

## Special Feature

# Hypoallergenic derivatives of major grass pollen allergens for allergy vaccination

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**Summary** Grass pollen-induced hay-fever and allergic asthma represent a major health problem in industrialized countries. Whereas the symptoms of these allergic conditions can be controlled by pharmacotherapy, specific immunotherapy vaccination is the only causative approach towards the treatment of these type 1 allergies. Specific immunotherapy is based on administration of increasing amounts of the disease-causing allergens in the form of allergen-containing extracts. However, the extracts used for immunotherapy consist of allergenic and non-allergenic components and may induce severe anaphylactic side-effects upon therapeutic administration. With recent developments in molecular biology of pollen allergens it has become feasible to produce modified hypoallergenic derivatives of recombinant allergens with abrogated or greatly reduced likelihood of anaphylactic side-effects as compared to extract-based treatments. We have demonstrated this concept through reducing the anaphylactic potential of major rye grass pollen allergens by introducing a few point mutations which leave the overall structural fold of the molecule unaltered. These modified forms are expected to make allergen-specific immunotherapy more widely used in the future.

**Key words:** grass pollen allergy, hay fever, hypoallergen, IgE, immunotherapy, recombinant allergen, type 1 allergy.

## Introduction

Type I allergic diseases, caused by immunologically mediated hypersensitivity represent a major health problem, affecting more than 25% of the world's population. Atopic allergies are determined by a pathogenic IgE response to otherwise harmless environmental antigens (allergens). According to a recent report, the prevalence of asthma and other allergic diseases has doubled in the last two decades.<sup>1–3</sup> Allergic individuals respond to a specific allergen exposure by producing T-cell cytokines, predominantly IL-4 and IL-5, which in turn stimulate the production of allergen-specific IgE antibodies and the recruitment of an eosinophil-rich inflammatory infiltrate. The production of allergen-specific IgE requires the help of allergen-specific T lymphocytes, which are stimulated by linear peptide fragments of antigen. These peptides are generated by APC through antigen processing and are displayed at the cell surface with molecules of the MHC complex. The production of specific IgE by B cells is thus stimulated by allergen-specific T helper cells, which usually belong to the Th2 type. The immediate symptoms of allergic disease such as rhinitis (hayfever) conjunctivitis, allergic asthma and anaphylaxis are caused by allergen-induced cross linking of allergen-specific IgE bound to high-affinity Fc receptors on the surface of mast cells and basophils triggering the immediate release of pre-formed and newly synthesized inflammatory mediators such

as histamine and leukotrienes. The late symptoms of allergy, (e.g. atopic dermatitis and chronic asthma) can result from IgE-mediated allergen presentation and thus activation of T cells and release of proinflammatory cytokines.<sup>4,5</sup>

## Grass pollen is the most predominant source of outdoor allergens

The biological role of pollen is to fertilize the female gametophyte. In fulfilling this role, pollen is released from the mature anther and distributed through the environment by the wind or by the action of insects. The risk of sensitization to wind-pollinated flowering plant species is high since these species produce copious amounts of pollen that are amenable to being carried in the atmosphere for considerable distances. As a consequence, allergy to pollens is one of the most commonly encountered sensitivities, being a major causative factor in seasonal rhinoconjunctivitis. In many parts of the world, the pollen of grass genera is responsible for pollen allergy in more individuals than any other flowering plant family. At least 40% of type 1 allergic patients are sensitized against grass pollen allergens. Grass pollen is thus the most frequent elicitors of allergic symptoms.<sup>6</sup> Individuals sensitive to the allergens from one grass are often sensitive to those of a number of other grasses.<sup>7,8</sup>

Rye grass and closely related grasses are distributed in cool temperate regions in North America, Europe and southern parts of Australia. In warm temperate and subtropical regions pollen of Bermuda grass (subfamily *Chloridoideae*) is an important source of allergens. Allergens of Bermuda grass show low levels of cross reactivity with those from Pooidae grasses.<sup>9</sup>

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Grass pollen molecules that provoke allergic reactions have been identified and characterized. The number of grass pollen proteins or glycoproteins ranging from 10 to 85 kDa show reactivity towards IgE from grass pollen allergic patient sera.<sup>10</sup> However, two unrelated proteins, group 1 and 5, have been identified and characterized as major allergens of grass pollen.<sup>11,12</sup> Group 1 and group 5 together account for most of the IgE-binding capacity of crude pollen extracts.

