide regions of Lol p 5 are underlined. B, schematic representation of mutated Lol p 5 variants; for example: mut 1 contains mutation in D1, mut 2 contains mutation in D5 and so on. Note that mutants 3, 8 and 9 are truncated in the C terminal region and are therefore depicted as being shorter then Lol p 5 in this figure. C, CD analysis of purified rLol p 5 and rLol p 5 mutants (mut 3, mut 4, mut 6, mut 9). Results are expressed as the mean residue ellipticity (y-axis) at a given wavelength (x-axis).

domain D2 corresponds to a previously identified IgE-reactive synthetic peptide [16] and the four-amino acid block $K^{172}F^{173}T^{174}V^{175}$ of D2 is strictly conserved in group 5 grass pollen allergens. Consequently, we substituted in

mutant 4 all four amino acids of this block (KFTV → NLAA). The three-amino acid block $A^{204}V^{205}K^{206}$ of domain D3 was selected for site-directed mutagenesis only due to its strict conservation in group 5 allergens and their isoforms and was replaced by GAA in mutant 5. In contrast, mutations at the C terminus of the Lol p 5 molecule (D4 and D5) were again based on previous B cell epitope mapping studies. The synthetic peptide GAATAAAGGYKA corresponding to this region (Fig. 1 A) had strongly reacted with IgE from a subset of grass pollen-allergic patients' [16]. Furthermore, alanine-scanning studies had shown that replacement of the strictly conserved lysine residue K^{275} with alanine leads to loss of IgE reactivity of this synthetic peptide [16]. Therefore, K^{275} and the other conserved residue, G^{273}, were both either substituted by alanines as in mut 2 (Fig. 1 A) or deleted by introduction of a termination codon as in mut 3, which is truncated by 4 amino acids from the C terminal end (Fig. 1 A; Fig. 1 B).

Recombinant Lol p 5 and its nine mutants were expressed as histidine-tagged proteins and purified by nickel affinity chromatography. Purity of the expressed proteins was assessed by SDS-PAGE (Fig. 2 A). Immunoblot analysis showed that all Lol p 5 mutants were recognized by a rabbit serum raised against rLol p 5. However, mut 1 and mut 6- to mut 9, in which lysine at position 57 of domain D1 was replaced by alanine, showed loss of reactivity to the monoclonal antibody A7. This lack of antibody recognition, observed in immunoblots (Fig. 2 A) and ELISA assays (data not shown) indicated that antibody binding capacity of domain D1 had been destroyed by exchange of a single amino acid.

2.2 rLol p 5 and rLol p 5 mutants represent folded molecules containing a considerable amount of α-helical structure

The far-UV spectrum of the wild-type Lol p 5 protein (Fig. 1 C) was characterized by minima at 222 and 208 nm and a strong maximum at 192 nm. Such a shape is typical of well-structured proteins with a considerable amount of α-helices. Overall the spectra of the Lol p 5 mutants, of which four (mut 3, mut 4, mut 6, mut 9) are displayed in Fig. 1 C, showed the same characteristics as the spectrum of the wild-type protein. It thus can be concluded that introduction of a few amino acid exchanges did not significantly alter the fold of the protein. Comparison of the α-helical content between the wild-type protein and the mutants showed a slight decrease in the secondary structure content for all mutants except mut 3, with the largest decrease observed for mut 4 (Table 1). The circular dichroism (CD) results are in good agreement with secondary structure predictions using the PHD package [18, 19] which classify the Lol p 5 protein as an all-α protein (% $\alpha > 45$ % and % $\beta < 5$ %).

2.3 Reduced IgE binding capacity of rLol p 5 mutants

The IgE binding capacity of rLol p 5 mutants containing single or multiple amino acid substitutions compared to wild-type Lol p 5 was evaluated using different methods with immobilised allergen or allergen in solution (immunoblots, dot blots and ELISA competition experiments). Fig. 2 A shows the IgE reactivity of six individual patient

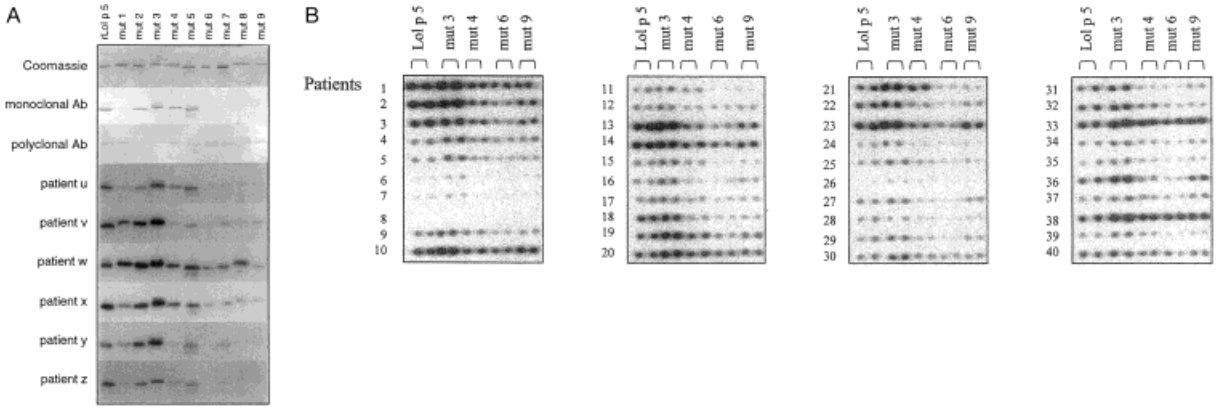


Fig. 2. SDS-PAGE and immunoblot analysis of purified rLol p 5 and rLol p 5 mutants. A, Immunoblot analyses of Lol p 5 (wild-type) and the nine mutated variants (mut 1 to mut 9) showing reactivities of the purified proteins to rabbit antiserum, a mouse monoclonal antibody A7 and to sera of 6 ryegrass pollen-allergic patients (u–z). B, IgE reactivity of sera from additional 39 grass pollen-allergic patients (1–7, 9–40) and a non-atopic person (8) to nitrocellulose-dotted duplicates of rLol p 5 wildtype and Lol p 5 mutants (mut 3, mut 4, mut 6, mut 9).

