

REVIEW

Genetic allergen modification in the development of novel approaches to specific immunotherapy

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Summary

In the recent past, multiple allergens from relevant allergen sources have been cloned, sequenced and produced as recombinant proteins. The availability of recombinant allergens with immunological characteristics similar to their natural counterparts has improved the diagnosis of allergic disorders and increased our knowledge of the biochemical, structural and immunological characteristics of proteins with allergenic potential. Moreover, the use of defined recombinant proteins as vaccines substituting currently used total protein extracts from allergen sources may improve specific immunotherapy (SIT) of Type I allergy. In addition to producing well-defined batches of wild-type allergens, the recombinant technology offers the possibility to easily and selectively modify their properties or functions. Diverse modifications of allergens can be genetically engineered, e.g. variants with reduced IgE-binding capacity, multi-mers of single allergens or hybrids consisting of different allergens. Furthermore, allergens can be genetically fused with proteins that promote immune responses, which counterregulate the disease-eliciting T-helper type 2-dominated immune response in allergic individuals and may therefore, improve the efficacy of SIT. This review will introduce different concepts of allergen modification using genetic engineering to improve vaccines for SIT of Type I allergy.

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Introduction

In industrialized countries, IgE-mediated diseases like allergic rhinoconjunctivitis, atopic asthma and atopic dermatitis represent increasingly relevant causes of morbidity and impaired quality of life. This situation demands efficient and safe therapeutic interventions. To treat allergic disorders, specific immunotherapy (SIT) is currently the only causative approach. During SIT, increasing doses of allergen extracts are administered subcutaneously or sublingually to the patient in order to induce clinical tolerance to natural allergen exposure. In contrast to symptomatic pharmacological treatment, SIT modulates the allergen-specific immune response and induces long-term remission. In addition, SIT prevents the so-called atopic march, i.e. the development of bronchial asthma as a consequence of non-treated allergic rhinitis (AR), as well as the onset of hypersensitivity reactions to additional allergen sources (reviewed in [1]).

SIT is the only treatment of Type I allergy that alters the disease-eliciting hypersensitivity reaction against aller-

gens. At the T cell level, successful SIT has originally been associated with reduced proliferative T cell responses to the administered allergens and immune deviation, i.e. the switch from the allergen-specific overwhelming T-helper (Th) type 2-like response typical for allergic patients to a more Th0/1-like response observed in non-allergic individuals [2–4]. Th1 cells produce high amounts of IFN- γ . Study of the immune mechanisms responsible for the observed reduced allergen-induced T cell proliferation in more detail revealed that continuous administration of allergens during SIT induced regulatory CD4⁺ T cells that produce suppressive IL-10 and/or TGF- β and actively inhibit the proliferation and cytokine synthesis of allergen-specific T cells [5–7]. The cytokines IFN- γ and IL-10 lead to the production of IgG1 and IgG4 antibodies, respectively [8]. Indeed, the levels of allergen-specific IgG antibodies in the serum increase during SIT [9–11]. These immunoglobulins are thought to bind to the allergen and thus to block the binding of IgE antibodies [12]. Consequently, IgE-mediated activation of mast cells and basophils as well as IgE-facilitated uptake of allergens by

B lymphocytes is impaired [13]. In summary, SIT counter-regulates the allergen-specific hypersensitivity reaction at different steps of the allergic cascade (reviewed in [14]). Although we still do not know which of these mechanisms is the most relevant for clinical improvement, we may consider the immunomodulation at the T cell level, i.e. the induction of IL-10-producing regulatory CD4⁺ T cells and of allergen-specific IFN- γ -producing T cells, as one basic principle of SIT. Regulatory CD4⁺ T lymphocytes and allergen-specific Th1 cells have also been shown to characterize the 'healthy' immune response to allergens in non-allergic individuals [15, 16].

