

Genetically Engineered Plant Allergens with Reduced Anaphylactic Activity

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Key Words

Type-I allergy · Immunotherapy · Recombinant allergens · Site-directed mutagenesis · Pollen allergens · T-cell epitopes · IgE epitopes

Abstract

Allergy immunotherapy is based on the administration of increasing amounts of the disease-eliciting allergens in order to yield allergen-specific non-responsiveness. Success of this therapy is associated with modulation of the immune response to allergenic molecules at the level of T-helper cells and the induction of blocking antibodies. The extracts used for immunotherapy are highly heterogeneous preparations from natural sources and contain additional components, mostly proteins which are not well defined. Recombinant DNA technology offers novel tools for production of pure and well-characterised allergens for specific immunotherapy. However, high IgE reactivity of pure recombinant allergens is associated with an increased risk of potentially life-threatening anaphylactic reactions. A major improvement in allergen-specific immunotherapy may be achieved by using genetically engineered recombinant allergens with reduced anaphylactic activity. Recently the site-directed mutagenesis technique has been applied suc-

cessfully to produce variants of major grass, birch and oilseed rape allergens with reduced IgE reactivity but retained T-cell reactivity. These modified allergens with reduced anaphylactic potential are novel candidates for safer and more effective allergen-specific immunotherapy.

Introduction

Allergen immunotherapy (IT) involves hyposensitisation by repeated parenteral injections of allergen extracts [1, 2] since 1911 [3]. IT has been shown to be effective in a number of clinical trials involving atopic individuals allergic to house dust mite [4], domestic cat [5], grass pollen [6] and ragweed pollen [7]. IT with grass-pollen extracts showed a significant decrease in antigen-induced eosinophil migration into the nasal cavity [8]. Furthermore, specific IT with grass-pollen extracts led to a decrease in the production of histamine-releasing factor by mononuclear cells in grass pollen-allergic individuals [9]. Specific IT in ragweed-allergic individuals decreased antigen-specific serum IgE and increased antigen-specific IgG [10]. IT is also associated with an increase in IgG and IgA found in the nasal secretions of allergic individuals [11].

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It has been proposed that elevated levels of 'blocking IgG' antibody are involved in IgE-mediated amelioration of allergic symptoms [12]. However, a number of studies also reported no increase in IgG levels that correlate with effective IT [2]. Recently, the function of CD4⁺ T-helper (Th) cells in regulating the allergic response has generated great interest [13]. Previously it was demonstrated that Th2 cells produce interleukin-4 (IL-4) required for induction of IgE synthesis [14]. In contrast Th1-type cells produce IL-2 and interferon- γ (IFN- γ). It is now considered that the ratio of the major IgE-switch factor, IL-4, and its regulator, IFN- γ , determines the level of IgE production. The parenteral administration of allergen is associated with an initial increase and then subsequent decrease in allergen-specific serum IgE and a decrease in mast cell and basophil activities [2]. Allergen IT also results in changes of the cytokine profile of T cells in the respiratory tract [15]. Despite the vast amount of existing knowledge on the immunobiology of the allergic response, none of the described mechanisms has been conclusively shown to be singularly responsible for the efficacy of allergen IT.

Currently available IT is based on crude protein extracts derived from natural allergen sources [1, 2]. Although clinical benefit for most allergic individuals has been observed with the current form of IT, it should be noted that severe side effects were also observed. The physiological events that lead to the development of the clinical symptoms of immediate hypersensitivity in a sensitive individual must be altered during IT for the procedure to be successful. The current form of pollen-allergy IT is associated with the risks of anaphylaxis and development of sensitisation to minor allergens. The use of modified, non-anaphylactic plant allergens for IT would overcome these problems.

The chemical modification of allergens to reduce the risk of anaphylaxis was first introduced by Marsh et al. [16]. The chemically modified allergens termed 'allergoids' were produced by polymerisation of allergens using formaldehyde [17] or glutaraldehyde [18]. In mice, allergoids obtained by polymerisation can down-regulate IgE [19]. Norman et al. [20] studied the efficacy and safety of long-term allergoid treatment and reported significant IgG antibody responses in ragweed-allergic patients. However, the chemical modification of allergens is difficult to define and standardise [11]. Moreover, the allergoids produced by a chemical polymerisation of crude protein extracts contain other irrelevant proteins in addition to allergens. In this review we will focus on genetic engineering strategies being developed to modulate recombinant plant allergens for reduced anaphylactic potential.