Table 1. Secondary structure estimations for rLol p 5 and Lol p 5 mutants

Protein	c [M][a]	α[%][b]	r.m.s.[c]
rLol p 5	2.80 ± 10^{-6}	40	0.118
mut 3	3.74 ± 10^{-6}	42	0.103
mut 4	3.13 ± 10^{-6}	28	0.111
mut 6	2.75 ± 10^{-6}	34	0.100
mut 9	3.01 ± 10^{-6}	33	0.105

a) c[M]: protein concentration used for the CD measurements.
b) α[%]: percent of α helices deduced from the secondary structure estimations.
c) r.m.s.: root mean square, deviation of experimental CD measurements from calculated CD values.

sera examined by immunoblot analysis. All the sera displayed IgE reactivity towards wild-type rLol p 5. However, the single amino acid exchange ($K^{57} \rightarrow A$) introduced in domain D1 (mut 1, mut 6–mut 9), which resulted in inhibition of reactivity to monoclonal antibody A7, also caused reduction in IgE binding in four out of the six sera. This indicated the importance of the putative linear B cell epitope D1 for IgE reactivity and further showed that replacement of the selected amino acid (K^{57}) was sufficient to reduce IgE recognition of Lol p 5. Our data further revealed the significant contribution of domain D2 to the IgE binding capacity of Lol p 5, since amino acid substitutions in this region (see mut 4) caused a notable reduction of IgE reactivity in almost all of the allergic patients sera tested (Fig. 2 A).

In contrast, mutants carrying amino acid substitutions (mut 2) or truncations (mut 3) at the C terminus of Lol p 5 exhibited IgE reactivity similar to wild-type rLol p 5 (Fig. 2 A) in most of the allergic patients tested. These data suggested that the C terminal part of Lol p 5 does not represent a major IgE binding epitope. Mutations in domain D3 (mut 5) resulted in variable changes of IgE reactivity depending on the sera used. The latter indicates the importance of D3 for IgE binding in a subset of patients.

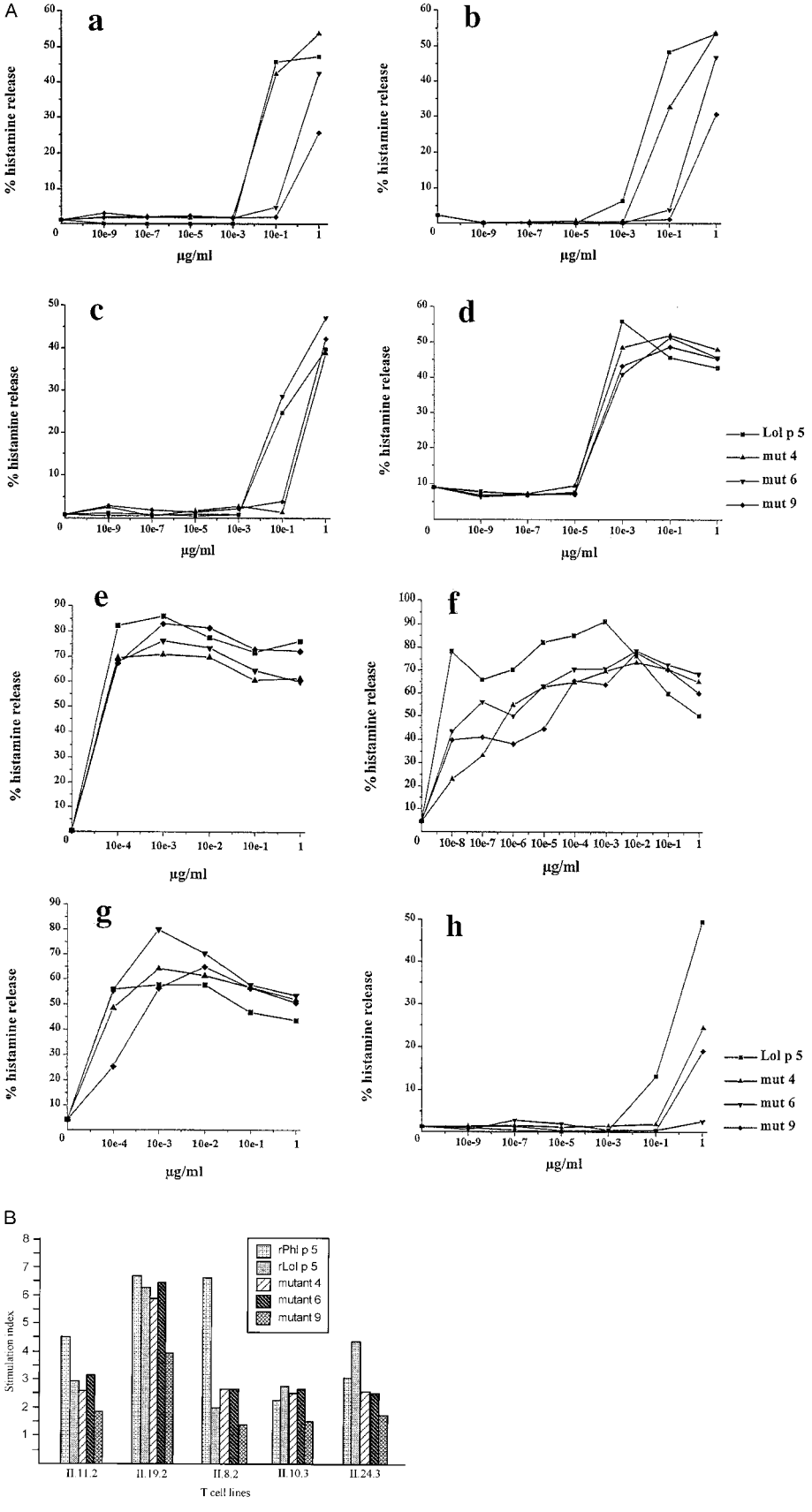
Two of the single mutants (mut 3 and mut 4) and two of the triple mutants (mut 6 and mut 9) were tested for IgE reactivity to non-denatured, dot-blotted proteins using sera from 39 additional grass pollen allergic patients. Mutants mut 4, mut 6 and mut 9 showed significantly lower IgE binding capacity than mut 3, which frequently even bound more IgE than rLol p 5 wildtype protein (Fig. 2 B).

