



Genetic engineering of pollen allergens for hayfever immunotherapy

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Type I allergy is an immunoglobulin E-mediated disease, representing a major health problem affecting more than 25% of the world's population. Grass, birch and ragweed pollen are a major source of environmental allergen. Specific immunotherapy is clinically proven to be an effective treatment for allergic conditions that involve the administration of crude extracts prepared from natural sources with potential life-threatening anaphylactic side effects. Recent developments in the molecular biology of pollen allergens have made it possible to design novel therapeutic approaches for improved and safer forms of specific immunotherapy. Hypoallergenic forms of major allergens with reduced immunoglobulin E epitopes have been produced using genetic engineering, whilst preserving other characteristics of the molecule that are able to induce a protective response. These modified forms are expected to make allergen-specific immunotherapy more widely used.

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The term 'hayfever' was coined by Bostock in the 19th century, who believed that it was caused by the smells from grasses and hay. Blackley demonstrated pollen as a causative agent in 1873. The name hayfever is therefore misleading as there are no symptoms of fever and it is not caused by hay. Hayfever is best defined as seasonal allergic rhinitis and allergic conjunctivitis caused by a Type I allergic reaction. Type I allergic diseases (allergic rhinitis or hayfever, rhinoconjunctivitis, allergic asthma and allergic dermatitis) represent a major health problem, affecting more than 25% of the world's population [1]. Atopic allergic diseases, such as hayfever and allergic asthma, have increased in prevalence during the past three decades, especially in western countries, such as the USA, UK, New Zealand and Australia and an upward trend has been forecasted [2]. These diseases are the most common cause of chronic ill health in western societies. Hayfever, characterized by inflammation and runny eyes and nose, is induced in the mucous membranes of the upper respiratory tract of susceptible individuals. If the lower respiratory tract, lungs or bronchials are affected, clinical symptoms, such as wheezing, cough and shortness of breath, are induced.

The term 'atopy' is used to describe immunoglobulin (Ig)E-mediated diseases. Atopic persons have a hereditary predisposition to produce IgE antibodies against common environmental allergens. It is now thought that there are three steps to induction and maintenance of allergy caused by an allergen, such as pollen: sensitization, immediate reaction and late reaction [3]. Initial contact with a minute amount of pollen-bearing allergen molecule releases soluble allergen on the mucosal surfaces of eyes and nose followed by allergen uptake by allergen-presenting cells, dendritic cells and priming of allergen-specific T-cells leading to activation allergen-specific B-cells. T-helper (Th)2 cells produce cytokines, such as interleukin (IL)-4 and IL-13 that favor Ig class switching of specific B-cells to IgE. Hence the production of IgE and sensitization of the individual against the pollen allergen. It is postulated that sensitization leads to the establishment of IgE⁺ memory B-cells and antigen-specific memory T-cells. Subsequent contact with pollen allergen will boost IgE⁺ memory B-cells that receive T-cell help to produce increased levels of allergen-specific IgE antibodies. These antibodies are loaded on to mast cells and basophils through specific

receptors, Fcε R1 and Fcε R11. Immediate reaction occurs when allergen cross links the effector (mast) cell bound to IgE leading to degranulation of the mast cell, which induces the release of biologically active mediators, such as histamine and leukotriene. This causes the display of the immediate symptoms of allergy – rhinitis, conjunctivitis and asthma. Late reaction occurs after hours and days and is caused by the presentation of allergens to T-cells, leading to their activation, proliferation and release of proinflammatory cytokines, such as IL-4, IL-5, IL-13. This phase is also characterized by eosinophil activation and the release of inflammatory mediators.

Current treatment

The symptoms of allergic disease (allergic rhinitis, rhinoconjunctivitis, asthma) can be controlled by pharmacotherapy and the pharmaceutical industry has focused on treatment of the symptoms rather than cause. Currently, antihistamine and topical corticosteroids are commonly used drugs to relieve symptoms or to suppress allergic inflammation respectively. Little progress has been made in the development of cure or therapy for atopic allergic diseases. Specific immunotherapy, first introduced in 1911 [4], is the only causative approach towards the treatment of Type I allergy [5]. Specific immunotherapy is based on the administration of increasing amounts of the disease-eliciting allergens in the form of allergen-containing extracts over a long period in order to desensitize the allergic subjects. The treatment can provide a long-lasting cure for allergic diseases without the need for continuous therapeutic intervention [6]. The efficacy of specific immunotherapy has been documented in numerous controlled clinical trials and a WHO position paper outlines guidelines for current practice of immunotherapy [7]. However, the underlying immunological mechanisms are still not clear [8–11].