Recombinant Plant Allergens

Molecular cloning of allergen genes led to determination of the nucleotide sequences and, indirectly, the amino acid sequences of these allergens. Allergen genes were cloned using various approaches, including screening of cDNA expression libraries with monoclonal antibodies and/or IgE from allergic patient sera [21], screening cDNA libraries with degenerate oligonucleotide probes or homologous probes. In addition, polymerase chain reaction (PCR) using degenerate oligonucleotide primers is also being used to clone allergen cDNAs. Molecular cloning has made possible the production of large amounts of pure recombinant allergens. These recombinant allergens are now being used to assess IgE reactivity. In some cases the IgE reactivity of the recombinant protein could not be distinguished from that derived from the natural allergen [22]. Recombinant birch and grass pollen allergens have been shown to retain high IgE reactivity [22, 23]. This high IgE reactivity which makes recombinant-expressed allergens suitable for allergy diagnosis is detrimental for their use in IT. Because of the purity of the recombinant allergen preparations, their anaphylactogenic potential is expected to be very high even at very low doses. Mohapatra et al. [24] suggested the chemical modification of the recombinant allergens (r-allergoids) as a means of reducing IgE reactivity. As with allergen extracts from natural sources, chemical modification of recombinant allergens is difficult to standardise and unlikely to be used in IT.

Purified recombinant proteins were used to analyse the three-dimensional structures of allergens [25, 26]. The availability of nucleotide and amino acid sequences of allergens have made it possible to search sequence homologies, and to map B- and T-cell epitopes using recombinant allergen fragments and synthetic peptides [27–29]. The development of PCR cloning techniques has made possible the rapid selection of large numbers of distinct clones encoding the same allergen. The cloning of multiple allergen genes indicated that major allergens could be encoded by families of genes. The major ragweed pollen allergen, Amb a 1, is encoded by a family of four closely related genes [30, 31]. Similarly major grass [30, 32–34] and birch pollen allergens [35] have been shown to be encoded by multigene families. Each isoallergen may show unique antigenic reactivity. For the birch pollen allergen, Bet v 1, it was shown that the isoforms can have different IgE or T-cell reactivities [36]. The observation of differences in IgE reactivities has led to the expectation that low IgE-reactive forms can potentially be used for IT. Ferreira et al. [36] examined this possibility with the major birch pollen allergen, Bet v 1.

These investigators identified a Bet v 1 isoform, Bet v 1d, that has low IgE reactivity but with high T-cell stimulatory capacity. It has been suggested that since Bet v 1 binds IgE in >95% of all birch pollen-allergic patients, treatment with high doses of this hypoallergenic form can potentially be used for birch pollen IT with a reduced risk of anaphylactic side effects [37]. Such low IgE-reactive forms have not been identified for other pollen allergens. Two isoforms of the major ryegrass allergen, Lol p 5a and b, show antigenic differences in the sense that Lol p 5b is not recognised by mAb A7 [32]. However, in spite of sequence divergence and differential mAb reactivity, both isoforms show high IgE reactivity. The binding site of mAb A7 on Lol p 5a has mapped on a 7 amino acid linear sequence. The corresponding sequence on Lol p 5b differs by a single amino acid indicating that this single amino acid may be critical for mAb recognition of Lol p 5a. The site-directed mutagenesis to exchange this single amino acid (Lys57) in Lol p 5a resulted in loss of IgE recognition of this isoform as well [38]. Thus the study of amino acid sequences and immunoreactivity of different allergen isoforms has provided valuable information that can be used to identify the sites in the allergen molecules which represent potential targets for site-directed mutagenesis for production of low IgE-reactive variants. Site-directed mutagenesis has been used successfully to produce low IgE-reactive recombinant forms of birch, grass and oilseed rape pollen allergens. We will now discuss these studies outlining the rationale and outcomes of engineering these allergenic proteins.

Non-Anaphylactic Forms of the Major Birch Pollen Allergen, Bet v 1

A member of the *Fagales* order, white birch (*Betula verrucosa*) is grown widely around the world, predominantly in the northern hemisphere. Sensitisation to tree pollen allergens, including those from birch contribute significantly to the development of allergies in sensitive subjects [39]. Although up to 12 birch pollen proteins are recognised by IgE from allergic individuals [40, 41], only 4 have been cloned and characterized. The first cloned birch allergen was the group-1 protein, Bet v 1 [35]. The major birch pollen allergen, Bet v 1, is recognised by IgE from 95% of birch pollen-allergic patients, with 60% binding exclusively to Bet v 1 [41]. A function has yet to be defined for Bet v 1; however, it shares amino acid sequence homology with plant pathogenesis-related proteins [35] and has been shown to possess RNAase activity [42]. The 24 isoforms of Bet v 1 identified differ in the level of binding to specific

