

Genetic Engineering of Allergens: Future Therapeutic Products

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Key Words

Allergen · Immunotherapy · Hypoallergen · Genetic engineering · Recombinant allergen

Abstract

Genetic engineering of allergens for specific immunotherapy should aim at the production of modified molecules with reduced IgE-binding epitopes (hypoallergens), while preserving structural motifs necessary for T cell recognition (T cell epitopes) and for induction of IgG antibodies reactive with the natural allergen (blocking antibodies). Common approaches for engineering of hypoallergens usually require knowledge of T and B cell epitopes and involve changing specific base pairs (mutated gene), introduction of a new piece of DNA into the existing DNA molecule (chimeric or hybrid gene), and deletions (truncated gene or fragments). DNA family shuffling has the advantage that it does not require a priori knowledge of structural and functional properties for efficient generation of hypoallergens. The combination of the hypoallergen concept with the Th1-inducing genetic immunization approach might be an attractive alternative for protein-based immunotherapy.

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Introduction

Allergen-specific immunotherapy (SIT) is an effective form of treatment and represents the only curative approach for type I allergy. It is capable of modifying the natural course of the disease by preventing the worsening of symptoms (from rhinitis to asthma) and the onset of sensitization against new allergens [1–3]. During immunotherapy, increasing doses of natural allergen preparations are given on a regular basis, and the beneficial effects for the patient depend on the amount of allergen used. Because treatment involves injecting allergens into a sensitized organism, the occurrence of typical allergic symptoms is frequent, with risks increasing with the concentration of allergen applied per injection.

The mechanism operating in SIT is still not completely understood. It seems that several immunological pathways are involved in the clinical improvement achieved by the common SIT schedules [reviewed in ref. 4–7]: (1) rise in allergen-specific IgG antibodies, in particular IgG4, which is supposed to block the allergen and IgE-facilitated allergen presentation to T cells [8–10]; (2) generation of IgE-modulating CD8+ T cells [11]; (3) reduction in the number of mast cells, eosinophils, and release of mediators [12, 13], and (4) modulation of allergen-spe-

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cific T cells: a shift from Th2 to Th1 cytokine pattern with a decrease of IL-4 and IL-5 production accompanied by an increase of IFN- γ (immune deviation) [14–16]. Moreover, the induction of an anergic state in peripheral T cells (immunologic tolerance), which is mediated by IL-10 and characterized by suppressed proliferative and cytokine responses against major allergens, represents an important aspect in successful SIT [14, 17, 18].

Concept for the Development of Recombinant Allergen-Based Vaccines

In the past few years, efforts have been concentrated on developing approaches to address three major problems associated with SIT: (1) presently SIT is performed with allergen extracts (containing mixtures of allergens, nonallergenic and/or toxic proteins, and other macromolecules), which are difficult to standardize; (2) systemic administration of allergens can cause severe IgE-mediated side effects during the treatment, and (3) therapeutically effective doses often cannot be achieved because of non-standardized extracts or side effects.

In principle, these problems could be overcome with the use of genetically engineered allergens [reviewed in ref. 19–23]. Cocktails of pure and standardized recombinant allergens can be formulated to replace natural extracts. Moreover, the selected recombinant allergens can be engineered to reduce the risk of IgE-mediated side effects. In this way, high doses of allergen can be administered to allergic patients increasing the efficacy of the treatment.

Based on the above considerations, genetic engineering of allergens for SIT should aim at the production of modified molecules with reduced IgE-binding epitopes (hypoallergens), while preserving structural motifs necessary for T cell recognition (T cell epitopes) and for induction of IgG antibodies reactive with the natural allergen (blocking antibodies).

The uptake of allergens by antigen-presenting cells (APCs) is mediated and facilitated by the interaction of the allergen with specific IgE [24–26] and leads to higher Th2 cytokines and IgE production [27]. Engineered allergens lacking IgE binding might avoid these pathways and preferentially target APCs that utilize phagocytosis or pinocytosis for antigen uptake (e.g. monocytes, macrophages and dendritic cells). This in turn induces a balanced Th0- or Th1-like cytokine production by T cells, low IgE and high IgG production by B cells. The presence of intact T cell epitopes on hypoallergens would also tar-

Table 1. Approaches for genetic engineering allergens

Full-length allergens with reduced IgE binding activity
Site-directed mutants
Conformational variants
Allergen fragments
Deletion mutants
Allergen oligomers
Allergen chimeras
Hybrids of allergens and homologues
Shuffled allergens

get T cells allowing the administration of higher doses to induce tolerance of allergen-specific T cells and alteration of cytokine production towards a Th1-like pattern.