Immune-based allergen immunotherapy modifies the cascade of underlying adverse immune responses in allergy and asthma in an antigen-specific manner. Since the target is to modify the immune response, specific immunotherapy has been termed as a 'vaccine' for allergic disease [12]. The mode of action of specific immunotherapy is complex. Specific immunotherapy has been shown to decrease the number of mast cells in tissue and also inhibits the release of pharmacologic mediators from mast cells and basophils [13]. The 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 [14]. 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 the induction of more Th1-like responses with increased allergen-induced interferon (IFN)-γ and IL-12 [15]. These changes may explain the marked inhibition of the late-phase reaction induced by immunotherapy. After several months or years of treatment, the intensity of the early, immediate, wheal and flare reaction is also reduced and total serum IgE levels are decreased.

Recently, there has been a resurgence in the classic blocking antibody paradigm of the mechanisms of immunotherapy [16]. Blocking antibodies, IgG, compete with IgE for allergen. They may also prevent the aggregation of IgE and its receptor (FcεRI) on the mast cells by altering steric confirmation. Blocking antibodies induced by immunotherapy regime can also inhibit the IgE-mediated presentation of allergens to T-cells and thus interfering with chronic manifestations of atopy [17].

There are drawbacks to conventional immunotherapy. One of the main problems of immunotherapy is that the administration of crude allergen extract could cause life-threatening anaphylactic shock. Moreover, allergen extracts for therapy are from natural allergen sources containing various allergenic and nonallergenic components and complex allergen extract can induce new IgE reactivities towards other components of the extract. The amount of these components may vary markedly from batch to batch therefore, it is very hard if not impossible to control the concentration of the antigen.

Pollen: source of most potent environmental allergens

Allergy to pollen is one of the most commonly encountered sensitivities, being a major causative factor in seasonal rhinoconjunctivitis. Pollen of grass, weed (ragweed) and tree (birch) are the source of most potent environmental allergens. 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 I allergic patients are sensitized against grass pollen allergens, thus making grass pollen the most frequent elicitor of allergic symptoms [18]. Individuals sensitive to the allergens from one grass are often sensitive to those of a number of other grasses [19]. Rye grass and closely-related grasses of subfamily *Pooideae* are distributed in cool temperate regions in North America, Europe and southern parts of Australia. In warm temperate and sub-tropical environments pollen of Bermuda grass (subfamily *Chloridoideae*) is an important source of allergens.

Pollen of trees, such as birch, alder, oak are the principal source of winter and spring allergens in temperate countries while ragweed pollen is the major cause of fall hayfever in the eastern part of North America [19].

Major pollen allergens have been identified & cloned

Molecules in pollen that provoke allergenic reactions have been identified and characterized [19]. These are proteins or glycoproteins of variable (5–80 kDa) size.

Two different proteins, group 1 and 5, have been described as the major allergens of grass pollen [20,21]. More than 95% of grass pollen-allergic patients have IgE recognizing these allergens [20,21]. Group 1 allergens, 30–35 kDa proteins, are localized in the cytoplasm of the pollen grain but are released rapidly upon hydration on the stigma surface or on human mucosal surfaces [20,21]. The cDNAs encoding for group 1 allergens from rye grass (*Lol p 1*), canary grass, timothy grass and velvet grass have been cloned [22]. Group 5 allergens are highly IgE-reactive proteins of molecular mass 28–32 kDa and