Bet v 1 monoclonal antibodies and IgE from birch pollen-allergic individuals [43–45]. Bet v 2 is a profilin, an actin-binding protein, recognised by 10–20% of patients allergic to tree pollen [46–47]. Both Bet v 1 and Bet v 2 are strongly cross-reactive with pollen allergens from other trees in the *Fagales* order [48–51]. Since Bet v 1 and Bet v 2 bind more tree pollen-specific IgE than any other pollen allergen from hazel, oak, birch, alder or hornbeam, they appear to be the most allergenic pollen proteins from these trees. Together, these two allergens harbor most of the IgE epitopes of the *Fagales* pollen allergens [52], indicating that these birch pollen allergens alone may be effective in diagnosing and treating *Fagales* pollen allergen [53–55]. Bet v 3 and Bet v 4 have been identified as calcium-binding proteins [56, 57].

Most studies that endeavour to engineer recombinant allergens for birch pollen IT have focussed on Bet v 1 due to its high level of allergenicity and the strong cross-reactivity with other *Fagales* pollen allergens. The allergenic integrity of recombinant Bet v 1 (rBet v 1) was shown in in vitro and in vivo experiments. Not only does rBet v 1 possess an IgE-binding capacity comparable to the native protein, but it also retains the IgE epitopes that the native protein shares with other allergens [23, 58, 59]. rBet v 1 has also been shown to stimulate Bet v 1-specific T-cell clones [23], induce the release of histamine from basophils [60] and produce an immediate-type wheal reaction in skin prick tests [61, 62]. Furthermore, rBet v 1 was also shown to induce Bet v 1-specific IgE antibodies upon injection into mice and rhesus monkeys [63, 64]. These tests demonstrate the comparable allergenicity of rBet v 1 with the native form, and pave the way for the use of the recombinant allergen in further understanding and manipulation of the allergy process. rBet v 1 has been proposed as an ideal diagnostic reagent for birch pollen-induced allergy. A prerequisite to the use of rBet v 1 as a IT reagent is the need to reduce its IgE reactivity and anaphylactic potential. For engineering recombinant rBet v 1, the aim is to reduce allergen-specific IgE recognition and histamine release, and to maintain T-cell reactivity.

Two approaches have been developed to reduce the anaphylactic potential of rBet v 1. The first approach is based on the knowledge that Bet v 1 forms conformational IgE epitopes [65, 66]. Since conformational epitopes are formed by the correct folding of the polypeptide chain, disrupting the overall tertiary structure of the protein may disrupt the IgE epitopes, and thus prevent allergen-specific IgE recognition. This concept was tested by Vrtala et al. [67]; two fragments of Bet v 1, representing amino acids 1–74 and 75–160, were generated in *Escherichia coli* (fig. 1). These fragments were designed to end (aa 1–74) and begin (aa

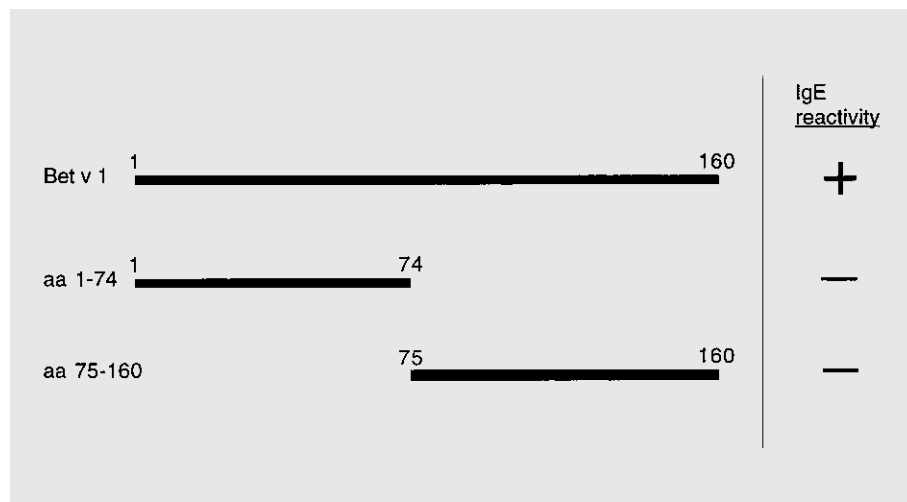


Fig. 1. Schematic representation of two non-anaphylactic fragments of the major birch pollen allergen, Bet v 1 [67]. The two fragments represent amino acids 1–74 and 75–160 of the native Bet v 1 protein.

