Genetic Engineering of Allergens for Immunotherapy

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Abstract: Allergen-specific immunotherapy was introduced into clinical practice at the beginning of the 20th century and its efficacy in the treatment of seasonal allergic rhinitis has been confirmed in many clinical studies which have shown that it can prevent the onset of new sensitizations to different allergens and reduces the development of asthma in patients with allergic rhinitis. Progress in molecular cloning and characterization of allergens have made it possible to produce single recombinant allergens whose immunological properties have been tested *in vitro* and *in vivo* and have demonstrated that they retain properties resembling their natural counterpart. Several rational approaches are being developed to improve the efficacy of SIT by reducing immunoglobulin IgE-mediated adverse reactions. Some of these molecules have been tested in the clinic, demonstrating the feasibility of using biotechnology-derived products as new standardized, improved and safer therapeutic compositions.

The immune systems of higher animals play a central role in body defence against a large variety of infectious microorganisms. Macromolecules, including proteins, polysaccharides, and complex lipids, expressed by infectious agents are recognized, attacked and memorized as foreign antigens by the immune system to prevent the multiplication of infecting microorganisms. More recently, protein antigens which are recognized by the immune system with no correlation to infections have been considered in the immunological research field (allergens) as potent activator causing pathological damage to the host organisms and, occasionally, leading to serious systemic reactions (IgE-mediated allergic reaction).

It is well established that allergic diseases, such as hay fever, rhinoconjunctivitis, allergic bronchial asthma, allergic dermatitis, eczema, urticaria, angioedema and anaphylaxis, are global health problems affecting more than 25% of the population living in the industrialized countries [1] with a significant worldwide increase in the prevalence of asthma and allergic rhinitis since 1960 [2]. However, some recent studies indicate that this increase has now slowed down [2].

Overall, allergic disorders remain one of the most common causes of chronic diseases compromising the quality of life and increasing healthcare costs. Allergy can be induced by both outdoor and indoor sources. The main contributors to outdoor allergies are pollens [3], whereas indoor allergies are dominated by house dust mites, animal dander, cockroaches and fungal spores [4]. Peanuts, tree nuts, soybean, milk, fish, egg and fruits (such as the Rosacee) have also been identified as sources of allergens [3].

IgE sensitization involves the development of allergen-specific IgE antibodies and their binding to surface receptors on mast cells and basophils. Once a patient is sensitized, further exposure to the allergen can trigger the allergic reaction inducing a cascade of events leading to the release of inflammation mediators like histamine and leukotriens which are responsible for the immediate symptoms of allergy and the production of pro-inflammatory cytokines (IL-3, IL-5) which determine the recruitment and activation of eosinophils [5]. The route and dosage of antigen exposure are important external factors affecting the mode of antigen presentation [6].

In addition, it has been shown that IgE antibodies act as regulators of the immune response since they are involved in the uptake and processing of allergens mediating their presentation on specific Th cells [7].

The only treatment which can modify the natural outcome of the disease and restore normal immunity against allergens is specific immunotherapy (SIT) [8]. Although it has been used in clinical practise for almost a century, the molecular mechanisms involved in successful SIT are not fully understood and a variety of factors seem to influence the immune response such as the concentration of the allergen, the type of antigen presenting cell [6], the structure of the allergen [1] and the type of adjuvant used for the formulation of the vaccine [9].

So far, immunotherapy has been performed by s.c. injection or mucosal administration of a mixture of proteins from natural sources without considering the individual sensitisation profile of the patients and, possibly, inducing new IgE specificity [10]. Therefore, one of the major disadvantages of this strategy is that crude extracts contain a mixture of proteins which are difficult to standardize. Extraction procedures are not easy to standardize with huge differences among manufacturers, exhibiting a considerable heterogeneity regarding the presence of individual allergens [11]. In addition, the administration of crude extracts can lead to serious adverse reactions due to the activation of the immune system. The use of purified allergens can overcome many of these problems and has the advantage of making it possible to produce genetically modified molecules with reduced allergenicity and standardizable quantity and quality as a new improved and safer tool for SIT (see Table **1**) [12].