In view of these findings, it may be advantageous to particularly support the development of regulatory and allergen-specific Th1 cells during SIT to guarantee clinical efficacy. Exposure of patients to high doses of allergens was shown to favour the development of these T cell subsets [17–19]. However, administration of allergens to allergic patients bears the risk of inducing IgE-mediated side-effects, and thus the dosage of administered allergens is limited. At present, crude protein extracts from allergenic sources are used that contain allergens relevant for the patient, together with other allergenic and non-allergenic proteins. Although progress has been made to improve the quality and standardization of protein extracts by endeavours such as the European Union CREATE network [20] and the introduction of a new European Guideline for Allergen Products (EMA/CHMP/BWP/304831 2007 – <http://www.emea.europa.eu>), the use of allergen extracts for SIT still has some disadvantages. The complex protein mixture makes it difficult to determine the exact content of single allergens. Because of the production process, the concentration of individual allergens may vary between different batches. Highly labile allergens might even be totally lost during the production process and not be present in the extract. Together, this situation does not allow a reliable risk assessment of possible side-effects during the administration of these extract-based vaccines. Consequently, small and safe doses are administered over a long time period to achieve therapeutically effective amounts. The availability of recombinant allergens with the same immunological characteristics as their natural counterparts can overcome several disadvantages of allergen extracts (reviewed in [21]). Briefly, under controlled production procedures, recombinant allergens can be produced as molecules with known molecular, immunological and biological characteristics in consistent quality and unlimited amounts. Thus, the content of individual allergens in a vaccine can be formulated precisely and the potential loss of allergens destroyed during the production process of allergen extracts can be excluded. Recombinant allergens have been demonstrated to improve allergy diagnosis [22, 23]. In addition, recombinant allergens have been used as vaccines for SIT of grass pollen allergy and have ameliorated

symptoms of AR [24]. A recent study demonstrated that a recombinant allergen-based vaccine was as effective for treatment of birch pollen allergy as the complex allergen extract [25].

In addition to improving the diagnosis and therapy of Type I allergy, the availability of recombinant wild-type allergens has also markedly contributed to our knowledge of the immunological and structural features of proteins with allergenic potential. For example, recombinant allergens with a known amino acid sequence were used to identify relevant T and B cell epitopes [26–28]. This immunological characterization is important for the creation of hypoallergenic variants of allergenic proteins. The rational design of allergen variants can be easily achieved by genetic engineering, i.e. the manipulation of allergen genes resulting in desired modifications at the protein level (reviewed in [29]). Recombinant allergen variants with reduced allergenicity and conserved immunogenicity can be produced. Otherwise, fusion proteins of allergens and molecules supporting a desired immune response, e.g. Th1 responses, can be generated to develop allergen variants with an immunomodulatory capacity. Below, different approaches of genetic allergen modification to improve vaccines for SIT will be discussed in more detail.

Hypoallergenic variants of allergens

Variants of allergens with low/no IgE-binding capacity and thus, a reduced capacity of inducing IgE-mediated side-effects, and retained T cell stimulatory activity have been termed 'hypoallergenic'. As compared with native allergens, hypoallergens are attractive molecules to be administered in higher doses without increasing the risk for anaphylactic events. The administration of higher doses of allergens has been demonstrated to support the development of IL-10- and IFN- γ -producing T cell subsets *in vitro* and *in vivo* [17–19].

IgE binding to allergens often depends on their tertiary structure because many IgE epitopes are conformational (discontinuous) epitopes, meaning that amino acid residues separated in the primary structure become neighbouring residues forming an epitope by correct folding of the protein (Fig. 1). Alterations of the amino acid sequence of allergens may influence protein folding and can result in the reduction or the total prevention of IgE binding. A first approach to persistently alter the native conformation of allergens was chemical modification of entire allergen extracts using formaldehyde [30]. This treatment resulted in so-called allergoids consisting of large molecules of thousands of kilodaltons, which contain the individual allergens and other proteins contained in the extract. Allergoids showed a considerable decrease in IgE-mediated adverse reactions and have been used as vaccines in SIT for several years [31–33]. However, the



Fig. 1. Schematic illustration of IgE and T cell epitopes of allergens. Epitopes recognized by IgE antibodies (indicated in grey) are frequently of a conformational nature formed by originally separated amino acid residues that become neighbouring residues after proper folding of the protein. In contrast, T cell epitopes that result from antigen processing are linear peptides. Putative residues comprising the respective epitope are underlined in the amino acid sequence of the allergen.

