

ENGINEERING ALLERGENS

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INTRODUCTION

Allergen-specific immunotherapy (SIT) is one of a few efficient treatments and the only curative approach for allergies to defined allergens. The mechanisms underlying successful immunotherapy are assumed to operate at the level of T helper cells, leading to a modulation of the immune response to allergens. The disadvantages of its clinical application are the risk of anaphylactic and other IgE-mediated side effects and the difficulties in standardization of the allergenic extracts. Developments in molecular biology should lead to improvements in conventional SIT and allow a safer and more targeted form of treatment. Possible options comprise the use of better standardized extracts and of recombinant allergens. Moreover, recombinant molecular technology has made it possible to develop novel forms of allergen vaccines. In this context, the concept of hypoallergens has gained much attention. Hypoallergens are defined substances with decreased IgE reactivity and allergenicity but conserved immunogenicity (T cell reactivity) (Durham and Till 1998; Frew 2003). Thus, current approaches attempt to engineer allergens, aiming at a reduction of their IgE binding epitopes while maintaining the T cell activating capacity (T cell epitopes) and the structural motives necessary for IgG antibody (blocking antibodies) induction.

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Earlier studies showed that chemical modification of allergens (e.g. polyethylene glycol, glutaraldehyde, formaldehyde, etc) could be used to decrease allergenicity. Some of these preparations (allergoids) are currently available in the clinical practice. Experience with these hypoallergenic allergoids encouraged the idea of designing hypoallergenic vaccines based on synthetic peptides or recombinant proteins. Presently, four major groups of hypoallergens can be distinguish-

shed: synthetic peptides, chemically modified allergens, naturally occurring hypoallergens, and genetically engineered hypoallergens. We are presenting here a brief summary on engineering of allergens. Although chemical modifications have been mostly used in combination with natural allergens, the possibility of chemically modifying recombinant allergens could be an interesting approach for producing hypoallergens. Therefore, we included a short overview on approaches for chemical modification of allergens.

Chemical modification of allergens

■ **Allergoids:** Allergoids are chemically modified allergens or allergen extracts displaying strongly reduced IgE binding activity but retained immunogenicity. Common modifications are carbamylation or treatment with glutaraldehyde or paraformaldehyde. These chemical modifications seem to destruct some B cell epitopes of an allergen, thus hindering antigen internalization via Fcε or Fcγ receptors. B cells can internalize IgE-antigen complexes via FcεRII, so that even B cells not specific for the particulate allergen can efficiently take up and present allergen-derived peptides to Th cells, leading to an upregulation of the allergic response. Consequently, the destruction of B cell epitopes by chemical modification hinders antigen presentation by B cells and prevents further amplification of the allergic reaction (Kahlert, Grage-Griebenow *et al.* 2000). Furthermore, chemically modified antigen preferentially elicits induction of Th1-type cytokine synthesis patterns *in vivo* (Yang, Gieni *et al.* 1993). These observations suggest that allergoids are a promising basis for the development of new vaccines for SIT, but the undefined properties and the suboptimal batch-to-batch reproducibility of the allergoid preparations remain to be optimized.

■ **Maleylation:** Scavenger receptors (SRs) belong to the family of pattern recognition receptors and bind to a variety of polyanionic ligands. Internalization of proteins through scavenger receptors results in increased immunogenicity with a dominant Th1-type immune

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response. Many proteins can be targeted to SRs by chemical modification with acetic acid or maleic anhydrides, resulting in an increase of their negative charges. Furthermore, maleylation disrupts the B cell epitopes but not the T cell epitopes of a protein, making this application ideal for allergens used in specific immunotherapy (Abraham, Singh *et al.* 1995; Singh, Bhatia *et al.* 1998; Bhatia, Mukhopadhyay *et al.* 2002).