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Table 1. Engineered Recombinant Allergens *vs* Natural Extracts

Engineered Recombinant Allergens	
1)	Recombinant allergens and their hypoallergenic derivatives represent molecules which can be standardized and purified in a highly reproducible way (batches to batches consistency)
2)	They can be modified to suit different treatment strategies
3)	Vaccines with engineered recombinant allergens can be tailored according to the patient's sensitization profile
4)	Engineered allergens display improved safety
Natural Extracts	
1)	Unknown concentration of single protein components
2)	Variations between different preparations and customers
3)	Contamination with different allergenic sources
4)	Local and systemic reactions
5)	Allergens cannot properly be extracted from raw material
6)	Induction of new IgE sensitizations
7)	The whole spectrum of allergens is not standardized

In the last few decades, several molecules with allergenic activity have been isolated and characterized using biochemical and recombinant DNA technologies. So far more than 950 allergenic molecules have been described and subdivided into 143 families (http://www.meduniwien.ac.at/ allergens/allfam/, update 24/09/2008). Allergens are usually distributed among a few protein families and possess a restricted number of biochemical functions [3]. The use of purified (recombinant or natural) molecules in specific diagnoses has revealed the antibody reactivity profile of the allergic patient and allowed the disease-eliciting molecules to be identified [13]. The identification of the individual allergen reactivity profile has been termed 'component-resolved diagnosis' and allowed the definition of species-specific and cross-reactive allergens [13-16].

Allergenic molecules, in general, contain several IgE binding regions (epitopes). The ability to cross-link mast cell-bound IgE epitopes is a prerequisite to trigger the cascade of events leading to the allergic symptoms. Epitopes can be defined by a continuous stretch of amino acids (continuous epitopes) or by conformational structures (discontinuous IgE epitopes) which are usually located on surface exposed parts of the allergen. The disruption of these structures has been pursued in an attempt to reduce the IgE epitope density and, consequently, the anaphylactic activity of the allergen [17]. Independent strategies have been proposed and recombinant hypoallergenic allergen derivatives have been produced for several allergen sources. In general, they exhibit reduced allergenic activity in comparison to the corresponding wild-type allergen, and retain T cell reactivity and immunogenicity, as demonstrated by *in vitro* experiments and in animal models. Their properties will be discussed in this review (Table **2**).

Table 2. List of Hypoallergenic Derivatives Produced *via* Biotechnological Approaches

	Source	Allergen	Strategy	Reference
Recombinant derivatives	Birch pollen	Bet v 1	fragmentation	Vrtala *et al.*
	Birch pollen	Bet v 1	polymerization	Vrtala *et al.*
	Birch pollen	Bet v 1	folder variant	Kahlert *et al.*
	Birch pollen	Bet v 1	site specific mutagenesis	Holm *et al.*
	Parietaria pollen	Par j 1	disulphide bond variant	Bonura *et al.*
	Olive pollen	Ole e 1	site specific mutagenesis	Marazuela *et al*
	Grass pollen	Phl p 7	disruption on the 3D structure	Westritschnig *et al.*
	Grass pollen	Phl p 12	tail to head reassembly	Westritschnig *et al.*
	Grass pollen	Lol p 5	site specific mutagenesis	Swoboda *et al.*
	Grass pollen	Phl p 6	fragmentation	Vrtala *et al.*
	Grass pollen	Phl p 5	point and deletion mutants	Schramm *et al.*
	House Dust Mite	Der f 2	disulphide bond variant	Takai *et al.*
	Carrot	Dau c 1	polymerization	Reese *et al.*
B cell epitope	Birch pollen	Bet v 1	B cell derived epitope	Focke *et al.*
	Grass pollen	Phl p 1	B cell derived epitope	Focke *et al.*
Hybrid molecules	Parietaria pollen	Par j1 + Par j 2	hybrid formation	Bonura *et al.*
	Bee Venom	Api m 1 + Api m 2 + Api m 3	hybrid formation	Karamloo *et al.*
	Grass pollen	Phl p 2 + Phl p 6	hybrid formation	Linhart *et al.*
	Grass pollen	Phl p 1+ Phl p 2 + Phl p 5 + Phl p 6	hybrid formation	Linhart *et al.*
	Fagales	Bet v 1 ; Cor a 1 ; Aln g 1	Dna shuffling	Wallner *et al.*
Fusion molecules	Birch pollen	Bet v 1/S-layer protein	fusion protein	Bohle B *et al.*

SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis of amino acids within IgE epitopes has been shown to be a highly effective strategy in reducing IgE reactivity of grass and tree pollen allergens. Using overlapping dodeca-peptides, the dominant T cell epitopes of the timothy grass pollen allergen Phl p 5 b have been identified. By site-directed mutagenesis outside these regions, point and deletion mutants have been generated by DNA recombinant technology leading to the generation of variants that exhibit reduced IgE reactivity and histamine-release capacity [18]. Two genetically engineered forms of the white birch pollen major allergen Bet v 1 with point mutations directed at molecular surfaces have been characterized. Four and nine point mutations have led to a significant reduction of the binding to human serum IgE and a decrease of anaphylactic potential. The rBet v 1 mutants were able to induce proliferation of T cell lines derived from allergic patients and induce mouse IgG antibodies capable of blocking the binding of human serum IgE to rBet v 1 [19]. Based on B- and T-cell epitope mapping studies and on sequence comparison of group 5 allergens from different grasses, point mutations were introduced in highly conserved sequence domains of Lol p 5, the group 5 allergen from ryegrass. The Authors were able to select Lol p 5 mutants with low IgE-binding capacity and reduced allergenic activity as determined by basophil histamine release and by skin prick testing in allergic patients. Circular dichroism analysis showed that these mutants exhibited an overall structural fold similar to the recombinant Lol p 5 wild-type allergen. In addition, Lol p 5 mutants retained the ability to induce proliferation of group 5 allergen-specific T cell lines and clones, thus demonstrating that the mutagenesis approach can be successful in producing engineered recombinant allergens for pollen immunotherapy [20]. The C-terminal region of Ole e 1, a major allergen from olive pollen, is a dominant IgE-reactive site. One and two deletion mutants were generated and produced in *Pichia pastoris*. Immunological properties of the recombinant derivatives were evaluated by ELISA *in vitro* and allergenicity was also investigated in a mouse model of allergic sensitization. Immunization of mice with the mutant induced IgG1 antibodies which cross-reacted with Ole e 1 and Ole e 1-like allergens from ash, lilac and privet pollens [21].

DISRUPTION OF 3D STRUCTURE

The disruption of the conformational epitopes approach has been successful in producing low or even absent IgE binding forms of several allergens. Conformational changes can be achieved by using different strategies which express the allergens as (1) fragments, (2) disrupting conserved cysteine residues, (3) cutting and pasting allergenic sequences in a different order from the original w.t. sequence and (4) folding variants of the wild type allergen. The expression of Bet v 1 as two separate fragments led to highly reduced IgE reactivity. In particular, the fragment mix failed to elicit a positive skin prick test in 18 out of 23 patients in comparison with 0/23 with the w.t. allergen [22]. Both fragments, covering the full Bet v 1 sequence, induced human lymphoproliferative responses similar to rBet v 1 wild type. Immunization of mice and rabbits with rBet v 1 fragments induced IgG Abs, which cross-reacted with complete Bet v 1 and Bet v 1-related plant allergens and strongly inhibited the IgE binding of allergic patients to these allergens [23]. With a similar strategy and on the basis of IgE epitope mapping data, three allergen fragments comprising aa 1-33, 1-57, and 31-110 of the major timothy grass pollen allergen Phl p 6 were produced by expression in *Escherichia coli* and chemical synthesis. Circular dichroism analysis showed that the purified fragments lack the typical alpha-helical fold of the complete allergen. The lack of structural fold was accompanied by a large reduction of IgE reactivity and allergenic activity of the three fragments as determined by basophil histamine release in allergic patients [24]. The major house dust mite allergen, Der f 2, was engineered to reduce its capacity to induce skin test reactivity and histamine release from peripheral blood basophils in allergic patients by disruption of the disulfide bond that linked the N- and C-terminal sequences of the molecule. Such a derivative showed reduced allergenicity but retained T cell reactivity [25]. Using a similar strategy, conformational changes in the *Parietaria* major allergen (Par j 1) were introduced by replacing conserved cysteine residues, resulting in the loss of IgE reactivity [26]. Studies in an animal system demonstrated that disulphide bond variants of the Par j 1 allergen can induce a strong IgG1 response capable of blocking the binding of natural occurring human IgE [27]. Three-dimensional structure information from X-ray crystallography of grass pollen allergen Phl p 7 was the rational approach for designing a candidate protein for allergy vaccination. In three recombinant variants, amino acids essential for calcium binding were mutated and IgE binding was greatly reduced to the variant containing two mutations in each of the two calcium-binding sites. Recombinant mutants showed altered structural fold and loss of IgE reactivity. Another strategy which has been proposed is a tail-to-head reassembly of non-IgE-reactive fragments of an allergen. This concept was tested for the treatment of profilin-allergic patients. It was tested by swapping the C terminus of the Phl p 12 allergen at its N terminus and the Phl p 12 N terminus at its C terminus and then expressing it in *E. coli* as a recombinant protein. The modified molecule exhibited reduced IgE binding capacity and allergenic activity but preserved T cell reactivity in allergic patients [28]. A new approach was proposed using folding variants of Bet v 1 allergen. The physicochemical and immunological characteristics of this variant were investigated by comparing them to natural Bet v 1 and the correctly folded recombinant Bet v 1 using several immunological and biochemical techniques. They showed reduced IgE reactivity and decreased capacity to activate basophils but retained T cell reactivity and strong immunogenicity [29].