75–160) in a region of Bet v 1 devoid of T-cell epitopes [23]. The two fragments together should therefore harbor all T-cell epitopes of Bet v 1. Western and dot blot analyses of the fragments incubated with sera from 42 birch pollen-allergic individuals showed almost no IgE binding. Of the few sera that showed weak binding, IgE recognition was estimated to represent less than 1/1,000 of the reactivity of the complete Bet v 1 protein. Furthermore as observed by CD spectra, the two fragments showed a dramatic change in conformation from the complete Bet v 1 molecule. When combined in solution, the fragments did not revert to the conformation of the complete Bet v 1 molecule. The ability of the fragments to induce T-cell proliferation showed that together the fragments do indeed contain all T-cell epitopes present on the complete Bet v 1 protein. As another indicator of the reduced anaphylactic activity of the two fragments, histamine release induced from basophils by the fragments was reduced 1,000-fold from that induced by rBet v 1. Finally, skin prick tests on 6 birch pollen-allergic individuals showed no wheal reaction to either fragment.

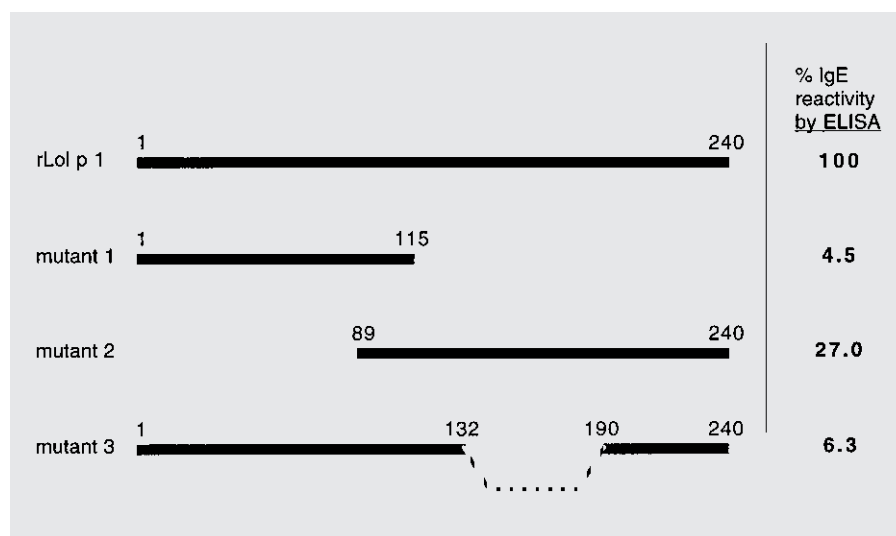
The second approach reported for the engineering of rBet v 1 is based on the contribution of certain amino acids to IgE binding and protein allergenicity. After predicting the functional residues in Bet v 1, site-directed mutagenesis was used to alter six amino acids at various positions across the molecule [68]. These amino acid substitutions altered IgE binding, dramatically reducing the reactivity of the mutant allergen. The sera from 70% of birch pollen-allergic patients showed almost no IgE binding to the Bet v 1 mutant. Furthermore, the mutant showed no histamine release, good T-cell proliferation and a 1,000-fold reduction in the ability

to induce a type-1 wheal reaction in skin prick tests. These two Bet v 1 fragments and the Bet v 1 mutant demonstrated dramatically reduced anaphylactic activity indicating that these engineered allergens have the potential to be used as a safer form of birch pollen IT than that applied currently.

Engineering Grass Pollen Allergens for Reduced IgE reactivity

Grass pollen allergy occurs world wide affecting about 75% of all allergic individuals [69]. The major grass pollen allergens are proteins classified as group 1 and group 5 which together account for almost all of the IgE-binding capacity of crude pollen extracts. Approximately 90% of the grass pollen-allergic patients show concordant immunoreactivity to both group-1 and group-5 proteins. Among the grass genera, perennial ryegrass (*Lolium perenne* L.) is one of the main sources of allergens, due to its wide distribution and copious pollen production. We have earlier reported molecular cloning and bacterial expression of both group-1 (Lol p 1) and group-5 (Lol p 5) allergens from ryegrass [21, 70] and showed that recombinant forms of Lol p 1 and Lol p 5 retain high IgE reactivity. In addition both group-1 and group-5 allergens exist in different isoforms showing sequence variations, but sharing similar allergenic epitopes [21, 22, 33]. Group-1 allergens comprise glycosylated proteins with apparent molecular masses of 28–35 kD [69]. The group-5 allergens are non-glycosylated proteins of molecular mass ranging from 28 to 31 kD [21]. There is no sequence similarity between group-1 and group-5 proteins.