The IgE binding capacity of mut 4, mut 6 and mut 9 was also analyzed in ELISA competition experiments, where reaction between proteins and IgE antibodies occurs in solution and which therefore closely reflects the *in vivo* situation. Thereby the mutants were compared with rLol p 5 wild-type protein regarding their ability to inhibit serum IgE binding to immobilized wild-type allergen. Seventeen of the sera tested by dot blot analysis were preadsorbed with an excess of the wild-type or the mutant proteins (mut 4, mut 6, mut 9) and then exposed to ELISA plate-bound wild-type Lol p 5. In Table 2 inhibition of IgE binding achieved after preincubation of the sera with the mutants is expressed as percentage inhibition obtained after preadsorption with the wildtype protein. Although individual IgE reactivity of different patients sera to the different mutants was observed, three mutants were identified (mut 4, mut 6, mut 9) which exhibited a much lower inhibition capacity than the wild-type protein. The percentage inhibition of IgE binding found for mutant 4 was in the range of 25.5 %–72.2 % (average 43.1 %), for mutant 6, 24.4 %–61.9 % (average 38.4 %) and for mutant 9, 30.9 %–96.6 % (average 56.4 %) of that of rLol p 5 wild-type protein (Table 2).

2.4 Reduced ability of rLol p 5 mutants to induce basophil histamine release

To analyze the biological activity of the Lol p 5 mutant proteins, granulocytes from eight grass pollen-allergic individuals were incubated with different concentrations of rLol p 5 wildtype and rLol p 5 mutant proteins (mut 4, mut 6, mut 9) (Fig. 3 A). We found that mut 4 induced less histamine release than rLol p 5 wild-type in 7 out of 8 patients (Fig. 3 A; patients b–h). However, in one patient, (Fig. 3 A; patient a) rLol p 5 and mut 4 induced comparable release of histamine. Mut 6 exhibited a strongly reduced activity in the basophil histamine release tests in four patients (Fig. 3 A; patients a, b, f and h). For these patients we observed a hundredfold or more reduction of allergenic activity but in certain patients mut 6 induced comparable (patients e and g) or even stronger (patient c) histamine release than rLol p 5 wild-type (Fig. 3 A). The histamine release induced by mut 9 was at least tenfold reduced as compared to rLol p 5 wild-type in each of the 8 patients tested, thus identifying mut 9 as a mutant with a consistent tenfold reduction of allergenic activity (Fig. 3 A).

Similar to the *in vitro* assays, we also found intersubject variability regarding the allergenic activity of rLol p 5 wild-type and mutants in the histamine release assay. Even the threshold concentration of wild-type rLol p 5 for the stimulation of peripheral basophils varied significantly between the patients and ranged between 10^{-9} μg/ml


A
a
b
c
d
e
f
g
h
% histamine release
µg/ml
Lol p 5
mut 4
mut 6
mut 9
B
rPhl p 5
rLol p 5
mutant 4
mutant 6
mutant 9
Stimulation index
II.11.2
II.19.2
II.8.2
II.10.3
II.24.3
T cell lines

◀ *Fig. 3.* Biological activity of Lol p 5 mutant. A, Induction of histamine release from basophils of grass pollen-allergic patients stimulated with purified rLol p 5 and rLol p 5 mutants (mut 4, mut 6, mut 9). Granulocytes of eight grass pollen-allergic patients were incubated with various concentrations (μg/ml) of purified recombinant allergens (x-axis) and the percentage of total histamine release is expressed on the y-axis. B, T cell proliferation by the Lol p 5 mutants. Induction of proliferation in Phl p 5-specific T-cell lines (TCL) by rPhl p 5, rLol p 5 and rLol p 5 mutants 4, 6, and 9.

(patient f) and 10^{-2} μg/ml (patients a, c and h). Nevertheless our results demonstrate that it was possible to select for each patient out of the panel of three mutants (mut 4, mut 6 and mut 9) one mutant with an at least tenfold reduced allergenic activity. Since mutant 9 showed a tenfold or more reduced allergenic activity in all patients tested, it may be considered as the most suitable candidate molecule for therapeutic applications.

2.5 Ability of recombinant Lol p 5 mutants to induce immediate-type skin reactions

The capacity of the wild-type Lol p 5 protein and of two of the single mutants (mut 3 and mut 4) and two of the triple mutants (mut 6 and mut 9) to elicit immediate skin reactions was evaluated in several allergic patients by *in vivo* skin prick tests. Fig. 4 and Table 3 show the results obtained with two of the grass pollen-allergic patients (b, c), a birch pollen-allergic patient (d) and a non-atopic individual (a). The two triple mutants, mut 6 and mut 9, exhibited reduced capacity to induce allergic skin reactions in all the grass pollen-allergic patients tested, including patients b and c (see Fig. 4 and Table 3). In contrast, the two single mutants, mut 3 and mut 4, induced wheal reactions comparable to those induced by rLol p 5 wild-type in some of the patients (e. g. patient b), but caused reduced reactions in other patients (e. g. patient c). These results again reflect the variability in intersubject reactivity to certain of the mutant allergens. No false positive skin reaction was observed when a birch pollen-allergic individual, without grass pollen-allergy and a non-atopic individual were skin prick tested with the proteins. Timothy grass pollen and birch pollen extract induced immediate skin reactions in sensitized patients, but not in the non-atopic individual (Fig. 4, Table 3), and histamine hydrochloride caused wheal and flare reactions in all the individuals tested (Fig. 4, Table 3).