OLIGOMERIC AND HYBRID ALLERGEN MOLECULES

An alternative strategy for reducing the IgE reactivity of recombinant allergens is to generate oligomeric or hybrid forms combining multiple copies of a single or independent allergens in a unique molecule [30]. Tandem fusions of the cDNAs of major birch pollen allergen Bet v 1 led to the expression of hypoallergenic oligomers (Bet v 1 trimer). The oligomer generated showed a 100-fold reduction in allergenic activity as measured by histamine-release and SPT [22]. In addition, oligomerization seems to influence the immunological properties of the derivative with a Th1 profile of cytokine production and increased T cell reactivity [31]. The development of hybrid vaccines that comprise recombinant forms of different grass allergen molecules has

also been explored [32]. In this construct, the cDNA coding for the hybrid molecule was generated by a PCR based strategy, fusing the cDNAs coding for the major grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6. This hybrid molecule induced a stronger immune response in mice and rabbits and these antibodies were capable of blocking the binding of the IgE of allergic patients to grass pollen allergen [33]. A hybrid molecule expressing two hypoallergenic derivatives of the two major allergens of the *Parietaria* pollen (Par j 1 and Par j 2) has been generated by head to tail fusion. *In vitro* and *in vivo* assays showed highly reduced anaphylactic activity but increased immunogenicity [34]. A similar approach was used with the major allergens of bee venom. Phospholipase A2 (Api m 1), hyaluronidase (Api m 2) and melittin (Api m 3) fragments with overlapping amino acids were assembled in a different order in the Api m (1/2/3) chimeric protein, which preserved entire T cell epitopes, whereas B cell epitopes of all three allergens were abrogated. The hybrid protein showed 100- to 1000-fold reduction in SPT reactivity. Treatment of mice with Api m (1/2/3) led to a significant reduction of specific IgE development towards native allergen, representing a protective vaccine effect *in vivo* [35]. Another interesting observation about the formulation of recombinant oligomeric forms of allergens is emerging from the carrot major allergen, Dau c 1. Comparison of the immunological features of oligomeric forms of the wild type allergen versus mutant forms revealed that destruction of native conformation rather than oligomerization is the appropriate strategy for reducing the allergenicity of Bet v 1-homologous food allergens [36]. A novel application which has been suggested is to design a mosaic gene generated by fragmentation of the Phl p 2 sequence and reassemble the resulting peptides in an altered order, with a truncated Phl p 6 allergen, to produce a hybrid protein. The resulting hybrid retained the reduction of IgE reactivity and allergenic activity of its components. However, immunization with the hybrid molecule demonstrated an increased immunogenicity of this molecule, leading to higher levels of allergen-specific IgG antibodies compared to the single components [37].

HYPOALLERGENS GENERATED *VIA* DNA SHUFFLING

DNA shuffling is a technology that enables the generation of a large number of gene variants by reassembling random fragments of related genes. This approach has been used to create shuffled forms of the major Fagales pollen allergens to generate molecules that are suitable for specific immunotherapy not only against birch pollen allergy but also against allergies caused by other cross-reactive tree pollens.

Multi-vaccine forms of birch pollen allergen were generated by shuffling fragments of Bet v 1 with homologous allergens, Cor a 1 (major allergen of hazelnut pollen) and Aln g 1 (major allergen of alder pollen), resulting in two chimeric proteins showing low IgE-binding capacity but T-cell immunogenicity higher than that of the parental allergens. These hypoallergenic chimeras could efficiently substitute a mixture of extracts used for treating patients with tree pollen-induced spring pollinosis worldwide [38].