Approaches for Genetic Engineering of Allergens for SIT

Genetic engineering comprises the targeted modification of a protein in order to alter its function or properties in a predictable manner and usually requires complete understanding of the relationship between structure and function/properties for precise and effective manipulation. Alteration of the gene can involve changing specific base pairs (mutated gene), introduction of a new piece of DNA into the existing DNA molecule (chimeric or hybrid gene), and deletions (truncated gene or fragments). The advent of recombinant allergens has enabled their selective alteration in order to produce second-generation molecules with properties specifically modulated for use in more efficient and safer approaches of immunotherapy. To date, cDNA sequences of 60 pollen allergens from 27 plant species have been deposited in the allergen databank (<http://www.allergen.org>). We will discuss here several approaches (table 1) and examples of engineered pollen allergens (table 2). With the exception of the DNA shuffling approach [56], which bypasses the need to identify amino acid residues or motifs that are important to structure and function, altering allergen genes requires knowledge of B and T cell epitopes and in some cases also of the three-dimensional structure of the allergen.

Full-Length Allergens with Reduced IgE-Binding Activity (Hypoallergens)

Our findings that certain naturally occurring isoforms of Bet v 1, the major birch pollen allergen, display high T cell antigenicity and low/no IgE binding activity

prompted us to suggest the use of full-length hypoallergenic variants for immunotherapy [28, 29]. Hypoallergenic variants of allergens can also be obtained by targeting B cell epitopes to produce site-directed mutants or conformational variants.

Site-Directed Mutants. Various approaches have been used to identify antibody-binding sites of allergens. After identification of crucial amino acid residues or motifs involved in IgE recognition, dominant epitopes can be targeted using site-directed mutagenesis [30].

Our findings showing that Bet v 1 and other closely related tree pollen allergens consist of a mixture of closely related isoforms displaying striking differences in their ability to bind IgE [28, 31] constituted the basis for engineering a Bet v 1 hypoallergen [32]. The patterns of amino acid substitutions in tree pollen isoallergens and their IgE-binding activities were analyzed using a computer algorithm developed to predict functional residues in proteins [33]. This 'sequence space' method relies on the translation of sequences into vectors. Each sequence is represented as a vector point in a multidimensional space (sequence space), with residue position and residue type as the basic dimensions. At each position in the sequence a summary is given of which amino acid type occurs at what frequency in the protein family, in our case the group I of tree pollen allergens. Differences between isoforms can be picked by this algorithm by defining a 'functional direction' in the sequence space. In this case, functional axes that separate isoforms according to their allergenicity were defined manually by assigning +1 for high IgE-binding and -1 for low/no IgE-binding activity. From the combined information of the IgE reactivity patterns of tree-pollen-allergic patients to the fourteen isoallergens, we obtained a list of eight most common residues/amino acid positions. We chose to replace the amino acid residues occurring at positions 113, 57, 125, 112, 30, and 10 of Bet v 1a by those present in the same positions of low IgE-binding isoforms by *in vitro* site-directed mutagenesis. A Bet v 1 mutant carrying six point mutations was thus produced, which displayed extremely low IgE-binding activity in all patients tested. *In vivo* (skin prick) tests indicated that the potency of the six-point mutant to induce typical urticarial skin reactions in allergic individuals was dramatically reduced (100- to 1,000-fold) compared to Bet v 1a. T cell clones (TCC) established from the peripheral blood of birch-pollen-allergic patients and reactive with Bet v 1a were also activated by the six-point mutant (table 3). From these experiments, we concluded that it is possible to modulate the IgE-binding properties of allergens without significantly changing their recognition by allergen-specific T lymphocytes [32].

The observation that IgE recognition of a group of calcium-binding allergens found in various pollens was influenced by bound calcium led to the idea of disruption of EF-hand calcium-binding domains for engineering hypoallergenic mutants. In this way, calcium-binding deficient mutants of Bet v 4 [34] and Bra r 1 [35] with reduced IgE-binding activities were generated. However, no data are available concerning T cell recognition of calcium-binding allergens and their engineered counterparts (table 3).

B and T cell epitope mapping and sequence comparison of group 5 allergens from different grasses provided the basic information for introducing point mutations in highly conserved sequence domains of Lol p 5. Hypoallergenic forms of Lol p 5 were produced that contained all relevant T cell epitopes [36].