were initially identified as IgE-reactive fusion proteins in grass pollen cDNA expression libraries [21]. It has also been shown that group 5 allergens can be distributed as micronic particles in the atmosphere and can act as triggers for grass pollen-induced allergic asthma [23]. Group 1 and 5 together account for most of the IgE-binding capacity of crude pollen extracts. In the case of rye grass extracts, nearly two thirds of IgE-binding capacity is accounted for by Lol p 5 [24]. This has been confirmed in our laboratory by antisense-mediated silencing of Lol p 5 production in pollen of transgenic rye grass plants [25]. Major allergens from birch, alder, hornbeam, hazel and white oak tree pollen were found to be of 15–17 kDa in size while Japanese red cedar pollen allergens range from 37 to 50 kDa. Weed pollen allergy is mainly attributed to ragweed (*Ambrosia artemisiifolia*) and mugwort (*Artemisia vulgaris*). Though a number of proteins from ragweed pollen ranging from less than 10 to 60 kDa have been found to be IgE binding, two allergens, Amb a 1 and Amb a 2, of approximately 40 kDa have been identified as major allergens of ragweed pollen [26]. Three allergens from mugwort pollen, Art v 1, Art v 2 and Art v 3, have been studied in some detail. Recently, analysis of the 3D structure of Art v 1, the major mugwort pollen allergen, indicate that it is a secreted glycoprotein of approximately 11 kDa with an N-terminal cysteine-rich domain homologous to plant defensins [27]. Although over 60 allergen molecules from pollen of more than 25 plant species have been studied (their sequences are available from the allergen data bank [101]), molecular and structural analyses of allergens from various sources indicate that there are no apparent common motifs or biological properties that predict their allergenic nature.

Approaches to improve safety & efficacy of immunotherapy

Recombinant pollen allergens

As mentioned above, with the advent of molecular biology, isolation and cloning of cDNAs encoding most of the clinically important environmental allergens has been possible [19]. These recombinant allergens have been shown to retain their IgE reactivity hence can be used in both *in vivo* (skin testing) and *in vitro* (allergen-specific IgE antibody detection) diagnostic tests [20–22]. Recombinant allergens offer several advantages in the diagnosis and therapy of allergic diseases [28,29]:

- They can be produced in large quantities in a standardized way
- They can be used to identify disease-eliciting allergens and thus help to establish a reactivity profile that could allow immunotherapy to be tailored to individual patients
- Recombinant technology also allows modification of allergen to produce hypoallergen with reduced or nil allergenic derivatives, with the expectation that fewer injections of high allergen doses will be required with a lower risk of side effects
- 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

Use & modification of naturally occurring allergens

High IgE reactivity, which makes recombinant allergens suitable for allergy diagnosis is detrimental for their use in immunotherapy because of the purity of the recombinant allergen preparation – their anaphylactogenic potential is expected to be very high even at very low doses. Therefore, it is necessary to reduce IgE reactivity of the recombinant allergen for therapeutic use. Various approaches have been used (TABLE 1) as described below:

Allergoids: chemical modification of allergen

Chemical modification (using formaldehyde, glutaraldehyde and potassium cyanate) of allergens to reduce allergenicity as measured by *in vivo* (skin tests) and *in vitro* (histamine release) tests without loss of T-cell immunogenicity of native allergen have been used for immunotherapy [30–32]. Similar chemical modification of recombinant allergens as a means to reducing IgE reactivity has been suggested, but chemical modification of recombinant allergens such as allergen extract from natural source is difficult to standardize and therefore unlikely to be used in immunotherapy [33]. In addition, use of allergoids not only decreases binding of the allergen by IgE, it also reduces immunogenicity.

Expression of naturally occurring hypoallergenic forms

Molecular cloning of allergens revealed that major allergens could be encoded by gene families. In fact, major allergens of weed, grass and tree pollen have been reported to belong to a multigene family [34–36]. 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 [37]. The observation of differences in IgE reactivities has led to the expectation that low IgE reactive forms can potentially be used for immunotherapy. This possibility has been explored with Bet v 1 [37]. The isoform, Bet v 1d was found to have low IgE reactivity and high T-cell-stimulatory capacity. Since Bet v 1 is a major birch tree pollen allergen as demonstrated by binding of IgE in more than 95% of birch pollen allergic patients, treatment with high doses of this hypoallergenic form can potentially be used for birch pollen immunotherapy [38]. However, no such low IgE reactive forms have been identified for other pollen allergens.