NON ANAPHYLACTIC B CELL EPITOPES

This strategy is based on the idea of generating non allergenic B cell derived peptide vaccines capable of inducing a protective effect. On the basis of the experimentally determined B cell epitopes of Phl p 1 allergen, five synthetic peptides were synthesized. The peptides, as well as an equimolar mixture, lacked allergenic activity in grass pollen allergic patients. When used as immunogens in mice and rabbits, the peptides induced protective IgG antibodies, which recognized the complete Phl p 1 wild-type allergen and group 1 allergens from other grass species [39]. Using a similar strategy non-anaphylactic peptides derived from the Bet v 1 allergen were synthesized. In a mouse model, peptide vaccination induced Bet v 1-specific IgG and prevented IgE-mediated allergic sensitization to Bet v 1. The protective role of peptide-induced blocking antibodies was demonstrated by the inhibition of allergic patients IgE binding to the allergen and by the blocking of allergen-induced basophil degranulation [40].

FUSION PROTEINS

The gene sequence encoding the major birch pollen allergen, Bet v 1, was fused with the gene encoding the bacterial cell surface (S-layer) protein of Geobacillus stearothermophilus (rSbsC-Bet v 1). This fusion recombinant protein contained all relevant Bet v 1-specific B and T cell epitopes, but was significantly less efficient in releasing histamine than rBet v 1. This construction combines reduced allergenicity with immunomodulating capacity since the chimera induced IFN-gamma synthesis in Bet v 1-specific Th2 cell clones and increased IL-10 production in these cell [41]. In addition, it was shown that immature monocyte-derived dendritic cells (mdDC) are capable of responding to rSbsC-Bet v 1 with a significant up-regulation of co-stimulatory molecules, functional maturation, and the synthesis of IL-10 and IL-12. In parallel, a substantial number of naive T cells developed into IL-10-producing CD25(+)Foxp3(+)CLTA-4(+) cells capable of active suppression [42].

CLINICAL TRIALS WITH RECOMBINANT ENGINEERED ALLERGENS

Despite the large number of potential candidates described in this review, only a few recombinant vaccines have been tested in clinical trials. The first study with genetically engineered hypoallergens was based on the tree pollen allergen Bet v 1 [43]. A multi-centre, double-blind, placebo-controlled and randomized trial involved 124 patients allergic to birch pollen who each received a single pre-seasonal injection with either two recombinant fragments of Bet v 1 or Bet v 1 trimer (as described above). Patients were not only able to tolerate high doses but also showed a strong protective antibody (IgG) response against both native Bet v 1 allergen and cross-reactive allergens, such as group 1 allergens of alder and hazelnut tree pollen, and food allergens of apple, carrot and celery [43]. In addition, the symptom medication scores (SMS) were significantly lower for these patients than for the patients treated with birch pollen extract [44].

Similar results were obtained with a folding variant of rBet v 1 (rBet v 1-FV) that had been prepared to reduce the

allergenic activity of the allergen. In a double blind randomized placebo controlled study, patients with birch pollen allergic rhinoconjunctivitis with or without asthma were treated with r Bet v 1-FV (n=108) or Placebo (n=103). After 1.5 years of therapy, a highly significant and clinically relevant reduction in the SMS in the active group compared to the placebo group was observed. The mean levels of allergen specific IgG4 in the first year of the study increased significantly in patients on active treatment and this response was further boostered in the second year, clearly indicating the immunogenic activity of the active treatment. In the placebo group, IgG4 remained relatively constant at a low level during the two year study period [45].

These results show that immunotherapy with genetically modified hypoallergens is effective in targeting the immunological mechanisms of allergies, and could be clinically useful for improving respiratory and pollen-associated oral syndrome induced by birch pollen allergen.

CONCLUSION

A tremendous amount of information derived from the use of recombinant DNA technologies has been accumulated in the last few decades since the isolation of the first recombinant allergen in the 1990s which opened the way to molecular allergology. A large set of experimental data performed with recombinant allergens have shown that these molecules can indeed replace natural allergen extracts. In addition, allergen–based vaccine studies conducted for birch [43] and grass pollen [46] allergy have demonstrated that these new biotechnology-based molecules are suitable reagents for curing allergic diseases, with perfectly standardized preparations satisfying the highest pharmaceutical standards and, for the first time, suggesting it may possible to perform patient-tailored treatments. Moreover, the possibility of designing hypoallergenic derivatives with reduced allergenicity but retained immunogenicity for the most common allergen sources represents an exciting new option which could reduce side effects and increase the efficacy of the treatment.

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