Fig. 2. Diagrammatic representation of Lol p 1 variants with reduced IgE-binding capacity, as demonstrated by ELISA [28]. The mutants represent portions of the native Lol p 1 protein: mutant 1, amino acids 1–115; mutant 2, amino acids 89–240; mutant 3, the entire Lol p 1 molecule without amino acids 133–189.



Group-1 allergens are located in the cytoplasm of grass pollen, while group-5 proteins are present in amyloplasts [21]. No low IgE-reactive isoform of group-1 or group-5 grass pollen allergens have been identified so far. These grass pollen allergens have been the subject of genetic engineering to reduce their IgE reactivities. Tamborini et al. [28] reported production of low IgE-reactive forms of the ryegrass group-1 allergen, Lol p 1, while Swoboda et al. [38] and Schramm et al. [71] produced low IgE-reactive mutants of ryegrass allergen, Lol p 5, and timothy grass allergen, Phl p 5, respectively.

Low IgE-Reactive Forms of Lol p 1

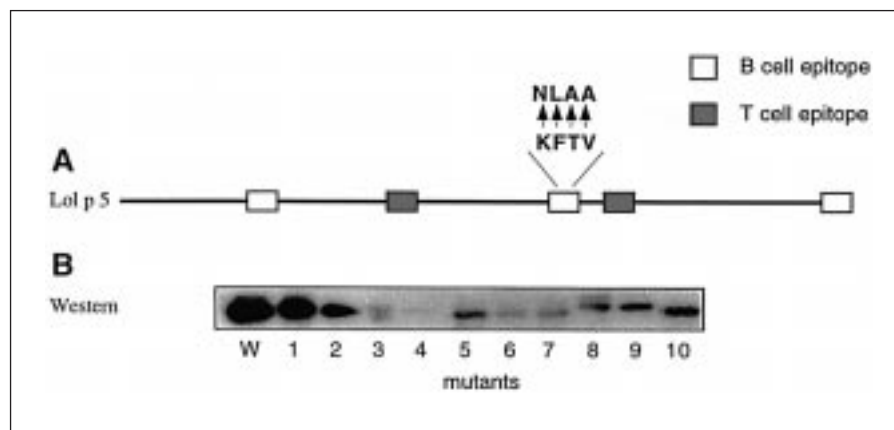
Lol p 1 is a glycosylated protein of 35 kD, containing intramolecular disulphide bridges. An IgE-reactive region corresponding to 29 C-terminal amino acids of Lol p 1 was identified by proteolytic fingerprinting [72]. Tamborini et al. [28] produced three deletion mutants lacking large portions of protein internally or at terminal regions. A schematic representation of the mutants is shown in figure 2. Mutants 1 and 3 showed significant reduction in IgE reactivity. In a histamine release assay using 0.1 mg/ml of recombinant proteins, rLol p 1, induced a maximum release of 70% of total histamine, and mutant 2 produced a maximum release of 50% total histamine, while the other two mutants did not trigger any histamine release up to a test concentration of 10 mg/ml. No information regarding skin prick test reactivity or T-cell proliferation induction ability of the above-described mutants is available. However, these stud-

ies pointed to the use of molecular biology techniques for production of grass group-1 allergen molecules with reduced anaphylactic potential.

Hypoallergenic Lol p 5 Mutants

In order to reduce the anaphylactic potential of the major ryegrass pollen allergen, Lol p 5, we tested whether substitution of selected amino acids might reduce its IgE reactivity while maintaining relevant T-cell epitopes. Lol p 5 is a highly immunoreactive protein with up to 60% of the IgE against total grass pollen allergens in individual sera directed against epitopes on this protein [21, 29, 32]. Identification of the structures critical for IgE recognition of Lol p 5 is a prerequisite for generation of low IgE-reactive forms by site-directed mutagenesis. We have previously carried out Lol p 5 IgE epitope mapping using overlapping recombinant fragments and synthetic peptides [21, 29, 32] and demonstrated that Lol p 5 contains continuous IgE-reactive structures throughout the molecule. In addition, series of overlapping synthetic peptides to map dominant T-cell epitopes on Lol p 5 were also used [73]. We have also reasoned that amino acid residues strictly conserved between all Lol p 5 isoforms and group-5 homologues from other grasses might be critical for IgE reactivity. Based on the knowledge about T-cell epitopes and putative, continuous IgE-reactive epitopes on Lol p 5, we have produced modified forms of Lol p 5 by introducing either single or multiple amino acid substitutions. T-cell epitope-mapping studies showed that two peptides, Lol p 5 105–116 and Lol p 193–204, were