Table 2. Inhibition of IgE reactivity to rLol p 5 after preincubation of sera with Lol p 5 mutants as determined by ELISA

Patient	mut 4	mut 6	mut 9
1	39.9 %	36.6 %	39.7 %
2	50.4 %	43.3 %	49.4 %
3	44.9 %	42.6 %	63.3 %
5	54.0 %	49.5 %	54.3 %
10	31.4 %	28.5 %	63.3 %
13	25.6 %	24.4 %	30.9 %
16	28.8 %	26.6 %	41.5 %
18	59.3 %	52.0 %	51.6 %
21	56.5 %	27.6 %	33.0 %
22	44.4 %	35.3 %	42.5 %
23	41.0 %	42.2 %	96.6 %
25	72.2 %	61.9 %	76.5 %
27	25.9 %	24.8 %	58.9 %
29	43.2 %	45.0 %	73.8 %
31	37.3 %	42.4 %	73.6 %
32	47.7 %	42.9 %	35.2 %
36	30.0 %	27.7 %	74.8 %
average inhibition	43.1 %	38.4 %	56.4 %

2.6 Purified rLol p 5 and rLol p 5 mutants induce proliferative responses in Phl p 5-specific T cell lines and clones

Five T cell clones with specificity for different epitopes of the Lol p 5-homologous allergen from timothy grass, Phl p 5 [13], were exposed to purified rPhl p 5b, rLol p 5, mut 3, and mut 4 and mut 9 (Table 4). All five Phl p 5b-specific T cell clones showed significant proliferative responses when exposed to rLol p 5 (Table 4). Proliferation indices obtained with rLol p 5 ranged between 1.87 and 25.16 and were of the same magnitude as those obtained with Phl p 5, indicating that rLol p 5 contained the corresponding T cell epitopes of Phl p 5 (Table 4). Mutations introduced in mut 3, mut 4 and mut 9 did not affect the T cell epitopes recognized by four of the clones (II10.3 : 21E7, II19.1 : 10C6, II19.1 : 21D9 and II17.3 : 12F5) and mut 3, mut 4 and mut 9 indeed induced proliferative responses comparable to Lol p 5 in these four T cell clones (Table 4). However, the epitope of mut 9

Table 3. Induction of immediate skin reactions with rLol p 5 and rLol p 5 mutants[a)]

Individual	1 μg/ml					5 μg/ml								
	rLol p 5	mut 3	mut 4	mut 6	mut 9	rLol p 5	mut 3	mut 4	mut 6	mut 9	timothy	birch	histamine	NaCl
a	0	0	0	0	0	0	0	0	0	0	0	0	56.76	0
b	19.6	15.9	19.6	0	7.1	28.3	28.3	33.2	1.8	15.9	33.2	0	19.6	0
c	38.5	19.6	15.9	15.9	15.9	56.76	50.3	33.2	28.3	23.8	122.8	4.9	15.9	0
d	0	0	0	0	0	0	0	0	0	0	0	33.2	23.8	0

a) A non-atopic individual (a), two grass pollen-allergic patients (b, c) and a birch pollen allergic-patient without anti-rLol p 5-specific IgE antibodies (d) were skin prick tested with recombinant allergens (wild type rLol p 5, mutant 3, mutant 4, mutant 6, and mutant 9), timothy grass and birch pollen extract, histamine hydrochloride and sodium chloride. The mean wheal areas (mm^2) are displayed.

recognized by clone II17.3:19A1 contained a single amino acid exchange. Obviously this mutation did not affect the proliferative response of II17.3:19A1, because the T cell clone was still able to proliferate after stimulation with mut 3, mut 4 and also mut 9.

We further studied whether rLol p 5 wildtype and rLol p 5 mutants were able to induce proliferative responses in Phl p 5-specific T cell lines. Four of the Phl p 5-specific T cell lines showed proliferative responses to rLol p 5 which were comparable to those obtained with rPhl p 5 whereas one (II.8.2) showed weaker response to rLol p 5 than to Phl p 5 (Fig. 3 B). All mutants tested (mut 4, mut 6 and mut 9) were able to induce lymphoproliferative responses in almost all of the Phl p 5-specific T cell lines albeit sometimes to a lower magnitude than rLol p 5 wildtype protein (Fig. 3 B).

3 Discussion

We demonstrate that it is possible to reduce the allergenic activity of one of the most potent environmental allergens, the major rye grass allergen, Lol p 5, by few point mutations which leave the overall structural fold of the molecule unaltered. CD analysis demonstrated that rLol p 5 wild-type and rLol p 5 mutants exhibited a similar fold containing a considerable amount of α-helical structure.