production process involving cross-linking of proteins by formaldehyde may result in batch-to-batch variations and the concentration of disease-relevant allergens in allergoids is difficult to determine. Moreover, allergoids cannot be taken up and presented by all types of antigen-presenting cells (APC) [34] and have been shown to lose the availability of some T cell epitopes of major allergens, resulting in the loss of immunological activity [35, 36]. These disadvantages may be overcome by developing stable hypoallergenic variants from recombinant allergens. On the one hand, changing certain steps in the production process of recombinant wild-type allergens can result in stable non-IgE-binding folding variants, as has been demonstrated for the major birch pollen allergen [37]. The Bet v 1-folding variant preserved its T cell-stimulating capacity and displayed significantly reduced IgE-binding and mediator-releasing activity. On the other hand, DNA technology can be used to alter the DNA sequence encoding the wild-type allergen in order to create a protein with a reduced/abolished IgE-binding capacity. Different concepts of changing DNA sequences of allergens have resulted in engineering of recombinant hypoallergens. For example, wild-type allergens can be separated into different fragments. These fragments can then either be produced as individual proteins or re-fused in an exchanged sequence to generate a new molecule [38–40] (Fig. 2). For instance, the administration of two hypoallergenic fragments with retained T cell-activating capacity of the major birch pollen allergen to birch pollen-allergic patients induced blocking IgG antibodies specific for natural Bet v 1 and improved the symptoms in actively treated individuals [41, 42]. Another frequently used approach to engineer hypoallergens is site-directed mutagenesis, which focuses on the selective exchange of single amino acid residues or motifs relevant for IgE

binding (Fig. 2). Site-directed mutagenesis has been applied to generate hypoallergenic variants of several respiratory and food allergens [28, 43–49]. The targeted exchange of only a few selected amino acid residues to destroy IgE-binding epitopes is an elegant way to retain all or most of the relevant T cell-activating regions. These T cell epitopes are necessary to modulate the allergen-specific T cell response and to guarantee the induction of blocking IgG antibodies.

Allergen–allergen fusion molecules

Many allergen sources, e.g. grass pollen, insect venoms or house dust mites, contain more than one major allergen. Thus, to replace a natural extract, a cocktail of recombinant allergens must be used to cover the B and T cell epitope spectrum desired for SIT of patients sensitized to multiple major allergens [24]. Alternatively, hybrid molecules consisting of different allergens can be engineered (Fig. 2). The sensitization pattern of each patient can be determined by applying a component-resolved diagnosis. The investigation of a high number of allergic individuals reveals the most disease-relevant allergens for a population. A single hybrid molecule replacing a cocktail of individual disease-relevant allergens potentially facilitates the production, standardization and consistent pharmacological quality of a vaccine. As a further advantage, the combination of individual allergens in one hybrid molecule may increase the immunogenicity of single allergens with a low immunogenicity [50]. Dimers or trimers of one individual allergen have been shown to induce its immunogenicity and reduce its allergenicity [51, 52]. Allergen–allergen hybrid molecules may combine different allergens from one complex allergen source or allergens from different sources, e.g. homologous