■ **Allergen-immunostimulatory sequences Conjugates (AIC):** Synthetic oligodeoxynucleotides (ODNs) that contain unmethylated CpG motifs (hexameric consensus sequence consisting of a central GpG dinucleotide flanked by two 5'purines and two 3'pyrimidines) can deviate a normal Th2 response to allergens toward a non-allergic Th1 response. The induction of Th1-polarized immune responses results from the ability of CpG-ODNs to induce activation and secretion of IL-12 and IL-18 by macrophages and dendritic cells. Both cytokines trigger IFN- γ production by NK and T cells (Chu, Targoni *et al.* 1997; Roman, Martin-Orozco *et al.* 1997; Tighe, Takabayashi *et al.* 2000; Krieg 2002). Additionally, CpG-ODNs induce the maturation of immature dendritic cells, enabling them to activate antigen-reactive naïve T cells (Santeliz, Van Nest *et al.* 2002). Approaches linking purified natural Amb a 1, the major allergen from ragweed, with a 22-mer ISS-ODN enhanced its immunogenicity and reduced its allergenicity, characterized by an induction of a Th1 response in Amb a 1-ISS treated mice, whereas Amb a 1 alone induced a Th2 response. Furthermore, the Amb a 1-ISS conjugate was effective both in the prevention of a Th2 reaction and in the conversion of a preexisting Amb a 1-specific Th2 response towards a Th1 response (Tighe, Takabayashi *et al.* 2000).

Recombinant production of naturally occurring hypoallergenic isoforms

Many allergens show a considerable degree of heterogeneity in that they are encoded by gene families and naturally occur as a complex mixture of different isoforms. Sequence polymorphisms have been described for ragweed Amb a 1, hazel Cor a 1, birch Bet v 1, group I and group 5 grass allergens, apple Mal d 1, celery Api g 1, olive Ole e 1, group 1 and group 2 dust mite allergens, latex Hev b 7, cow dander Bos d 2 and others.

Several studies concentrated on the possibility of distinct allergenicity but similar T cell immunogenicity of different isoallergens. In a classical example, Bet v 1 isoforms were divided into three groups: isoforms with high IgE-binding properties (isoforms a, e, and j), intermediate IgE-binding activity (isoforms b, c, and

f), and low/no IgE-binding activity (isoforms d, g, and l). Interestingly, the T cell activating potency of the isoforms tested did not correlate with their IgE-binding capacity. Such naturally occurring hypoallergens with high immunogenicity would be ideal candidates for SIT (Ferreira, Hirthenlehner *et al.* 1997).

Genetically engineered hypoallergens

Genetic engineering manipulates allergen genes in order to produce modified proteins with altered properties, aiming at the generation of hypoallergens with low allergenicity but conserved immunogenicity. Site-directed mutagenesis, gene deletion, gene fragmentation, oligomerization, and the creation of hybrids and chimeras are common techniques used.

■ **Site-directed mutants:** If the epitopes of an allergen involved in IgE-binding are known, its sequence can be specifically altered by site-directed mutagenesis, thus modifying or disrupting the crucial amino acids or motifs recognized by specific antibodies. Many alterations target elements crucial for maintaining the three dimensional structure of the protein such as disulfide bonds or calcium binding domains.

Based on a computer algorithm used to predict functional residues in proteins, six amino acid residues of the high IgE binding isoform Bet v 1a were replaced by those present in the same positions of low IgE binding isoforms of Bet v 1 and some closely related tree pollen allergens. This molecule displayed significantly reduced IgE binding but normal T cell activation compared to Bet v 1a (Ferreira, Hirtenlehner *et al.* 1996; Ferreira, Ebner *et al.* 1998).

As calcium-binding proteins are known to change their conformations in the presence or absence of Ca²⁺, other studies presumed that mutating the EF-hand domains of calcium binding allergens would lead to conformational changes in discontinuous IgE epitopes. Indeed, mutations in the EF-hand domains of the two calcium-binding allergens Bet v 4 and Bra r 1 caused a dramatic decrease in their IgE-binding activity (Engel, Richter *et al.* 1997; Okada, Swoboda *et al.* 1998). Studies on the T cell activating capacity of these mutants still have to be performed.

Other approaches targeting the disulfide bonds of Par j 1, the major allergen from pellitory, and of Der p 2 resulted in molecules of altered three dimensional structure with a greatly reduced IgE-binding capacity (Smith and Chapman 1996; Bonura, Amoroso *et al.* 2001).

The use of such full-length recombinant proteins with reduced IgE antibody binding is a promising tool for the development of safer and more effective vaccines for specific immunotherapy. A potential disadvantage of these molecules might be the solubility because denatured or unfolded proteins can potentially form aggregates.

■ **Deletion mutants:** Knowledge of the T- and B cell epitopes of an allergen allows the specific deletion of DNA segments encoding epitopes involved in IgE binding while preserving T cell epitopes. This approach was successfully used for Phl p 5b, a timothy grass pollen allergen, for the American cockroach allergen Per a 1, and for ryegrass Lol p 1. Deletion mutants of Phl p 5b showed reduced IgE-binding properties, no histamine-releasing activity, and reduced skin reactivity. No significant impairment of T cell reactivity was observed (Tamborini, Faccini *et al.* 1997; Schramm, Kahlert *et al.* 1999; Wu, Lee *et al.* 2002).