In contrast to a previous study where deletion of IgE-reactive domains was necessary to reduce the allergenic activity of the group 5 allergen from timothy grass, Phl p 5 [20], we found that the exchange of a few amino acids within the IgE-reactive domains of Lol p 5 could reduce IgE recognition. For example, a considerable loss

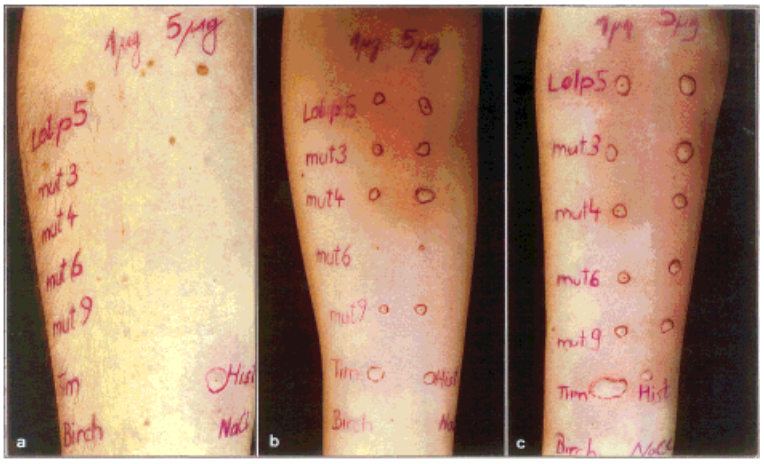


Fig. 4. Induction of immediate skin reactions with rLol p 5 and rLol p 5 mutants. Two grass pollen-allergic individuals (b, c) and a non-atopic individual (a), were skin prick tested with two different concentrations (1 μg/ml; 5 μg/ml) of recombinant allergens (Lol p 5, mut 3, mut 4, mut 6 and mut 9), timothy grass pollen (Tim), birch pollen extract (Birch), histamine hydrochloride (Hist) and sodium chloride (NaCl). The wheal reaction is marked with a ball point pen.

Table 4. Induction of proliferation in Phl p 5-specific T-cell clones (TCC) by rPhl p 5, rLol p 5 and rLol p 5 mutants 3, 4, and 9

	Stimulation indices				
TCC	II.17.3:19A1	II.10.3:21E7	II.19.1:10C6	II.19.1:21D9	II.17.3:12F5
Epitope	aa 193–204	aa 31–45	aa 106–117	aa 238–249	aa 160–174; aa 208–221
rPhl p 5	1.72	4.15	28.90	5.08	1.80
rLol p 5	1.87	3.19	25.16	3.72	3.60
mut 3	2.07	5.96	23.58	4.52	3.98
mut 4	2.20	2.86	35.33	5.37	1.96
mut 9	1.96	3.39	37.46	6.19	1.78

a) Stimulation indices are indicated for five designated T cell clones. The Phl p 5 epitopes that these clones recognize are indicated.

of IgE reactivity was found in case of mutant 4, in which four contiguous amino acids, KFTV, were replaced by the altered sequence, NLAA. This was a surprising result, considering that out of a total of 301 amino acid residues of Lol p 5, only four had been modified. The observation that many of the allergic sera exhibited reduced IgE reactivity to mutant 4 suggests that the IgE epitope disrupted by the four-amino acid replacement represents one of the immunodominant B cell epitopes on the Lol p 5 molecule. Mutants 6 to 9 carried multiple mutations, as depicted in Fig. 1 B, and exhibited strongly reduced IgE binding. It was, however, remarkable to note that the reduction in IgE binding capacity of the triple mutants (mut 6 to mut 9) was not significantly higher than that of mutant 4, where only a single domain had been altered. Furthermore, as observed for short synthetic peptides [16], intersubject variability of IgE binding was also found with the different Lol p 5 mutants.

The lowered IgE reactivity of the mutants also resulted in a reduction of their allergenic activity. Using basophil histamine release experiments and skin prick testing in grass pollen-allergic patients we found that those mutants which had low IgE binding capacity (*i. e.* mut 4, mut 6, mut 9) exhibited reduced biological activity. It was thus possible to identify at least one out of the three mutants that exhibited a tenfold or more reduction of histamine release activity. The most consistent reduction of allergenic reactivity was observed for mut 9 which in all 8 patients tested in the basophil histamine release had an at least tenfold reduction of allergenic activity as compared to rLol p 5 wild type.

The initiation and maintenance of allergic reactions are strongly influenced by allergen-specific T cell responses that not only regulate IgE production but also affect the activation and differentiation of allergic effector cells [21]. The regulation of such T cell responses has been suggested as an effective therapeutic approach to treat allergies [22]. One such approach is to use T cell epitope, peptide-based therapy. Unfolded peptides have the advantage that they are unable to cross-link allergen-specific IgE and the IgE receptor on the surface of mast cells and basophils [23, 24], and are thus unable to induce the release of histamine and IL-4 [25]. Initial clinical trials involving immunotherapy of cat-allergic patients with Fel d 1 peptides were reported, giving some promising results. However, Simons et al. [26] have reported that Fel d 1 peptide therapy did not reduce immediate or late phase skin reactivity in cat-allergic individuals. We suggest that mutant allergens retaining the overall fold of the wild-type allergen and comprising a more complete repertoir of T cell epitopes may offer advantages over T cell peptide-based immunotherapy. In contrast, on complete allergens, multiple T cell epitopes are recognized individually by different patients, due to the large diversity of major histocompatibility complex (MHC) and T cell receptors (TCR) which exist among individual patients [4, 26, 27]. The Lol p 5 mutants (mut 4, mut 6, and mut 9) described in the present study exhibited reduced allergenic activity but maintained the ability to induce lymphoproliferative responses in Phl p 5-specific T cell lines and T cell clones. It may thus be expected that Lol p 5 mutants could also be used for the treatment of allergies to Lol p 5-related allergens from other grass species. Several advantages are expected from the treatment with Lol p 5 mutants. Whereas allergen extracts represent complex mixtures of difficult, if not impossible to standardize, allergenic and non-allergenic components, it will be possible to select patients for reactivity to rLol p 5 in order to allow patient-tailored treatment according to the individual sensitization profiles. The reduction of

allergenic activity of the Lol p 5 mutants (*e. g.* mut 9) will likely reduce the rate of anaphylactic side effects compared to extract-based treatment by a factor of ten. It may well be considered that administration of the Lol p 5 mutants at higher doses than the levels of group 5 allergens currently present in immunotherapeutic grass pollen extracts may increase the efficacy of immunotherapy while keeping the rate of side effects at a comparable level. In view of the high allergenic activity and immunogenicity of group 5 allergens, we believe that the simultaneous reduction of IgE reactivity and preservation of the molecular fold represents a novel approach towards the design of hypoallergenic allergen variants. Our results thus contribute to the concept of using low-allergenic forms of major pollen allergens for allergen-specific immunotherapy, and thus to the development of safe and effective forms of allergy treatment.