Fig. 3. Site-directed mutagenesis of the major ryegrass pollen allergen Lol p 5 for generation of allergen variants with reduced anaphylactic potential [38]. **A** Linear representation of the Lol p 5 protein showing B- and T-cell epitopes, and four of the amino acids altered by site-directed mutagenesis to reduce IgE binding to the protein. **B** Western blot of 10 Lol p 5 mutant proteins incubated with sera from a ryegrass pollen-allergic patient showing reduced IgE binding to the allergen mutants. W = Wild type rLol p 5; 1–10 = rLol p 5 mutants.



able to significantly stimulate T-cell proliferation. Thus all the amino acid exchanges in the Lol p 5 molecule were designed to be outside these identified T-cell epitopes. We also observed that a four-amino acid block, K172–T173–F174–V175, is strictly conserved in group-5 grass pollen allergens. We have prepared a rLol p 5 mutant in which these four amino acids are exchanged with NLAA [38] (fig. 3A). The change in each position was determined by the ease with which a single mutagenic primer with minimal nucleotide exchanges can lead to a change in all four amino acids. In total we produced ten different Lol p 5 mutants that carried point mutations in single or multiple sites. The IgE-binding capacity of the rLol p 5 mutants compared to the wild-type rLol p 5 was evaluated by immunoblotting, histamine release assays and skin prick testing of allergic patients. Our data showed that only a few point mutations can significantly reduce IgE-binding capacity and anaphylactic potential of recombinant Lol p 5 (fig. 3B). The T-cell reactivity of the mutant rLol p 5 forms remained unaltered. We also observed differences in individual patients' response to individual mutants. This implies that each patient has to be tested against a number of allergen variants prior to deciding the suitability of a mutant for use in IT.

Hypoallergenic Variants of Phl p 5

Phl p 5 is a group-5 allergen of timothy grass (*Phleum pratense*). The molecular cloning of the Phl p 5 allergen was reported by Vrtala et al. [22]. In IgE-binding studies, it was demonstrated that the rPhl p 5 protein can block binding of patients' IgE to natural group-5 isoallergens in two-dimensional immunoblots. The purified rPhl p 5 was shown to induce specific histamine release from blood basophils of

grass pollen-allergic patients. Owing to its high IgE reactivity, Vrtala et al. [22] further proposed the usefulness of rPhl p 5 as a specific diagnostic reagent for grass pollen allergy. Recently, Schramm et al. [71] reported the production of reduced IgE-binding variants of Phl p 5. Site-directed mutagenesis was used based on the identification of dominant T-cell epitopes of Phl p 5 using overlapping dodeca peptides. By stepwise site-directed mutagenesis, the point and deletion mutations were performed within the putative IgE binding but outside the identified T-cell epitopes to reduce the IgE reactivity but not the T-cell reactivity (fig. 4). The IgE reactivity was tested by various in vitro techniques such as immunoblotting, ELISA inhibition, and histamine release. In addition, skin prick tests were performed to assess in vivo reactivity. Figure 4 shows the location of point mutations and deletions on the Phl p 5. The point mutant PM1 contained two amino acid exchanges within the identified N-terminal IgE-binding epitope (D49 to L; K50 to A). The introduction of these point mutations had no effect on IgE reactivity of this variant. The point mutant PM3 contained a new cysteine residue (A13 to C) in addition to wild-type cysteine residue, C186. Schramm et al. [71] expected that formation of an intramolecular disulphide bond between these cysteines should result in a drastic change in molecular conformation of Phl p 5 and hence its IgE reactivity. However, no change in IgE reactivity was observed. Further various deletion mutants were constructed, which lacked varying stretches of the internal sequences. Some of these deletion variants showed reduced IgE reactivity; the most significant results were observed in case of deletion mutants, DM2 and DM4. The histamine-release capacity was completely abolished in these two deletion mutants. Both DM2 and DM4 also showed dramatically reduced skin prick reactivity. The T-cell reactivity, however, was con-