Conformational Variants. The three-dimensional structure of an antigen and its recognition by different APCs seem to be crucial aspects in the development of distinct T cell cytokine patterns. Akdis et al. [27] demonstrated that bee venom phospholipase A₂ (PLA) preparations lacking the native conformation and antibody-binding activity were exclusively presented by monocytes and induced a Th1-biased cytokine profile leading to IgG4 production by B cells. In contrast, folded PLA with full antibody-binding activity was processed and presented by B cells, stimulated Th2-like cytokines and induced IgE antibodies. These findings give further support to the use of hypoallergens in immunotherapy.

Following this line, hypoallergenic variants of the major allergen of Par j 1 were produced [37]. Par j 1 is a member of the nonspecific lipid transfer proteins (nsLTPs) with a characteristic α - α - α - β structure, which is stabilized by four disulfide bonds [38]. Targeting these disulfide bonds by site-directed mutagenesis resulted in molecules with altered conformation and decreased IgE-binding activity, but at the same time able to stimulate T cell proliferation (table 3). Disruption of the native conformation by targeting disulfide bonds could be an approach generally used for engineering allergenic nsLTPs, including food-derived members.

Allergen Fragments

Allergens harbor a diversity of T and B cell epitopes. T cells recognize short linear peptides created by APCs through antigen processing and displayed at the cell surface with molecules of the MHC complex [39, 40]. Mapping of T cell epitopes of allergens showed the presence of multiple epitopes scattered over the whole sequence [reviewed in ref. 41]. IgE epitopes can be formed by a stretch of amino acids in a row (continuous epitopes) or by non-

Table 2. Engineered pollen allergens

Birch	Bet v 1	site-directed mutants [32] 2 fragments, 74 and 85 amino acids [42] trimer [50]
	Bet v 4	site-directed mutants [34] 1 fragment, 68 amino acids [46]
Alder	Aln g 4	2 fragments, 41 and 43 amino acids [47]
Ryegrass	Lol p 1	2 fragments, 115 and 151 amino acids [48]; deletion mutant, Δ 132–190 [48]
	Lol p 5	site-directed mutants [36]
Timothy grass	Phl p 5b	deletion mutants [49]
Parietaria	Par j 1	conformational variants, S-S bonds [37]
Oil seed rape	Bra r 1	site-directed mutants [35]

adjacent sequence elements brought together by folding (discontinuous or conformation-dependent epitopes). IgE recognition of continuous epitopes might also depend on their conformation, which might only occur in the context of the folded allergen molecule.

Thus, the disruption of the three-dimensional structure by fragmentation might represent a useful approach to reduce the anaphylactic potential of allergens. The three-dimensional structure of Bet v 1 was disrupted by expressing in two fragments of the cDNA in *Escherichia coli*, which corresponded to amino acids 1–74 and 75–160 [42]. The fragments exhibited random coil conformation and almost no allergenicity. Together, the fragments harbored all relevant T cell epitopes. Skin reactivity and histamine release were greatly reduced when compared to the native intact Bet v 1 allergen [43, 44]. Moreover, immunization of mice and rabbits with Bet v 1 fragments induced IgG antibodies that inhibited the binding of IgE from allergic patients to wild-type Bet v 1 [45]. Clinical trials will now show the efficacy of vaccines based on Bet v 1 fragments for the immunotherapy of patients allergic to birch pollen.

Fragments of the calcium-binding allergens Bet v 4 [46] and Aln g 4 [47], and an N-terminal fragment of Lol p 1 from ryegrass [48] also showed decreased IgE-binding activities. However, they were not investigated concerning T cell reactivity and immunogenicity (table 3).

Deletion Mutants

In principle, knowledge on IgE-reactive regions of allergens could be used to engineer hypoallergenic variants by deleting the corresponding DNA segment in the

gene. This approach was successfully used for the timothy grass pollen allergen Phl p 5b [49]. Epitope mapping was performed using overlapping recombinant fragments, and at least 4 continuous IgE-binding epitopes were identified. Deletions avoiding identified T cell epitopes were then performed within these IgE-binding regions. Some of these deletion mutants showed reduced IgE-binding properties, no histamine-releasing activity, and reduced skin reactivity. As the deleted fragments were relatively small, no significant changes in T cell reactivity were observed.

Based on the results obtained by proteolytic fingerprinting, a deletion mutant of ryegrass Lol p 1 was produced, which displayed decreased IgE-binding activity and did not trigger histamine release up to a concentration of 10 mg/ml [48]. The mutant was not tested in T cell proliferation assays and for skin reactivity.