Sequence alteration: deletion & site-directed mutagenesis

Reduction in IgE binding capacity by modification of B-cell epitopes without loss of T-cell epitopes is possible and promises increased efficacy and safety, by allowing T-cell interaction whilst avoiding the risk of IgE cross-linking and mast cell/basophil degranulation. This concept has been used to genetically engineer tree and grass pollen allergens for immunotherapy. Two approaches, deletion of some segments of the gene and precise mutation in the molecule have been attempted. These are based on prior knowledge of B- and T-cell epitopes of the allergen molecule.

Tree pollen allergen

In the case of birch tree pollen, 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 [39]. 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 1000-fold reduction in the ability to induce a Type I wheal reaction in skin prick tests demonstrating that these engineered allergens have the potential to be used as a safer form of birch pollen immunotherapy than that currently applied.

Grass pollen allergen

Group 1 allergen of grass pollen is one of the major grass allergens. Its C-terminal region of 29 amino acids was shown to be important for IgE binding [40]. Tamborini *et al.* produced three deletion mutants lacking large portions of protein internally or at terminal regions: C-terminal mutant containing amino acids 1–115, N-terminal mutant had amino acids 89–240 present, while internal deletion mutant had amino acids 133–189 lacking in the molecule [41]. Both N- and C-terminal mutants showed significant reduction in IgE reactivity and histamine release. However, skin prick reactivity or T-cell proliferation was not attempted in this study therefore limiting the use of these mutants for immunotherapy.

Reduced IgE-binding variants of timothy grass (*Phleum pratense*) allergen, Phl p 5, were attempted using site-directed mutagenesis [42]. Dominant T-cell epitopes of Phl p 5 were identified using overlapping dodecapeptides. Point and deletion mutations were obtained within the putative IgE-binding while maintaining the identified T-cell epitopes to reduce the IgE reactivity as tested both by *in vitro* (immunoblotting, enzyme-linked immunosorbent assay [ELISA] inhibition and histamine release) and *in vivo* (skin prick) tests. The point mutant 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 contained a new cysteine residue (A13 to C) in addition to wild type cysteine residue, C186. Schramm *et al.* expected that formation of an intramolecular disulfide bond between these cysteines should result in a drastic change in molecular conformation of Phl p 5 and hence its IgE reactivity [42]. However, no change in IgE reactivity was observed. Various deletion mutants of Phl p 5 were also constructed, which lacked varying stretches of the internal sequences. Some of these deletion variants showed reduced IgE reactivity. The histamine-release capacity was completely abolished in some of these deletion mutants. In addition, a significant reduction in skin prick reactivity was also observed while the T-cell reactivity remained unchanged [42].

Recently, we used group 5 allergen of rye grass pollen for site-directed mutagenesis study in order to reduce its allergenicity for specific immunotherapy of the grass allergy [43]. The basis of the study was to introduce site-directed mutations in highly conserved sequence domains of Lol p 5, the group 5 allergen of rye grass. Extensive IgE epitope mapping using overlapping recombinant fragments and synthetic peptides [44,45] and sequence comparison of group 5 allergens from other grasses were used to identify conserved IgE binding sites in group 5 allergens with little overlap with dominant T-cell epitopes [46]. Circular dichroism analysis showed that these mutants exhibited an overall structural fold similar to the recombinant Lol p 5 wild type allergen. In contrast to the timothy grass study where the deletion of IgE-reactive domains was necessary to reduce the allergenic activity of the group 5 allergen from timothy grass [42], we found that the exchange of a few amino acids within the IgE-reactive domains of Lol p 5 could reduce IgE recognition. The lowered IgE reactivity of the mutants also resulted in a reduction of their allergenic activity as demonstrated by basophil histamine release experiments and skin prick testing in grass pollen allergic patients. A tenfold or more reduction of histamine release activity was observed. In addition, these Lol p 5 mutants maintained to a high degree 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. Moreover, mutants generated could also be used 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 will likely reduce the rate of anaphylactic side effects by a factor of ten compared with extract-based treatment. 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.