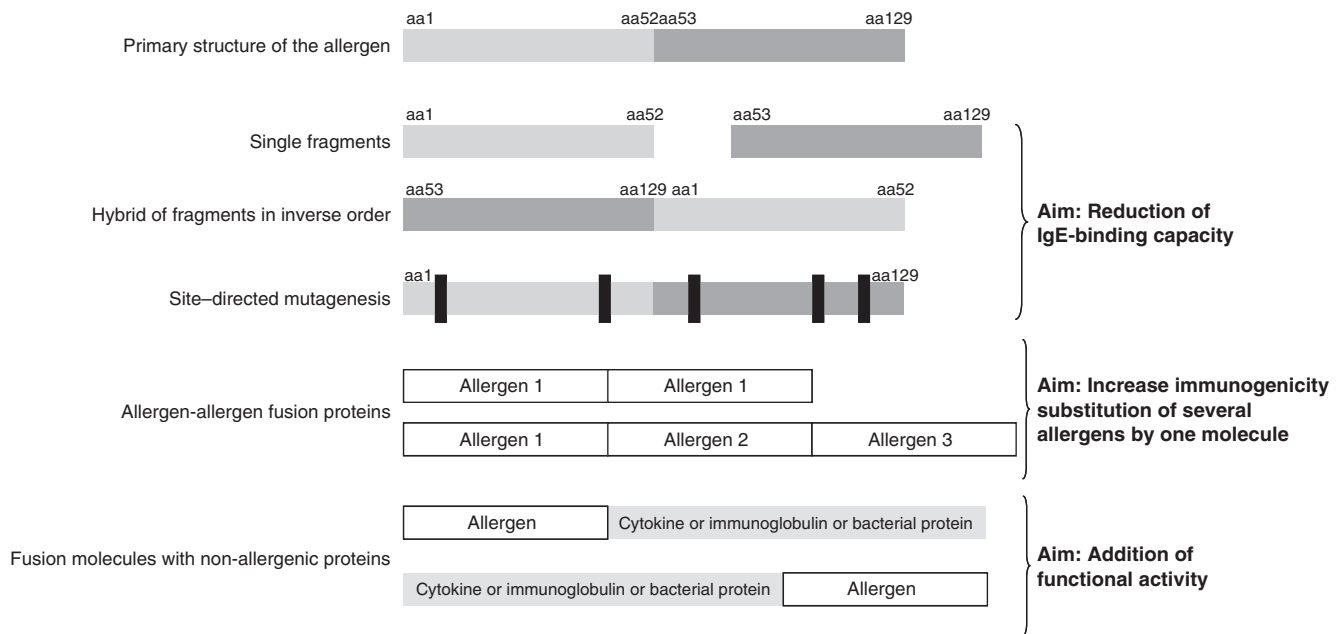


Fig. 2. Schematic overview of genetic modifications of allergens. Applying DNA technology the properties/functions of allergens can be modified in a desired manner. The different examples of genetic allergen modifications are described in the text.

proteins inducing clinically relevant cross-reactivity [50, 53–55]. Furthermore, instead of fusing full-length allergens, immunological active fragments, e.g. T cell epitopes, may also be combined in a hybrid molecule. Such approaches have also been applied to allergens derived from the same [56–58] or different sources [59, 60].

Allergen–immunoglobulin fusion proteins

Allergen exposure results in cross-linking of IgE antibodies bound to the high-affinity receptor Fc epsilon receptor 1 (FcεRI) on basophils and mast cells, which induces their activation and degranulation [61]. In addition to FcεRI, these effector cells also express Fc gamma receptor IIb (FcγRIIb), which contains an immunoreceptor tyrosine-based inhibitory motif within its cytoplasmic tail [62]. Co-aggregation of FcεRI to FcγRIIb was demonstrated to block the function of human effector cells [63]. Based on these insights the concept emerged to fuse an allergen with a truncated human Fcγ region. The allergen portion would bind to specific IgE on FcεRI whereas the Fcγ portion would co-aggregate FcγRIIb, resulting in the inhibition of allergic reactivity [64]. Indeed, such an allergen–Fcγ protein showed immunogenicity but no allergenicity in mouse models and blocked human effector cell degranulation (reviewed in [65]).

Besides the approach to inhibit the effector phase of Type I allergy, fusion proteins of allergens and immunoglobulins may also be used to target allergens to particular receptors expressed on the surface of APCs in order to enhance their uptake and presentation. In this context, a

fusion protein consisting of an allergen and the variable region of an antibody specific for the high-affinity IgG receptor FcγRI on monocytes and dendritic cells (DC) was shown to improve allergen presentation to T lymphocytes and modulated the human allergen-specific Th2 response *in vitro* [66]. Only recently, another concept to increase the immunogenicity of allergens was proposed by fusing them with a 'modular antigen transporter (MAT)' [67]. This MAT consists of a protein translocation sequence to improve cellular uptake and a truncated invariant chain to directly target the MHC class II antigen presentation pathway. Indeed, the modified allergen induced higher levels of allergen-specific IgG2a antibodies and Th1 cytokines as compared with the native allergen. Besides, the MAT modification transformed the allergen into a hypoallergenic form, causing reduced IgE-mediated degranulation of human basophils.

Allergen–cytokine fusion proteins

Successful SIT has been associated with the conversion of Th2 profiles into Th1 cytokine profiles. The differentiation of naïve CD4⁺ cells into IFN-γ-producing T lymphocytes is promoted by the presence of certain cytokines, in particular IL-12. Thus, an allergy vaccine could be substituted with human IL-12 to promote the induction of allergen-specific Th1 responses. Because the administration of IL-12 to patients might result in allergen-non-specific and probably overwhelming Th1 responses, its effects should be limited to allergen-specific cells. This principle was realized by fusing IL-12 to an allergen

(Fig. 2) [68]. The administration of the allergen-IL-12 fusion protein to mice enhanced IFN- γ synthesis and reversed the Th2-dominated allergic response in an allergen-specific manner. In parallel, administration of this fusion protein resulted in a reduced allergen-specific IgE production in the animals.