Allergen Oligomers

With the aim of engineering a recombinant allergen derivative with preserved structural features important for immune recognition (B and T cell epitopes) but with reduced anaphylactic potential, Vrtala et al. [50] constructed a Bet v 1 trimer. This oligomeric form consisted of three copies of full-length Bet v 1 cDNA linked by short oligonucleotide spacers in one open reading frame. Recombinant Bet v 1 monomer and trimer produced in *E. coli* showed comparable in vitro IgE-binding activity and strongly stimulated Bet v 1-specific TCC and peripheral blood mononuclear cells from birch-pollen-allergic patients. Interestingly, the Bet v 1 trimer exhibited extremely low histamine release from patients' basophils and skin reactivity [43, 44] compared to the monomer. When injected in mice and rabbits, the trimer induced IgG antibodies that inhibited human IgE binding to Bet v 1 monomer [50]. Several explanations were put forward as to why the IgE-binding Bet v 1 trimer showed a reduced anaphylactic potential: (1) lower affinity for IgE binding, (2) re-orientation of IgE epitopes preventing efficient cross-linking of Fc ϵ RI-bound IgE antibodies, and (3) microaggregation, steric hindrance and/or unfavorable charge interactions causing concealment of IgE epitopes required for efficient cross-linking.

Allergen Chimeras

An interesting approach to engineering of hypoallergens consists in producing hybrids or chimeras in which portions of homologous proteins are combined to form new hybrid molecules with defined features.

Hybrids of Allergens and Homologues. King et al. [51] reported an approach to genetically modify allergens in

Table 3. Evaluation of genetically engineered pollen allergens

Allergen	Engineered form	T-cell reactivity	Anaphylactic potential	Immunogenicity
Bet v 1	site-directed mutant	Bet v 1-specific TCC	negative SPT in 6/11 patients	ND
	2 fragments	Bet v 1-specific TCC PBMC	1,000-fold reduction in histamine release negative SPT in 18/23 patients negative IDT in 8/23 patients	induction of Bet v 1-reactive IgG Abs in mice and rabbits
	trimer (IgE-reactive)	Bet v 1-specific TCC PBMC	negative SPT in 15/23 patients negative IDT in 13/23 patients	induction of Bet v 1-reactive IgG Abs in mice and rabbits
Bet v 4	site-directed mutant 1 fragment	ND ND	ND ND	ND ND
Aln g 4	2 fragments	ND	ND	ND
Lol p 1	2 fragments deletion mutant	ND ND	reduced histamine release no histamine release up to 10 mg/ml	ND ND
Lol p 5	site-directed mutants	group 5-specific TCC and TCL	reduced histamine release and SPT	ND
Phl p 5b	deletion mutants	Phl p 5-specific TCC and TCL	reduced histamine release 10- to 100-fold reduction in SPT	ND
Par j 1	conformational variants	PBMC	negative SPT	ND
Bra r 1	site-directed mutant	ND	ND	induction of Bra r 1-reactive IgG Abs in mice

TCC = T cell clones; TCL = T cell lines; SPT = skin prick test; IDT = intradermal test; PBMC = peripheral blood mononuclear cells;
ND = not determined; Abs = antibodies.

which hybrids are prepared that consist of a small portion of the guest allergen of interest and a large portion of a homologous but weakly cross-reacting host protein. The idea is that the homologous host protein serves as a scaffold to maintain the native structure of the guest allergen of interest in order to preserve conformational-dependent B cell epitopes, but at a reduced density. The homologous allergens from yellow jacket venom Ves v 5 and from paper wasp Pol a 5 (59% sequence identity) show very limited cross-reactivity of antibodies from sensitized patients. Hybrids of these two molecules containing 10–49 residues of Ves v 5 showed 100- to 3,000-fold reduction in allergenicity in histamine release assays with basophils from yellow-jacket-sensitized patients. Mice immunized with these hybrids produced antibodies recognizing both Ves v 5 and Pol a 5 [51]. Thus, it remains to be established whether treatment with hybrids would lead to wasp sensitivity and/or protection in yellow-jacket-sensitive patients. Nevertheless, this approach could also be useful for pollen allergens as many of them show sequence homology with proteins from other sources and variable degrees of cross-reactivity.

Shuffled Allergens. As discussed above, directed mutagenesis has been widely used to generate hypoallergens. As other rational design methods, this method requires an

understanding of the relationship between structure and function/properties and is limited by the fact that substitutions of amino acids or segments must be appropriate for the specific position in which it is found in the protein. The effects of substitutions, even chemically conserved ones, are practically impossible to predict with the current system.