4 Materials and methods

4.1 Characterization of grass pollen-allergic patients

Grass pollen allergy was diagnosed on the basis of case history, skin prick testing and determination of specific IgE to grass pollen extracts (CAP-RAST measurements, Pharmacia, Sydney, Australia) or to purified recombinant grass pollen allergens [28]. Sera from patients with and without grass pollen-specific immunotherapy were included in the serological analyses.

4.2 Generation, expression and purification of recombinant Lol p 5 and rLol p 5 mutants

Point mutations were introduced into the Lol p 5 gene using a QuickChange site-directed mutagenesis kit (Stratagene, East Kew, Australia) according to the manufacturer's instructions. Primers (Table 5) were designed to mutate Lol p 5 at specific sites (Fig. 1 A). The coding sequences of Lol p 5 and its derivatives were introduced in-frame into the expression vector pQE31 (Qiagen, Clifton Hill, Australia). This vector allows expression of recombinant proteins with an N-terminal, hexa-histidine tag. Expression and harvesting of the proteins was carried out as outlined in the QIAexpressionist manual (Qiagen, Clifton Hill, Australia). Histidine-tagged proteins were purified using TALON metal affinity resins (Clontech, Palo Alto, CA).

4.3 CD analysis of rLol p 5 and rLol p 5 mutants

CD measurements were performed in MilliQ water pH 7.3 with concentrations in the range of 2.69×10^{-6} M–3.74×10^{-6} M. The investigations were carried out on a Jasco J-715 spectropolarimeter using a 0.1 cm pathlength cell equilibrated at 20 °C. Spectra were recorded with a 0.2 nm resolution at a scan speed of 50 nm/min and resulted from averaging three scans. The final spectra were baseline-corrected by subtracting the corresponding MilliQ spectra obtained under identical conditions. Results were expressed as the mean residue ellipticity [Θ] at a given wavelength. The data were fitted with the secondary structure estimation program J-700 (J-700 for Windows Secondary Structure Estimation, version 1.10.00, copyright 1993-94 JASCO Corp.) according to Yang et al. [29] using the finite-mode fitting procedure.

4.4 SDS-PAGE, immunoblot and dot blot analysis

For SDS-PAGE analysis, 1.3 μg purified recombinant proteins were separated on 15 % polyacrylamide gels (BioRad mini-Protean II cell, BioRad, Syndey, Australia). Gels were blotted onto nitrocellulose membranes (Schleicher & Schuell, Germany) using a Bio-Rad mini-Protean II cell. Membranes were either probed with a 1 : 5 diluted monoclo-

Table 5. The mutagenesis primers used in this study

Name	Sequence (5' → 3')
D1 fwd	CCTCCGGCGGACGCGTTCAAGATC
D1 rev	GATCTTGAACGCGTCCGCCGGAGG
D2 fwd	GCTGCTGGTGCCTACGCAGCCTGATCAGC
D2 rev	GCTGATCAGGCTGCGTAGGCACCAGCAGC
D3 fwd	CCACCGCCGCTGCTTGAGGCTACAAAGC
D3 rev	GCTTTGTAGCCTCAGGCAGCGGCGGTGG
D4 fwd	CCACCAACGATAACTTGGCCGCCGCCTTCGAGAGTGC
D4 rev	GCACTCTCGAAGGCGGCCAAGTTATCGTTGGTGG
D5 fwd	CCTCGAGGCCGGGGCCGCGCAGGCCTACG
D5 rev	CGTAGGCCTGCGCGGCCCCGGCCTCGAGG

nal anti-Lol p 5 antibody (A7) [11], a 1:50 diluted rabbit serum raised against rLol p 5 or 1:5 diluted patient sera. Antibody reactivity of blotted proteins was analysed as described by Singh et al. [11]. For dot blot analysis, 1.5-ml aliquots containing 0.15 μg of the recombinant proteins were dotted in duplicates on nitrocellulose membranes (Schleicher & Schuell). Membranes were then exposed to 1:10 diluted patient sera or, for control purposes, to serum from a non-allergic individual. The 1:10 serum dilution was chosen after pilot serum titration experiments to ensure excess of immobilized antigen. Bound IgE antibodies were detected with ^{125}I-labeled anti-human IgE (RAST, Pharmacia, Uppsala, Sweden) as described by Singh et al. [11].

4.5 ELISA competition experiments

For ELISA inhibition experiments, sera were diluted 1:5 in Tris-buffered saline containing 0.05 % Tween-20 and 0.05 % BSA (TBST, 0.5 % BSA) and preabsorbed with 7 μg/ml of either rLol p 5 wild-type, rLol p 5 mutants or, for control purposes with BSA, at 4 °C overnight. The serum dilution and concentration of competitor was chosen according to pilot titration experiments to ensure an excess of the competitor. ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with rLol p 5 (4 μg/ml in 0.1 M sodium bicarbonate pH 9.6) at 4 °C overnight. Plates were washed with TBST, blocked with 1 % BSA in TBST (200 μl/well) at 37 °C for 3 h and incubated overnight at 4 °C with 100 ml/well of the preabsorbed sera. After washing with TBST (200 μl/well), an alkaline phosphatase-coupled mouse monoclonal anti-human IgE antibody (Pharmingen, San Diego, CA) diluted 1:1000 in TBST, 0.5 % BSA was used to detect bound IgE antibodies. Following incubation for 1 h each at 37 °C and 4 °, plates were washed with TBST and the color reaction was started with an alkaline phosphatase substrate (Sigma Diagnostics, St. Louis, MO). The absorbance was measured in an ELISA reader (Dynatech, Denkendorf, Germany) at 405 nm. All determinations were carried out in duplicate and results are expressed as mean values. Inhibition of IgE binding after preincubation of the sera with the mutants is expressed as the percentage of the inhibition achieved by preincubation with the wildtype protein.