Group 1 allergens are glycoproteins with a molecular mass of around 30–35 kDa and can be seen as a prominent protein band in SDS-PAGE gels of soluble protein extracts. Group 1 grass pollen allergens are recognized by more than 95% of grass pollen allergic patients and represent a highly expressed pollen protein.<sup>11</sup> These glycoproteins are located in the cytoplasm of the pollen grain but are released rapidly upon hydration on the stigma surface or on human mucosal surfaces.<sup>13</sup> The cDNAs encoding for group 1 allergens from ryegrass (*Lol p 1*), Canary grass, Timothy grass and Velvet grass have been cloned.<sup>13</sup>

Sequencing of the group 1 pollen allergens from eight grass species has revealed significant levels of amino acid conservation, including the presence of seven strictly conserved cysteine residues within these proteins. The grass group 1 grass pollen allergens show conservation of six of their seven conserved cysteine residues and 25% overall amino acid homology to expansins, a group of proteins that induce plant cell wall extension (creep).<sup>14</sup> The degree of cysteine residue conservation suggests a common pattern of disulphide bond formation and protein folding between the two groups of proteins.<sup>15</sup> An expansin-like activity has also been demonstrated for the purified group 1 allergen from maize pollen.<sup>15</sup> Amino acid sequence homology has also been shown between the group 1 allergens and three functionally important sequence motifs of the C1 (papain-like) family of cysteine proteinases.<sup>16</sup> Recently, Grobe *et al.* have also shown that the group 1 pollen allergen from timothy grass demonstrates similar biochemical and functional properties to cathepsin B,<sup>17</sup> a member of the papain (C1) family of cysteine proteinases.

Group 5 allergens are non-glycosylated highly IgE-reactive proteins of molecular mass 28–32 kDa recognized by more than 95% of the grass pollen allergic sera. The group 5 allergens were initially identified as IgE-reactive fusion proteins in grass pollen cDNA expression libraries.<sup>12</sup> Recombinant group 5 allergens retain IgE reactivity equivalent to that of natural forms. In the case of timothy grass group 5 allergen, Phl p 5, the controlled titration of purified natural versus recombinant allergens in an ELISA inhibition experiment showed that both recombinant and natural form could cause equivalent inhibition of IgE binding to natural allergen.<sup>18</sup> Inhibition ELISA experiments with recombinant *Lol p 5* versus natural *Lol p 5* demonstrated similar equivalence. In the case of ryegrass extracts, nearly two-thirds of IgE-binding capacity is accounted for by *Lol p 5*. This has been confirmed in our laboratory by antisense-mediated silencing of *Lol p 5* in transgenic rye grass plants.<sup>19</sup>

### **Immunotherapy is the only causative approach towards the treatment of type 1 allergy**

Pharmacotherapeutic approaches that are currently available for the treatment of allergic disorders and asthma are

non-specific and are aimed primarily at neutralizing effector molecules and inflammatory mediators such as histamine and leukotrienes, or at inhibiting the function of inflammatory cells such as eosinophils and Th2 lymphocytes. While these approaches provide relief from symptoms, these therapies fail to alter the natural underlying inflammatory cascade leading to allergic diseases and asthma, and discontinuation of medications results in redevelopment of symptoms on repeat exposure to offending allergens. Moreover, there remains a group of patients who respond inadequately to conventional pharmacotherapy.<sup>20</sup>

In contrast immune-based allergen immunotherapies modify the cascade of underlying adverse immune responses in allergy and asthma in an antigen-specific manner. Immunotherapy can provide a long-lasting cure for allergic diseases without the need for continuous therapeutic intervention.<sup>21–23</sup> Immunotherapy is thus the only causative approach towards the treatment of type 1 allergy and only treatment that has potential to alter the natural course of allergic disease. Immunotherapy with allergens has been shown to be effective in many studies for the clinical indications for allergic asthma and rhinitis. Immunotherapy is known to prevent development of asthma in patients with allergic rhinitis.

Specific immunotherapy is based on the administration of increasing amounts of the disease-eliciting allergens in order to yield allergen-specific non-responsiveness.<sup>21–23</sup> Since immunotherapy aims to modify immune response, it has been termed as a 'vaccine' for allergic disease.<sup>24</sup> Success of this therapy is associated with a modulation of the immune response to allergenic molecules at the level of T helper cells and possibly by the induction of blocking antibodies that may prevent the activation of CD4 T cells.<sup>22,23</sup> Many studies on the mechanism of allergen immunotherapy have shown modulation of T-cell responsiveness with variable inhibition of Th2 cell responses (IL-4 and IL-5) to allergen, and induction of more Th1-like responses with increased allergen-induced IFN- $\gamma$  and IL-12. The mechanism of this immune switching is not clear. Factors that can influence Th1 and/or Th2 responses include the nature of the allergen, and the dose and the type of APC.<sup>25</sup> B cell- or dendritic cell- (DC) mediated presentation of a low dose of allergens favour Th2 responses while high dose processing and presentation by macrophages favour Th1 responses. Use of different adjuvants can also influence Th1/Th2 balance. Immunotherapy may also influence the course of allergic disease by lowering recruitment of inflammatory cells, activation or cell mediators. Grass pollen immunotherapy in adult patients has been shown to result in a decrease in the number of cutaneous mast cells,<sup>26</sup> and reduced histamine and prostoglandin-2 levels in nasal secretions following allergen challenge.<sup>27</sup>