Allergen–adjuvant fusion proteins and encapsulated allergens

Another concept to support SIT-induced immune deviation is the combination of allergy vaccines with novel adjuvants. Adjuvants serve as antigen-delivery systems. Accordingly, recombinant allergens were linked to carbohydrate particles or encapsulated in biodegradable poly(D,L-lactic-co-glycolic) acid nanospheres as allergen carriers. These approaches were shown to reduce the Th2-mediated responses in mouse models [69, 70]. Adjuvants may also foster certain immune responses, e.g. Th1 responses. In this regard, different candidates have already been tested in clinical trials, such as monophosphoryl lipid A, a non-toxic component of lipopolysaccharide, and immunostimulatory sequences of bacterial DNA [33, 71]. These molecules represent danger signals for the innate human immune system and are recognized by Toll-like receptors on APC, in particular DC and macrophages/monocytes. Upon ligand binding, these cells synthesize various pro-inflammatory and inflammatory cytokines including IL-12 and IL-18, which promote the induction of Th1-responses [72, 73]. In addition, immature DC start to mature, characterized by up-regulation of MHC class II molecules and different co-stimulatory molecules, e.g. CD80, CD86 and CD40, which are important for effective T cell activation. Studies with immunostimulatory DNA chemically linked to allergens demonstrated that allergen–adjuvant conjugates are more effective as compared with the mixture of allergen and adjuvant [74]. In analogy, genetic engineering allows to fuse allergens and proteins with adjuvant activity. For example, allergens have been fused with the mannose-binding protein to support immune deviation [75], subunit B of cholera toxin, an efficient mucosal carrier, to support tolerance induction [76], or with diphtheria toxin protein to confer specific toxicity to cells [77].

We have analysed a fusion protein consisting of the major birch pollen allergen and a surface layer (S-layer) protein derived from non-pathogenic Gram-positive bacteria [78]. S-layer proteins also represent danger signals for the human immune system and the S-layer-Bet v 1 fusion protein-induced DC expanded from birch pollen-allergic patients to mature and to synthesize IL-10 and IL-12 [79]. These DC promoted the differentiation of autologous naïve CD4⁺ T cells into IFN- γ -producing cells in an IL-12-dependent manner. In parallel, a substantial number of naïve T cells developed into IL-10-producing

regulatory T cells that actively suppressed the proliferative responses of anti-CD3-stimulated CD4⁺CD25⁻ T cells. In addition to linking the innate and adaptive immune system, the Bet v 1-S-layer fusion protein displayed less IgE-binding and reduced histamine-releasing capacity as compared with recombinant Bet v 1. Thus, the novel fusion protein showed reduced allergenicity combined with immunomodulatory capacity. Another feature of the S-layer-Bet v 1 fusion protein is its retained ability to self-assemble into monomolecular lattices of 0.5–2 μ m. These particulate structures might affect allergen uptake and presentation. Indeed, we found that B lymphocytes are inappropriate APCs of the self-assembled form of the fusion protein, which may be related to its size, by impairing pinocytosis, and/or its reduced IgE-binding capacity, by impairing IgE-facilitated uptake *via* Fc ϵ RII (CD23) [80]. These findings are in line with previous data on the uptake and presentation of allergoids [34]. However, in contrast to allergoids, the recombinant S-layer-Bet v 1 fusion protein retained all frequently recognized T cell epitopes of the major birch pollen allergen [35, 80].

Conclusions

The availability of recombinant allergens has provided a broad range of possibilities to genetically manipulate the properties or functions of allergens in an intentional direction. One general aim is to design hypoallergenic derivatives in order to reduce the risk of anaphylactic side events and to allow the administration of higher doses during SIT, which is thought to improve its efficacy. Another concept is to equip allergens with certain characteristics, e.g. targeting particular receptors to enhance their immunogenicity or to support immune responses that foster the down-regulation of allergen-specific Th2 responses. The most promising candidate vaccines combine both concepts and display reduced allergenicity, increased immunogenicity and immunomodulatory capacity. Although some of these novel allergy vaccines have been evaluated in clinical trials, most of the approaches showing promising results in human *in vitro* studies and/or animal models have not yet been clinically evaluated. Thus, we still lack the information on whether these novel vaccines are therapeutically effective. No modified allergen variant has so far been investigated in parallel with its unmodified original. Moreover, nothing is known about the potential risks associated with the administration of the different molecules fused with allergens. Therefore, future work should focus on parallel testing of modified and wild-type allergens in clinical trials. Such studies will demonstrate which modifications indeed improve SIT and will contribute to decipher the precise immune mechanisms underlying this causative treatment of allergic disorders.

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