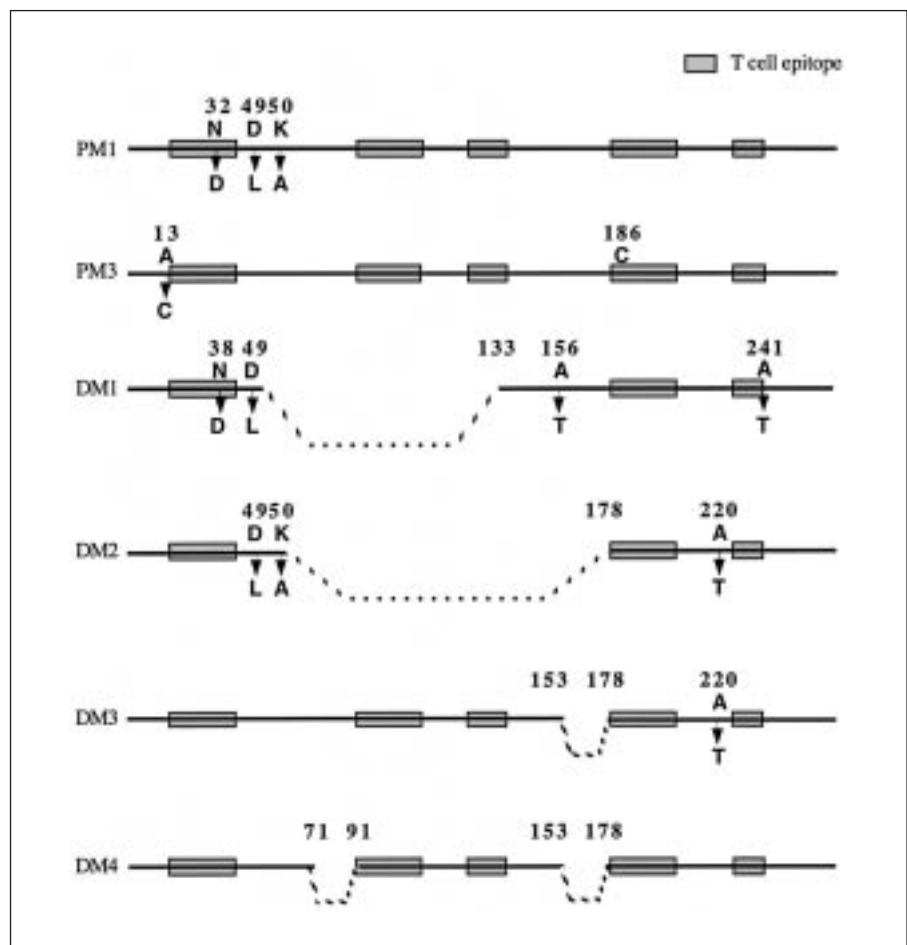


Fig. 4. Generation of variants of the major grass pollen allergen, Phl p 5, using site-directed mutagenesis and deletions [71]. Mutated amino acids, their altered form and the positions of the deletions are indicated.

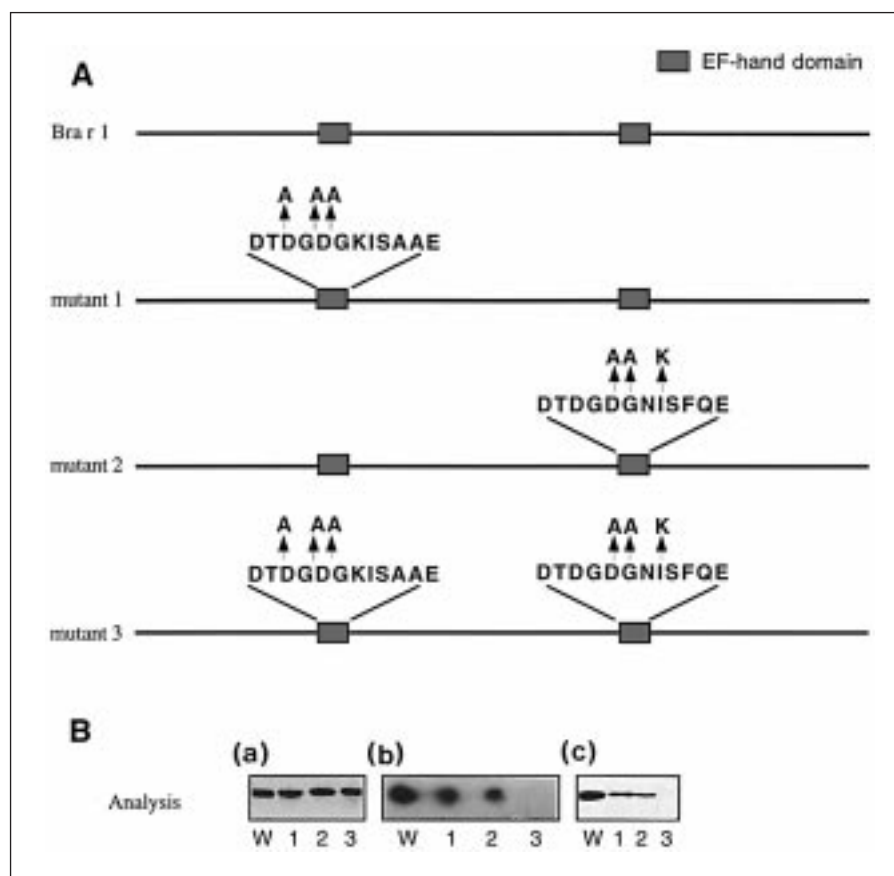
served in most of the Phl p 5 variants. DM4 was considered as the best allergen variant since it showed significant reduction of IgE-binding capacity with an overall conserved T-cell reactivity due to relatively small deletions [71].