Recently, there has also been a resurgence in the classic blocking antibody paradigm of mechanism of immunotherapy.<sup>28</sup> Blocking antibodies, IgG, compete with IgE for allergen and they may also prevent the aggregation of IgE and its receptor (*Fc $\epsilon$ RI*) on the mast cells by altering steric confirmation. Blocking antibodies that are induced by the immunotherapy regime can also inhibit the IgE-mediated presentation of allergens to T cells and thus it interferes with chronic manifestations of atopy.<sup>29</sup>

Specific immunotherapy (SIT) is a clinically proven, effective treatment for allergic conditions including rhinoconjunctivitis

and asthma. However, the reagents used for immunotherapy are crude extracts prepared from natural sources and contain additional, ill-defined components and are very difficult (if not impossible) to standardize in terms of allergen dose. The potential side-effect of injecting allergen into any specifically IgE-sensitized individual is anaphylaxis. Despite these limitations, immunotherapy is still considered to be the most viable therapeutic option. Recent developments in molecular cloning and the availability of amino acid sequences of major allergens have made it possible to design novel therapeutic approaches for improved and safer forms of allergen immunotherapy.

One such approach is to use T-cell epitope peptide-based therapy aiming to regulate T-cell responses.<sup>30</sup> 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 and are thus unable to induce the release of histamine and IL-4.<sup>30</sup> Initial clinical trials involving immunotherapy of cat-allergic subjects with Fel d 1 peptides reported some promising results. However, it has also been reported that Fel d 1 peptide therapy did not reduce immediate or late-phase skin reactivity in cat-allergic individuals,<sup>31</sup> thus limiting its use in immunotherapy. Smaller peptides of the major allergen of bee venom were shown to be promising immunotherapy reagents.<sup>32</sup> It is worth noting here that bee venom has one major allergen with a limited T-cell epitope repertoire while grass pollens have more than one major allergen with a wide repertoire of dominant T-cell epitopes. Thus the design of T-cell peptide-based immunotherapy for grass pollen would be very complex. In addition, multiple T-cell epitopes of grass allergen are recognized individually by different patients, due to the large diversity of MHC and TCR which exist among individual patients.<sup>33</sup>

### Modification of allergens for immunotherapy

A major problem with specific allergen immunotherapy is that the allergen extracts that are currently used for vaccination are unfractionated preparations from natural sources and contain besides the allergens, additional components, mostly proteins to which the subject is not allergic. These allergen extracts may lack important allergens and may show a high level of variability in the ratio of individual allergen components.<sup>24</sup> One of the major problems with conventional immunotherapy is the likelihood of life-threatening systemic adverse reactions such as anaphylactic shock. Use of alum-precipitated allergen preparations with slow release characteristics can help in reducing these side-effects. Aqueous allergen preparations, however, have higher potency with associated higher risk of adverse reactions.

Marsh was first to introduce chemical modification of allergens to reduce the risk of anaphylaxis.<sup>34</sup> The chemically modified allergens termed 'allergoids' were produced by polymerization of allergens using formaldehyde,<sup>35</sup> or glutaraldehyde.<sup>36</sup> In mice, allergoids obtained by polymerization were shown to down-regulate IgE levels.<sup>37</sup> Norman *et al.* studied efficacy and safety of long-term allergoid treatment and reported significant IgG antibody responses in ragweed-allergic subjects.<sup>38</sup> However, the chemical modification of allergens is problematic because it is difficult to define and standardize.<sup>21</sup> Moreover, the allergoids produced by a

chemical polymerization of crude pollen extracts contain other irrelevant proteins in addition to allergens. In addition, the allergoids not only have reduced IgE but also have reduced immunogenicity.