Other pollen allergens

Small, water-soluble Ca^{2+} -binding proteins of approximately 9 kDa has been identified as pollen allergens in grass [47,48], tree [49,50] and mustard crop [51]. These (Bra r 1, Cyn d 7 and Bet v 4) allergens not only show significant IgE cross-reactivities, but also share significant sequence similarities extending outside the Ca^{2+} -binding domains and thus, could be considered as cross-reactive allergens [49,50]. These Ca^{2+} -binding protein with two EF hands show preferential expression in pollen [50,51]. and IgE reactivity of these allergens is greatly influenced by bound Ca^{2+} . The hypothesis that perturbing the Ca^{2+} -binding activity of the proteins should generate non-IgE-binding

forms was tested by Engel *et al.* using site-directed mutagenesis to produce non- Ca^{2+} -binding variants of the birch pollen allergen, Bet v 4 [49]. However, the disruption of Ca^{2+} -binding site I did not significantly affect IgE reactivity for most of the patients tested demonstrating that this domain may not be important in IgE reactivity. On the other hand, the loss of Ca^{2+} -binding activity of site II of Bet v 4 led to a reduction in IgE reactivity for most of the patients, indicating functional domain. In our laboratory, we performed site-directed mutagenesis experiments on the mustard crop allergen, Bra r 1 and showed that both EF-hand domains constitute functional Ca^{2+} -binding sites [51]. Furthermore, we also demonstrated that disruption of both Ca^{2+} -binding sites resulted in decreased IgE binding to Bra r 1. Based on these results, we proposed that such variants of Ca^{2+} -binding proteins with greatly reduced IgE could be used as ideal immunotherapy reagents [52].

Conformational changes

The concept that 3D structure or folding is important for IgE binding has also been explored to obtain nil or low IgE forms of grass and tree pollen allergen for specific immunotherapy.

Grasses belonging to subtribe *Poodeae* are extensively used in modern-day agriculture. Group 1 allergen is exclusively expressed in pollen of these grasses. As mentioned above group 1 and 5 are the two major allergens accounting for more than 90% of IgE reactivity in most allergy sufferers. Isolation of group 1 allergen genes from a number of grasses has revealed significant levels of amino acid conservation and the presence of seven strictly conserved cysteine residues within these proteins, suggesting a pattern of disulfide bond formation and protein folding between the protein. Though importance of intramolecular disulfide bonds in maintaining conformation of IgE epitopes on the molecular surface has been suggested in number of studies, the role of cysteine residues in allergenicity of group 1 allergens has not been demonstrated [53]. Recently, we studied the effect of conformational changes in Lol p 1 on IgE reactivity by the specific replacement of the seven cysteine residues in the molecule via site-directed mutagenesis. Cysteine residues were replaced with serine residues at 72, 77, 83 and 139 amino acid positions of rLol p 1 resulting in a reduction in IgE binding of Lol p 1 [54]. Up to a 60% reduction in IgE binding was observed. Our study showed that although not all cysteine residues may be critical to maintain IgE reactivity of this molecule, one of the cysteine residues (at 77) in Lol p 1 protein plays a role in IgE binding. However, disruption of disulfide bonds of Phl p 1 fail to affect IgE reactivity [55]. Moreover, one would like to think that this type of altered molecule could not be used as a safer molecule for immunotherapy because disruption of disulfide bonds will lead to denatured protein with a disrupted secondary structure.

Birch tree pollen allergen, Bet v 1, is known to have conformational IgE epitopes that are formed by the correct folding of the polypeptide chain [56,57]. The concept that changing the overall tertiary structure of this protein may disrupt the IgE epitopes and thus prevent allergen-specific IgE recognition was studied by

Vrtala *et al.* by expressing Bet v 1 as two fragments while preserving all T-cell epitopes of Bet v 1 [58]. Western and dot blot analyses of the fragments with sera from a number of birch pollen-allergic individuals showed almost no IgE binding towards these fragments. The CD spectra analysis of these two fragments showed a dramatic change in conformation from the complete Bet v 1 molecule. In addition mixing these fragments in solution failed to revert the changed conformation to the original Bet v 1 molecule. The ability of these fragments to induce T-cell proliferation was retained and together the fragments were shown to contain all T-cell epitopes present on the original Bet v 1 protein. Moreover, these fragments were shown to have reduced anaphylactic activity as demonstrated by histamine release induced from basophils and skin prick tests. A 1000-fold reduction in histamine release from basophils was observed as compared with the complete rBet v 1 and skin-prick tests on six birch pollen-allergic individuals showed no wheal reaction to the either fragment indicating such approach potentially could be used in birch pollen-specific immunotherapy.