4.6 Histamine release experiments

Granulocytes were isolated from heparinized blood samples of grass pollen-allergic individuals and a non-atopic individual [30]. Cells were incubated with various concentrations of wild-type rLol p 5 and rLol p 5 mutants or, for control purposes, with a monoclonal anti-human IgE antibody (Immunotech, Marseille, France). Histamine released into the supernatant was measured by radioimmunoassay (Immunotech, Marseille, France). Total histamine was determined after freeze thawing of the cells. Results are expressed as mean values of triplicate determinations, and represent the percentage of total histamine.

4.7 Skin prick tests

After written consent was obtained from the volunteers, skin prick tests were conducted in accordance with the recommendations of the European Academy of Allergy and Clinical Immunology [31]. Skin prick tests were performed with 20-μl aliquots of two concentrations (1 μg/ml); 5 μg/ml) of the recombinant proteins diluted in PBS.

4.8 T cell proliferation assays

T cell lines and clones with specificity for the Lol p 5-homologous allergen from timothy grass, Phl p 5, were established from PBMC of grass pollen-allergic patients as described [32]. T cell epitopes recognized by the clones were determined with synthetic 12-mer peptides spanning the complete Phl p 5b sequences as described [32]. For the proliferation assay, T cells from each line or clone were seeded at a density of 2×10^4 cells/well in a 96-well culture plate in triplicate under stimulation with the allergens (rPhl p 5b, rLol p 5, rLol p 5 mutants) in concentrations of 10 μg/ml and 5×10^4 irradiated autologous PBMC/well. After 48 hours of culture, 1 μCi [^{3}H] thymidine (Amersham, Freiburg, Germany) was added to each well and cells were incubated for additional 16 h. Cells were then harvested using a 96-well cell harvester (Wallac ADL, Freiburg, Germany) and counts per minute (cpm) were measured in a microbeta scintillation counter. The stimulation index (SI) was calculated as the quotient of the cpm obtained by allergen stimulation (T cells, irradiated autologous PBMC and allergen) and the unstimulated control (T cells and irradiated autologous PBMC without allergen).

Acknowledgements: Financial assistance from International Collaboration Program of The University of Melbourne and the Australian Research Council is gratefully acknowledged. We thank Ute Siemann and Minoo Gannadan for excellent technical assistance. The Austrian team was supported in part by grants Y078 GEN, F01804, F01805, F01809, F01811 of the Austrian Science Fund and by the ICP program of the Austrian Federal Ministry for Education, Science and Culture.

References

1 **Miyamoto, T.,** Increased prevalence of pollen allergy in Japan. In **Godard, P., Bousquet, J. and Michel F. B.** (Eds.) *Advances in Allergology and Clinical Immunology.* The Parthenon Publishing Group, Cornforth. 1992, pp 434–437.

2 **Bousquet, J., Lockey, R., Malling, H. J., et al.,** Allergen Immunotherapy: Therapeutic vaccines for allergic diseases. A WHO position paper. *J. Allergy Clin. Immunol.* 1998. **102:** 558–562.

3 **Durham, S. R. and Till, S. J.,** Immunologic changes associated with allergen immunotherapy. *J. Allergy Clin. Immunol.* 1998. **102:** 157–164.

4 **Ebner, C., Siemann, U., Bohle, B., Willheim, M., Wiedermann, U., Schenk, S., Klotz, F., Ebner, H., Kraft, D. and Scheiner, O.,** Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clone specific for Phl p 1, a major grass pollen allergen. *Clin. Exp. Allergy* 1997. **27:** 1007–1015.

5 **Ball, T., Sperr, W. R., Valent, P., Lidholm, J., Spitzauer, S., Ebner, C., Kraft, D. and Valenta, R.,** Induction of antibody responses to new B-cell epitopes indicates vaccination character of allergen immunotherapy. *Eur. J. Immunol.* 1999. **29:** 2026–2036.

6 **Vrtala, S., Hirtenlehner, K., Vangelista, L., Pastore, A., Eichler, H.-G., Sperr, W., Valent, P., Ebner, C., Kraft, D. and Valenta, R.,** Conversion of the major birch pollen allergen, Bet v 1 to two non-anaphylactic T-cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J. Clin. Invest.* 1997. **99:** 1673–1681.

7 **Zeiler, T., Taivainen, A., Rytkonen, M., Rautiainen, J., Karjalainen, H., Mantyjarvi, R., Tuomisto, L. and Virtanen, T.,** Mapping of the antigenic epitopes of Lol p VB using gene fragmentation. *J. Allergy Clin. Immunol.* 1997. **100:** 721–727.

8 **Smith, A. M. and Chapman, M. D.,** Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. *Mol. Immunol.* 1996. **33:** 399–405.

9 **Takai, T., Yokota, T., Yasue, M., Nishiyama, C., Yuuki, T., Mori, A., Okudaira, H. and Okumura, Y.,** Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. *Nat. Biotech.* 1997. **15:** 754–758.

10 **Okada, T., Swoboda, I., Bhalla, P. L., Toriyama, K. and Singh, M. B.,** Engineering of hypoallergenic mutants of the *Brassica* pollen allergen, Bra r 1, for immunotherapy. *FEBS Lett.* 1998. **434:** 255–260.

11 **Singh, M. B., Hough, T., Theerakulpisut, P., Avjioglu, A., Davies, S., Smith, P. M., Taylor, P., Simpson, R. J., Ward, L. D., McCluskey, J., Puy, R. and Knox, R. B.,** Isolation of cDNA encoding newly identified major allergenic protein of ryegrass pollen: intracellular targeting to the amyloplast. *Proc. Natl. Acad. Sci. USA* 1991. **88:** 1384–1388.