Another approach is the use of recombinant-expressed allergens for specific immunotherapy. As mentioned above, with the advent of molecular biology, isolation and cloning of cDNA encoding, most of the clinically important environmental allergens have been possible.<sup>13</sup> Recombinant allergens are easy to purify and standardize and there is the exciting possibility of patient-tailored immunotherapy based on individual sensitization profiles. However, recombinant-expressed allergens cannot be used for specific immunotherapy because of their IgE reactivity. Moreover, due to purity of the recombinant allergen preparations, their anaphylaxis-inducing potential is expected to be very high, even at very low doses.<sup>39</sup> Site-directed mutagenesis techniques offer the exciting possibility of engineering allergens with low IgE binding to reduce the risk of anaphylaxis, but contain most of the T-cell epitopes for immunotherapy,<sup>40–42</sup> thus providing safer vaccine.<sup>22,23</sup> The use of recombinant allergens should circumvent the problem of standardization of crude extracts by allowing production and purification of many of the major allergens in ways that eliminate variation between batches.

Another important benefit of using modified allergens is the possibility of retaining immunogenic features that are necessary for the induction of a protective immune response (i.e. induction of blocking antibodies) and to alter the Th cytokine balance in a favourable way. Safer and more efficacious vaccines would increase patient compliance and ultimately lead to more widespread use of immunotherapy. Modified recombinant polypeptide that by virtue of its primary structure did not have the capability of producing IgE-binding structures could be an ideal candidate for immunotherapy. These polypeptides should be achieved with a few key changes that keep the general structure intact and hence allow the modified allergen to be processed in the same way by antigen processing cells as the natural allergen. It has been reported that isoforms of major birch pollen allergen with few amino acid variations show significant differences in IgE reactivities.<sup>43</sup> However, no naturally occurring low IgE reactive isoforms of grass pollen allergens could be identified.<sup>44,45</sup>

### Hypoallergenic variants of grass group 1 allergens

Site-directed mutagenesis to replace cysteine residues within rDer p 2 and rDer f 2 led to drastic reductions in the allergenic reactivity of both recombinant house dust mite allergens.<sup>46,47</sup> Unlike the group 2 house dust mite allergens, the presence of intramolecular disulphide bonds has not been proven for the group 1 grass pollen allergens. However, the presence of seven strictly conserved cysteine residues in the protein backbone, along with sequence and predicted structural homologies to functional proteins, implies that the group 1 grass pollen allergens possibly have a conserved molecular structure, which may be stabilized by disulphide bonds.

In a recent study de Weerd *et al.*<sup>48</sup> have studied the importance of the cysteine residues of rye grass group 1 allergen rLol p 1 to the overall IgE binding capacity of this

recombinant allergen.<sup>48</sup> Seven different rLolp1 cysteine mutants were produced. However, only one cysteine variant C77S showed the lowest level of human IgE antibody binding of the proteins immunoscreened with sera from a number of grass pollen allergic subjects. The results of the present study suggest that residue C<sup>77</sup> of rLol p 1, and also perhaps those situated at amino acid residue positions 72, 83 and 139 may contribute to the formation of a structure that is critical for human IgE recognition of rLol p 1. However, even after replacement of cysteine residues the modified rLol p 1 retained about 40–60% of the IgE reactivity depending upon the patient sera. It is apparent from these results that other factors, perhaps the presence of linear IgE epitopes, may also play a role in human IgE recognition of rLol p 1.

Studies designed to delineate the IgE epitopes of Lol p 1 have used either synthetic peptides, corresponding to linear segments of this allergen, or a pool of monoclonal antibodies to block human IgE binding to linear peptides.<sup>49–52</sup> Evidence for the existence of at least one conformational IgE epitope for Lol p 1 was obtained from a study using recombinant-expressed Lol p 1 fragments to inhibit human IgE binding to rLol p 1.<sup>52</sup> In this study it was reported that two fragments of Lol p 1, corresponding to amino acids 1–168 and 169–240, could inhibit human IgE antibody binding to rLol p 1 by 17 and 51%, respectively.<sup>52</sup> Since these fragments together represent the complete Lol p 1 molecule, and no known linear IgE epitope has been mapped across their point of junction, the remaining 32% of human IgE-binding not inhibited by these fragments suggests the presence of at least one conformational IgE epitope. The results of this study also suggested that the cysteine residues present within Lol p 1 may contribute to the formation of a conformational IgE-binding epitope on this allergenic protein. These cysteine replacement studies have indeed shown that a conformational epitope that involves C77 in its structure indeed accounts for about 40–50% of IgE reactivity of Lol p 1.<sup>48</sup>