Engineering of Low IgE-Binding Variants of Calcium-Binding Allergens

Calcium-binding allergens have been identified in diverse groups of organisms such as plants, fish and man. The distribution and biological properties of these allergens have been reviewed recently by Valenta et al. [74]. Calcium-binding allergens carry typical calcium-binding motifs, termed EF hands, within their sequence. The EF hands consist of two perpendicularly arranged α -helices and an interhelical loop, forming a single calcium-binding site. A three EF-hand birch pollen allergen, Bet v 3, was the first identified plant calcium-binding allergen [56]. Calcium-binding

proteins are known to exhibit different conformations in the presence or absence of Ca^{2+} [75]. Experiments using EGTA to deplete protein-bound Ca^{2+} indicated that IgE antibodies bound preferentially to calcium-bound allergen Bet v 3 [56]. A novel class of Ca^{2+} -binding pollen allergens with two EF hands has been identified in such diverse plant species as *Brassica rapa* [76], Bermuda grass [77, 78], birch [57, 79] and olive [80]. Members of this class of allergens are small, water-soluble proteins of about 9 kD that share significant sequence similarities extending outside the Ca^{2+} -binding domains. Furthermore, it was shown that Bra r 1, Cyn d 7, and Bet v 4 share significant IgE cross-reactivities [57, 79] and can thus be considered as relevant cross-reactive plant allergens. All these calcium-binding allergens show preferential expression in pollen [79, 81]. The finding that IgE reactivity of these calcium-binding allergens is greatly influenced by bound calcium led to the expectation that site-directed mutagenesis may be used to destroy the calcium-binding activity of the proteins. Engel et al. [57]

Fig. 5. Site-directed mutagenesis of the calcium-binding domains (EF hands) of Bra r 1. **A** Linear representation of the Bra r 1 protein showing the position of the EF-hand domains and the amino acids mutated to disrupt calcium binding, and IgE recognition of the Bra r 1 protein. **B** Coomassie blue-stained SDS-PAGE gel of wild-type Bra r 1 (W) and mutants 1, 2, and 3 (**a**); autoradiogram of a Western blot overlaid with ^{45}Ca showing reduced, or no calcium binding (**b**); Western blot of proteins incubated with sera from a Brassica pollen-allergic patient showing reduced or no IgE binding to the Bra r 1 mutants (**c**).



performed site-directed mutagenesis to produce non-calcium-binding variants of the birch pollen allergen, Bet v 4. It was found that the disruption of calcium-binding site I did not significantly alter IgE reactivity for most of the patients tested. On the other hand, the loss of calcium-binding activity of site II led to a noticeable reduction in IgE reactivity for most of the patients. In our laboratory, we have performed site-directed mutagenesis experiments on the oil seed rape allergen, Bra r1 [81], and showed that both EF-hand domains constitute functional Ca^{2+} -binding sites (fig. 5). Furthermore, we also showed that disruption of both calcium-binding sites resulted in decreased IgE binding to Bra r1. Such variants of calcium-binding proteins with greatly reduced IgE have been proposed as ideal IT reagents [74].

The examples detailed in this review illustrate that it has now become feasible to generate allergen variants with greatly diminished IgE reactivity but retained T-cell reactivity. In vitro proliferation of T cells in response to allergen mutant may not reflect its clinical efficacy in the allergic patient. Thus it will be important in future to confirm that im-

munisation with allergen variants can induce protective immune responses. Skin wheal reaction as a result of localised mast cell granulation is a good predictor of general mast cell degranulation and hence the anaphylactic potential of an allergen preparation. The engineered allergens described possess low or nil skin test reactivity. Thus these variants of pollen allergens with low anaphylactic potential are potential candidates for specific IT in the future.

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