Use of synthetic allergen molecules

Imaginative ideas as to whether new hypoallergenic forms can be produced by combining parts of different allergen while preserving B- and T-cell epitopes have been tested by producing recombinant allergen oligomers and hybrid allergen molecules (TABLE 1).

Hypoallergenic oligomers

Recently, Vrtala *et al.* explored the possibility of synthesizing hypoallergenic oligomers of Bet v 1, the major birch pollen allergen, by a recombinant DNA approach [59]. The objective was to preserve the overall structure including B- and T-cell epitopes of the molecule while generating a stable hypoallergenic oligomer of Bet v 1. Two types of recombinant oligomers, dimer and trimers were generated and demonstrated that the recombinant oligomers (trimer) preserved the structural features (shown by CD spectrum) and showed a 100-fold reduction in allergenic activity as measured by basophil histamine release and skin-prick tests. Some possible explanations of reduced allergenicity of the trimer could be that linking molecules may cause relative reorientation of epitopes without changing the secondary structure, resulting in less efficient cross-linking of FcεRI-IgE. The trimer may have different affinity, microaggregation, steric hinderance and unfavorable charge interaction. In addition, trimer also induced Th1 cytokine release and induced IgG antibodies capable of blocking IgE antibodies from Bet v1-allergic patients, therefore demonstrating that it is possible to synthesize hypoallergenic variants of a potent allergen while preserving immunological features – including the induction of blocking antibodies and favorable Th cytokine balance. Thus, the oligomer approach can be used to produce an hypoallergenic vaccine for the treatment of birch pollen allergy. No such approach has been tested for other pollen allergy. It will be interesting to know whether production of hypoallergenic oligomer will be applicable more widely, such as grass pollen allergy.

Table 1. Approaches used to genetically engineer pollen allergen for reduced anaphylactic potential.

Approach	Allergen	Pollen source	IgE reactivity	T-cell reactivity	Ref.
Use and modification of naturally occurring allergens					
Expression of hypoallergenic forms	Bet v 1	Tree (Birch)	Low	Retained	[37]
<i>Sequence alteration:</i>					
Deletion					
	Phl p 5	Grass (Timothy)	Low	Retained	[42]
	Lol p 1	Grass (Rye grass)	Low	nd	[41]
Site-directed mutagenesis					
	Bet v 1	Tree (Birch)	Low	Retained	[41]
	Phl p 5	Grass (Timothy)	No change	-	[42]
	Lol p 5	Grass (Rye grass)	Low	Retained	[43]
	Bet v 4	Tree (Birch)	No change (Domain 1)	nd	[49]
	Bra r 1	Crop (Mustard)	Low	nd	[51]
<i>Conformational changes:</i>					
Disruption of disulfide bonds					
	Lol p 1	Grass (Rye grass)	Variable	nd	[54]
	Phl p 1	Grass (Timothy)	No change	nd	[55]
Disruption via breakage					
	Bet v 1	Tree (Birch)	Nil to low	Retained	[58]
Use of synthetic allergen molecule					
Oligomers	Bet v 1	Tree (Birch)	Low	Retained	[59]
Hybrid allergen	Phl p 1, p 2, p 5, p 6 (Phl p 2 + Phl p 6; Phl p 6 + Phl p 2; Phl p 5 + Phl p 1)	Grass (Timothy)	Low	Retained	[60]

Ig: Immunoglobulin; nd: Not determined.