It is apparent that in order to produce hypoallergenic forms of group 1 allergens, it would be essential to target individual amino acids critical for IgE recognition of linear epitopes. A linear IgE reactive region corresponding to 29 C-terminal amino acids of Lol p 1 has been identified by proteolytic fingerprinting.<sup>50</sup> Tamborini *et al.* produced three deletion mutants lacking large portions of protein internally or at terminal regions.<sup>53</sup> Mature Lol p1 protein is made up of 240 amino acids. Mutant 1 contains amino acids 1–115, while mutant 2 is made up of a C terminal fragment 89–240. Mutant 3 carries a 58 amino acid internal deletion. Mutants 1 and 3 showed significant reduction in IgE reactivity. In 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 mutants described above is available. Recently we have produced a deletion mutant that shows only 3% of the IgE reactivity of the non-mutated form. These results show that it is now possible to produce hypoallergenic variants of group 1 allergen with significantly reduced anaphylactic potential.

### Hypoallergenic variants of group 5 allergens

Group 5 allergen of timothy grass (*Phleum pratense*), Phl p 5 was cloned by Vrtala *et al.*<sup>18</sup> Schramm *et al.*<sup>54</sup> reported production of various hypoallergenic forms generated by the introduction of point and deletion mutations within the putative IgE-binding but outside the identified T cell epitopes to reduce the IgE reactivity but not the T cell reactivity.<sup>54</sup> 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. The point mutants produced showed no change in IgE reactivity. However, some of the deletion variants showed reduced IgE reactivity; the most significant results were observed in the case of deletion mutants. The histamine release capacity was completely abolished in these two deletion mutants. Both mutants also showed dramatically reduced skin prick reactivity. The T cell reactivity, however, was conserved 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.<sup>54</sup>

Recently, by using Lol p 5a as a model we have demonstrated that it is possible to reduce the allergenic activity by few point mutations that leave the overall structural fold of the molecule unaltered.<sup>55</sup> Extensive IgE epitope mapping using overlapping recombinant fragments and synthetic peptides,<sup>56,57</sup> 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 (38). Using site-directed mutagenesis we introduced point mutations into the conserved IgE-bearing sites of Lol p 5a and generated nine Lol p 5a mutants.<sup>55</sup> Single amino acid residues were exchanged in five of the mutants. We also combined these single amino acid mutations in different ways to obtain mutants containing two or more amino acid changes. Circular dichroism analysis demonstrated that rLol p 5 wild type and rLol p 5a mutants exhibited a similar fold containing a considerable amount of  $\alpha$ -helical structure. Two of the mutants (single amino acid exchange from K57 to A; exchange from K172 F 173, T 174 V175 to NLAA, respectively) showed significant loss of IgE-binding capacity. These mutants also showed reduced allergenic activity as determined by basophil histamine release and by skin prick testing in allergic patients. The histamine release induced by one of the mutants was reduced at least tenfold as compared to rLol p 5 wild type in each of the eight subjects tested, thus identifying this mutant as a mutant with a consistent tenfold reduction of allergenic activity validates our approach towards allergen modification. Thus, our results have demonstrated that a few point mutations in the Lol p 5a sequence yield mutants with reduced allergenic activity that represent potential vaccine candidates for immunotherapy of grass pollen allergy. Further our experiments also showed that Lol p 5a variants generated exhibited reduced allergenic activity but maintained a high degree of ability to induce lymphoproliferative responses in Phl p 5-specific T cell lines and T-cell clones, indicating that Lol p 5a variants could also be used for treatment of allergies to group 5 allergens of other grasses. In view of the high allergenic activity and immunogenicity of group 5 allergens, this simultaneous reduction of IgE reactivity

and preservation of the molecular fold represents a novel approach towards the design of hypoallergenic allergen variants.

## Conclusion

Current allergy diagnosis identifies the source such as grass pollen but is unable to identify the disease-eliciting allergen molecule. Use of protein microarray technology in determination and monitoring of allergic patients IgE reactivity profiles to the allergens offers additional advantages of fast and reliable diagnosis to the disease causing molecule(s),<sup>58</sup> rather than the organism using very small quantities of a subject's serum. In addition, it has potential to provide overall IgE reactivity profile of a subject in a single test. New therapeutic agents for hay fever vaccine with enhanced safety and efficacy features will be based on developing hypoallergenic forms. Modified recombinant molecule or polypeptide that did not have the capability of producing IgE-binding structures could be an ideal candidate for immunotherapy. Such modified allergens should be achieved with a few key changes that keep the general structure intact and hence allow the modified allergen to be processed in the same way by antigen processing cells as the natural allergen. Alternative delivery route, oral immunotherapy, using either protein or DNA-based for improved patient compliance and reduced risk of systemic anaphylactic response is also likely to have an impact on future hay fever vaccines.

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