■ **Fragments:** While T cells recognize short linear epitopes created by antigen processing and presentation, B cell epitopes can be either formed by a linear stretch of amino acids (continuous epitopes) or by separated sequence elements brought together in the three dimensional structure of the folded molecule (discontinuous epitopes). Even the recognition of continuous epitopes might be conformation-dependent and occur only in the context of the correctly folded allergen. If the three-dimensional structure of an allergen is disrupted by fragmentation, conformation-dependent epitopes are abolished, reducing the anaphylactic potential of the allergen.

Vrtala *et al.* produced two recombinant Bet v 1 fragments comprising amino acids 1-74 and 75-160. These fragments lost their IgE binding capacity and showed reduced allergenic activity, probably due to a loss of their native conformation. Both fragments were able to stimulate T cells and induced the secretion of blocking IgG antibodies in mice and rabbits (van Hage-Hamsten, Kronqvist *et al.* 1999; Vrtala, Akdis *et al.* 2000).

Fragmentation of the major house dust mite allergen Der f 2, of birch pollen Bet v 4, of Aln g 4 from elder, and of ryegrass Lol p 1 resulted in decreased IgE binding activities. Studies on the T cell activating potential and immunogenicity of these molecules still have to be performed (Tamborini, Faccini *et al.* 1997; Twardosz, Hayek *et al.* 1997; Hayek, Vangelista *et al.* 1998; Takai, Mori *et al.* 1999).

■ **Oligomers:** Oligomerization is another approach to engineer a derivative of a recombinant allergen.

Dimers and trimers of Bet v 1, the major allergen from birch pollen, were recombinantly produced and analyzed in terms of structure, allergenic activity, and T- and B cell activating capacity. The trimer exhibited reduced allergenic activity and was able to induce Th1 cytokine release, even though it contained the same B- and T cell epitopes as the wild-type allergen. This is most likely caused by an impaired ability to cross-link FcεRI-bound IgE or by a decreased IgE-binding affinity of the trimer. Furthermore, it induced the formation of blocking IgG-antibodies when injected in mice and rabbits (Vrtala, Hirtenlehner *et al.* 2001).

■ Chimeras

➤ **Hybrids of allergens and homologues:** King *et al.* reported an approach to genetically modify allergens by producing hybrids consisting of a small portion of the allergen of interest and a large portion of a homologous but weakly cross-reacting host protein. The homologous host protein serves as a scaffold to sustain the native structure of the guest allergen, thus maintaining discontinuous B cell epitopes, but at a reduced density. The homologous allergens Ves v 5 from yellow jacket and Pol a 5 from paper wasp show 59% sequence identity. A hybrid of both allergens with Ves v 5 as the guest allergen and Pol a 5 as the host allergen showed a striking reduction in allergenicity, as determined by basophil histamine release assays (King, Jim *et al.* 2001).

➤ **DNA shuffling:** DNA shuffling is a powerful tool for directed evolution. It generates diversity by recombination, combining useful mutations/variations from individual genes. The DNA shuffling technique comprises the random fragmentation of a pool of related genes, followed by a random reassembly of the fragments and an amplification step. Recombination processes can take place in areas of sequence homology during the shuffling process, creating new genes that can be highly chimeric in structure. The proteins encoded by these new genes can then be screened for their hypoallergenic potential (reduced anaphylaxis, conserved T cell activating capacity, improved immunogenicity). This approach bypasses the need of a prior characterization of structural or functional characteristics (B- and T cell epitopes, three-dimensional structure) of the allergens of interest (Stemmer 1994; Crameri, Raillard *et al.* 1998).

Another promising approach not discussed here is the development of DNA-vaccines encoding engineered allergen genes. The combination of genetic immunization with the hypoallergen concept is an attractive alternative for the development of novel vaccines for the treatment of type 1 allergies (Leitner and Thalhamer 2003).

CONCLUSIONS

Although SIT is an effective treatment of type 1 allergies, it still implies a certain risk of anaphylactic and other IgE-mediated side effects. Future directions in specific immunotherapy must address these shortcomings. The use of recombinant engineered (hypo-) allergens is an attractive strategy for improving vaccine safety and permitting a more targeted form of treatment (Frew 2003).

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