Hybrid allergen molecule

It is well established that different grass pollen allergens display different immunogenicity and grass pollen immunotherapy using crude pollen extract induces variable responses in patients. With recombinant allergens, it is now feasible to diagnose and tailor treatment according to the patient's sensitization profile, preventing the induction of undesirable immune responses. However, treatment of allergy can nevertheless be hindered by differential immunogenicity of the individual allergen molecule, leading to variable immune responses. To overcome this problem Linhart *et al.* studied whether it is plausible to develop a combination vaccine based on recombinant hybrid molecules consisting of different grass allergen molecules [60]. Timothy grass pollen allergens were used to test this concept. Four major allergens of this grass (Phl p 1, Phl p 2, Phl p 5, Phl p 6) were expressed as recombinant hybrid molecules in three different combinations (Phl

p 2–Phl p 6, Phl p 6–Phl p 2, Phl p 5–Phl p 1) shown to possess most of the natural grass epitopes. Immunization with recombinant hybrid molecules induced not only stronger and earlier IgG responses as compared with individual allergens or allergen extracts but also IgG antibodies induced by the hybrid molecules were able to block the binding of allergic patients IgE to grass pollen allergen. The enhanced immune response was observed by linking a less immunogenic allergen with a highly immunogenic allergen, but also linking two different allergens with a low capacity to induce antibody. The results showed that by producing the hybrid molecule, it is possible to have a significant increase in immunogenicity of unrelated allergens with poor immunogenicity. Although the possible reason for such enhancement is unknown the approach deserves serious consideration for immunotherapy.

Other approaches

Genetic immunization

Genetic immunization for pollen allergy immunotherapy offers a challenging approach involving modifying the immune response to allergens by direct immunization with DNA encoding allergen protein. The concept of genetic immunization was first demonstrated in 1992 [61] and recently Johnston *et al.* [62] reviewed approaches towards improvement of this technology. DNA oligodeoxynucleotides, unmethylated CpG motifs are found in bacteria but are not common in vertebrates. These motifs have been used to induce a Th1 immune response either alone or in combination with allergen protein. Conjugates of CpG DNA to ragweed allergen, Amb a 1 have been shown to enhance immunogenicity by producing a Th1 profile in mice and reduce allergenicity [63]. DNA without CpG motifs or unconjugated allergen given with CpG was found not to be effective indicating that it is the conjugation of CpG DNA and allergen that is important at the antigen-presenting cell (APC) interaction. Similar results were obtained by Hartl *et al.* who immunized mice with plasmid DNA encoding Bet v 1 linked to CpG motifs [64]. The hybrid DNA vaccine induced an increased IFN- γ response by allergen challenged spleen cells compared with immunization with plasmid Bet v 1 DNA alone or recombinant protein. Direct comparison between CpG DNA therapy and conventional immunotherapy is lacking and hindered by contrasting views on the mechanisms of action of immunotherapy. Moreover, maintaining gene expression beyond a few weeks and targeting the DNA to a selected tissue site are among several difficulties to be overcome before this approach is accepted for pollen allergen immunotherapy.

Oral immunotherapy

Delivery of future allergen-specific immunotherapy may be based on current practices of subcutaneous injection but alternative routes are being investigated for improved patient compliance and reduced risk of systemic anaphylactic response. Oral administration of birch and ragweed pollen protein allergens in human trials showed that it can induce systematic and mucosal immune response when delivered directly to the gut mucosa in enteric-coated capsules making this approach simpler and easier to administer and effective in reducing allergy symptoms [65–67]. However, oral delivery requires much greater doses of antigen than that administered subcutaneously. As mentioned above, it is possible to produce large quantities of purified allergen by a recombinant approach, but whether these recombinant molecules are able to induce systemic response still needs to be investigated. Recently, using a highly purified recombinant form of the major rye grass pollen allergen, Lol p 5, we addressed this question using mice as an experimental model. Our results showed that oral delivery of this recombinant rye grass pollen allergen induced systemic antibodies able to block human IgE binding to this allergen [68]. In addition, induced antibodies were found to recognize homologous allergens from related grasses thus indicating that the oral route of delivery may provide an alternative protocol for improved efficacy of immunotherapy.