DNA shuffling or molecular breeding is a new approach that mimics natural evolution and can be performed without prior knowledge of structural or functional characteristics of the target molecules [52–56]. Basically, it allows the generation of large and complex libraries of novel chimeric genes, from which variants with desired properties can be selected using appropriate selection or screening methods. In the most widely used format, the initial step comprises of random fragmentation of a pool of homologous genes, typically carried out by treatment with DNase I. The resulting DNA fragments are subsequently reassembled through several denaturation-annealing cycles during which DNA polymerase extends the partially annealed fragments. At each step of this self-priming PCR, the gene fragments grow in length and are finally reassembled into full-length genes. Because of the sequence similarity of the starting genes, the regions of annealing in each step create crossovers, or points of

recombination, creating new genes that can be highly chimeric in structure. The starting genes can be a pool of DNA mutant sequences (e.g. mutants of a single gene obtained by random mutagenesis), or naturally existing homologues of a particular gene, also known as DNA family shuffling [54, 55]. The family shuffling mode permutes blocks of sequences containing conservative amino acid substitutions that have been selected for function during thousands or millions of years of natural selection. Consequently, the resulting libraries of shuffled chimeras are highly enriched in functional clones in comparison to libraries made by random mutagenesis.

The features of the DNA family shuffling method would allow the generation of allergen chimeras having T cell epitopes derived from several family members, but at the same time with reduced anaphylactic potential and improved immunogenicity [56, 57]. This could be an extremely efficient approach to produce optimal molecules for more efficient and safer forms of pollen and fungal allergen vaccines.

Engineered Allergens and Genetic Immunization

Genetic immunization is an attractive alternative for immunotherapy using allergenic proteins. It has been shown that intramuscular or intradermal injection of plasmid DNA encoding clinically relevant allergens can induce long-lasting immune responses with a Th1 bias and promote the formation of IFN- γ -producing CD4⁺ T cells. These IFN- γ -secreting Th1 cells can stimulate B cells to synthesize IgG2a antibodies and inhibit IgE production in mice [58–63]. Interestingly, genetic immunization induced Th1 type responses only after intradermal or intramuscular injection and not after inoculation with a gene gun [63–65]. This phenomenon was attributed to the fact that 50- to 100-fold higher doses of plasmid DNA are applied by intradermal or intramuscular immunization compared to gene gun-based delivery [66]. After subcutaneous administration of plasmid DNA encoding an allergen, transcripts were simultaneously detected in several tissues [60]. This raises the concern that the anti-allergic Th1-inducing delivery mode of plasmid DNA based on needle injection would result in a number of cells expressing the allergen in different tissues sufficient to trigger IgE-mediated side effects. Therefore, it would seem advantageous to combine the genetic immunization approach with the hypoallergen concept. In principle, immunization with plasmid DNA encoding hypoallergenic

variants (e.g. site-directed mutants, fragments, chimeras, etc.) instead of wild-type genes would fulfil the requirements of efficacy and safety in allergen-specific immunotherapy.

Conclusions

Although SIT is the only curative approach for type I allergies, in its present form, with the use of natural extracts that are difficult to standardize, SIT is accompanied by a risk of IgE-mediated side effects and shows variable clinical efficacy. Genetic engineering offers exciting possibilities for efficiently modifying allergen molecules that would allow overcoming the problems associated with SIT. The aim is to produce new allergen molecules with reduced allergenicity but preserved immunogenicity. The molecules with reduced allergenicity (hypoallergens) would not lead to anaphylactic reactions upon injection and would allow higher-dose administration of allergen, shown to be more effective in symptom reduction than low doses. Furthermore, hypoallergens would bypass IgE-facilitated antigen presentation that leads to Th2 cytokines and IgE production, and preferentially utilize phagocytic or pinocytic antigen uptake mechanisms leading to Th0- or Th1-like cytokine production, lower IgE and higher IgG production by B cells. Such engineered allergens are also recognized by T lymphocytes and can thus modulate the allergic immune response shown to be essential for successful therapy. Common approaches for genetic engineering of hypoallergens usually require knowledge of T and B cell epitopes and in some cases of the three-dimensional structure. DNA family shuffling has the advantage that it does not require a priori knowledge of structural and functional properties for efficient generation of molecules used as therapeutic agents. The combination of the Th1-inducing genetic immunization approach with the hypoallergen concept might be an attractive alternative in the future for protein-based immunotherapy.

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