12 **Ong, E. K., Griffith, I. J., Knox, R. B. and Singh, M. B.,** Cloning of a cDNA encoding a group-V (group-IX) allergen isoform from ryegrass pollen that demonstrates specific antigenic immunoreactivity. *Gene* 1993. **134:** 235–240.

13 **Vrtala, S., Sperr, W., Reimitzer, I., van Ree, R., Laffer, S., Muller, W.-D., Valent, P., Lechner, K., Rumpold, H., Kraft, D., Scheiner, O. and Valenta, R.,** cDNA cloning of a major allergen from timothy grass (*Phleum pratense*) pollen; characterization of the recombinant Phl p V allergen. *J. Immunol.* 1993. **151:** 4773–4781.

14 **Niederberger, V., Laffer, S., Froeschl, R., Kraft, D., Rumpold, H., Kapiotis, S., Valenta, R. and Spitzauer, S.,** IgE antibodies to recombinant pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Bet v 2) account for a high percentage of grass pollen-specific IgE. *J. Allergy Clin. Immunol.* 1998. **101:** 258–264.

15 **Ong, E. K., Knox, R. B. and Singh, M. B.,** Mapping of the antigenic epitopes of Lol p VB using gene fragmentation. *Mol. Immunol.* 1995. **32:** 295–302.

16 **Suphioglu, C., Blaher, B., Rolland, J. M., McCluskey, J., Schappi, G., Kenrick, J., Singh, M. B. and Knox, R. B.,** Molecular basis of IgE-recognition of Lol p 5, a major allergen of ryegrass pollen. *Mol. Immunol.* 1998. **35:** 293–305.

17 **Blaher, B., Suphioglu, C., Knox, R. B., Singh, M. B., McCluskey, J. and Rolland, J. M.,** Identification of T-cell epitopes of Lol p 9, a major allergen of ryegrass (*Lolium perenne*) pollen. *J. Allergy Clin. Immunol.* 1996. **98:** 124–132.

18 **Rost, B.,** PHD: predicting one dimensional protein structure by profile-based neural networks. *Methods Enzymol.* 1996. **266:** 525–639.

19 **Rost, B. and Sander, C.,** Prediction of protein secondary structure at better than 70 % accuracy. *J. Mol. Biol.* 1993. **232:** 584–599.

20 **Schramm, G., Kahlert, H., Suck, R., Weber, B., Stüwe, H.-T., Müller, W.-D., Bufe, A., Becker, W.-M., Schlaak, M. W., Jäger, L., Cromwell, O. and Fiebig, H.,** "Allergen engineering": variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. *J. Immunol.* 1999. **162:** 2406–2414.

21 **van Neerven, R. J. J., Ebner, C., Yssel, H., Kapsenberg, M. L. and Lamb, J. R.,** T-cell responses to allergens: epitope specificity and clinical relevance. *Immunol. Today* 1996. **17:** 526–532.

22 **Exley, M. A., Rogers, B. L. and Griffith, I. R.,** The use of peptides for allergy immunotherapy. In: **Roberts, A. M. and Walker, M. B. (Eds.)** *Allergic Mechanism and immunotherapeutic strategies. Allergic Mechanism and immunotherapeutic strategie* John Wiley and Sons Ltd. 1997. pp. 151–176.

23 **Metzger, H., Alcaraz, G., Hohman, R., Kinet, J.-P., Pribluda, V. and Quatro, R.,** The receptor with high affinity for immunoglobin E. *Annu. Rev. Immunol.* 1986. **4:** 419–470.

24 **Davis, M. M.,** T-cell receptor gene diversity and selection. *Annu. Rev. Biochem* 1990. **59:** 475–496.

25 **Brunner, T., Heusser, C. H. and Dahinden, C. A.,** Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J. Exp. Med.* 1993. **177:** 605–611.

26 **Simons, F. E. R., Imada, M., Li., Y., Watson, W. T. A., HayGlass, K. T.,** Fel d 1 peptides: Effects on skin tests and cytokine synthesis in cat-allergic human subjects. *Int. Immunol.* 1996. **8:** 1937–1945.

27 **O'Brien, R. M., Thomas, W. R., Nicolson, I., Lamb, J. R. and Tait, B. T.,** An immunogenic analysis of the T-cell recognition of the major house dust mite allergen Der p II: identification of high and low-responder HLA-DQ alleles and localization of T-cell epitopes. *Immunology* 1995. **86:** 176–182.

28 **Vrtala, S., Susani, M., Sperr, W. R., Valent, P., Laffer, S., Dolecek, C., Kraft, D. and Valenta, R.,** Immunologic characterization of purified recombinant timothy grass pollen (*Phleum pratense*) allergens. *J. Allergy Clin. Immunol.* 1996. **97:** 781–787.

29 **Yang, J. T., Wu, C. S. and Martinez, H. M.,** Calculation of protein conformation from circular dichroism. *Methods Enzymol.* 1986. **130:** 208–269.

30 **Valent, P., Besemer, J., Muhm, M., Majdic, O., Lechner, K. and Bettelheim, P.,** Interleukin 3 activates human blood basophils via high-affinity binding sites. *Proc. Natl. Acad. Sci. USA* 1989. **86:** 5542–5546.

31 **Dreborg, S.,** Skin-prick test. *Allergy* 1985. **40:** 55–60.

32 **Kahlert, H., Stüwe, H. T., Cromwell, O. and Fiebig, H.,** Reactivity of T-cells with grass pollen extract and allergoid. *Int. Arch. Allergy Immunol.* 1999. **120:** 146–157.

Correspondence: Mohan B. Singh, Plant Molecular Biology and Biotechnology Laboratory, Institute of Land and Food Resources, The University of Melbourne, Parkville, Vic 3010, Australia
Fax: +61-3-8344 05051
e-mail: mohan@unimelb.edu.au