Expert opinion

When pollen comes in contact with mucous membranes of the human respiratory system, a nonatopic person displays an immunological response by producing allergen-specific IgG antibodies and shows a Th1 response. Conversely, an atopic person would have an exaggerated response characterized by the production of allergen-specific IgE antibodies hence displaying elevated levels of IgE antibodies in their serum and other typical Th2 responses. Specific immunotherapy is the only causative approach for the treatment of Type I allergy. Current treatment involves the injection of increasing doses of crude extracts of pollen containing disease-eliciting allergen over a long period of time that can extend from months to years. Clinical efficacy of specific immunotherapy has been demonstrated in a number of studies but its efficacy varies with the allergen. Current diagnosis and specific immunotherapy does not take into account a patient's sensitization profile. In addition, systemic administration of allergens can lead to severe life-threatening anaphylactic reactions. Purity of allergenic component for diagnosis and specific immunotherapy and improved safety of immunotherapy are the desired key features of future therapeutic reagents.

Recombinant allergens offer an exciting possibility of patient-tailored immunotherapy based on individual sensitization profiles. Recombinant allergens are easy to purify and standardize however, these allergens can not 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. However, genetic engineering techniques offer exciting possibilities of modifying recombinant allergens with low IgE binding to reduce the risk of anaphylaxis whilst containing most of the T-cell epitopes for immunotherapy thus providing a safer vaccine. Another important benefit of using genetically-engineered allergens is the possibility of retaining immunogenic features necessary for the induction of a protective immune response (i.e., induction of blocking antibodies) and to alter the Th cytokine balance in a favorable way. Safer and more efficacious vaccines would increase patient compliance and ultimately lead to more widespread use of immunotherapy. Thus, these hypoallergenic allergens are potential candidates for specific immunotherapy in future.

Five-year view

Current allergy diagnosis analyzes the source, such as the general class of pollen (grass or tree or ragweed pollen), but are unable to identify the disease-eliciting allergen molecule. Use of microarray technology in determining and monitoring of allergic patients IgE reactivity profiles to the allergens has been recently demonstrated by Hiller *et al.* [69]. Such technology offers additional advantages of being a fast and reliable method of diagnosis of the disease-causing molecule(s) rather than the organism using very small quantities of patient's serum. In addition, it has potential to provide overall IgE reactivity profiles of a patient in a single test. With the use of such technology, it is easier to monitor the onset and

development of allergy in early childhood. Therefore, such technology will have significant impact on changing existing practice of allergy diagnosis.

New therapeutic agents for a hayfever vaccine with enhanced safety and efficacy features will be based on developing hypoallergenic forms. Modified recombinant molecules or polypeptides that by virtue of its primary structure did not have the capability of producing IgE binding structures could be an ideal candidate for immunotherapy. Such 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 APCs as the natural allergen. An alternative delivery route, oral immunotherapy, using either protein or DNA for improved patient compliance and reduced risk of systemic anaphylactic response is also likely to have impact on future hay fever vaccine. Whilst current immunotherapy is aimed at treating the established disease, the challenge is to develop a prophylactic vaccine against allergy and hypoallergenic derivatives will be required to avoid undesirable sensitization during prophylactic treatment.

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Key issues

- Type I allergic diseases, such as rhinitis or hayfever, rhinoconjunctivitis and allergic asthma, represent a major health problem affecting more than 25% of the world's population. Grass, tree and ragweed pollen are a major source of environmental allergens in different parts of the world. More than 400 million individuals are sensitized to grass pollen allergens alone.
- Current pharmacotherapy-based treatment focuses on the treatment of symptoms while specific immunotherapy is the only curative approach towards the treatment of Type I allergy.
- Although specific immunotherapy is a clinically proven, effective treatment for allergic conditions, the reagents used for immunotherapy are crude extracts prepared from natural sources and contain additional, ill-defined components so it is 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.
- Recombinant allergens offer an exciting possibility in the diagnosis of disease-eliciting molecules and patient-tailored immunotherapy based on individual sensitization profiles.
- Genetic engineering of allergen with nil or low IgE reactivity but retained T-cell reactivity offers a novel therapeutic approach to improve safety and efficacy of allergen-specific immunotherapy.
- Improved safety and efficacy would increase patient compliance and satisfaction.

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