

Strategies for converting allergens into hypoallergenic vaccine candidates[☆]

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Abstract

Specific immunotherapy is based on the administration of increasing doses of allergens to allergic patients with the aim of inducing a state of antigen-specific unresponsiveness. Specific immunotherapy is one of the few causative treatment approaches for Type I allergy but may cause numerous side effects, including local inflammatory reactions, systemic manifestations (e.g., asthma attacks) and in the worst case, anaphylactic shock which may lead to death. Several attempts have been made in the past to reduce the rate of side effects. They included the chemical modification of allergen extracts to reduce their allergenic activity and the adsorption of allergen extracts to adjuvants to prevent the systemic release of allergens after administration. During the last decade, cDNAs coding for the most relevant allergens have been isolated and the corresponding allergens have been produced as recombinant molecules. Using allergen-encoding cDNAs, the amino acid sequence of allergens or purified recombinant allergens several strategies can now be applied to produce allergen derivatives with reduced allergenic activity for allergy vaccination in a controlled and reproducible manner. Currently, allergen-encoding cDNAs are used to engineer recombinant hypoallergenic allergen derivatives. According to the amino acid sequences and experimental epitope mapping data, synthetic peptides representing T- or B-cell epitopes are produced and purified recombinant allergens are coupled to novel adjuvants for vaccine formulation. In this article, strategies for the production and evaluation of allergen derivatives with reduced allergenic activity for allergy vaccination are described. These new vaccines hold great promise to improve the current practice of allergen-specific immunotherapy and maybe also used for prophylactic vaccination in the future.

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1. Introduction

Type I allergy is a hypersensitivity disease affecting more than 25% of the population [1]. Allergic patients are characterized by the increased production of IgE antibodies against per se harmless antigens (i.e., allergens) from various sources (e.g., plants, animals, fungi, and food) [2]. The immediate symptoms of Type I

allergy (allergic rhinitis, conjunctivitis, asthma, dermatitis, and anaphylactic shock) are caused by allergen-induced crosslinking of mast cell-bound IgE antibodies and the release of inflammatory mediators (e.g., histamine and leukotrienes) [3,4].

The symptoms of Type I allergy can be reduced by pharmacological (i.e., anti-inflammatory) treatment, however, specific immunotherapy represents the only causative treatment of allergic patients [5]. Specific immunotherapy is based on the administration of increasing doses of allergens to allergic patients to induce a state of unresponsiveness [6,7]. Since the introduction of specific immunotherapy by Noon in 1911 [8], numerous clinical studies have proven the efficacy of this treatment but the underlying immunological mechanisms are still not completely understood [5,6].

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One major disadvantage of current immunotherapy is that the treatment is performed with natural allergen extracts which represent ill-defined mixtures of allergenic and non-allergenic components [5]. Natural allergen extracts are obtained from allergen sources. Their allergen contents may vary considerably depending on various factors (e.g., choice of allergen source, method of extraction) and there may be great batch to batch variations [9,10]. It is now well established that allergic patients show different IgE reactivity profiles to the individual allergens in a given allergen extract and it is hence not possible to perform patient-tailored treatment with such extracts [11]. On the one hand, it is possible that certain allergens are not present in the extract, thus excluding successful treatment in patients who are sensitized to these molecules. On the other hand, considerable evidence has accumulated that patients may become newly sensitized by extract-based treatment to allergens against which they were not allergic before treatment (reviewed in [12]).

Treatment according to the patient's individual sensitization profile requires the availability of a complete spectrum of allergen molecules for diagnostic and therapeutic purposes. The introduction of molecular biological techniques into the field of allergen characterization indeed gave rise to the production of many relevant recombinant allergens which equal the natural allergens regarding biochemical, biological, immunological, and structural properties [13,14]. Sequence analysis of recombinant allergens revealed high similarities between certain allergens from related and unrelated species [15]. As a consequence of these similarities and crossreactivities, it has become possible to use IgE competition studies to identify those recombinant allergens which contain most of the IgE epitopes present in natural allergen sources (e.g., pollens, food) [16–18]. Based on these developments, diagnostic tests based on purified recombinant allergens were obtained, which allow us to determine sensitization profiles of allergic patients [19]. According to component-resolved diagnosis (CRD), it is now possible to identify the disease-eliciting allergens for each patient and to perform patient-tailored, component-resolved immunotherapy (CRIT) treatment [20].

Treatment with purified recombinant allergens according to the patient's sensitization profile may solve the problems related to the use of ill-defined allergen extracts but does not address the second major disadvantage of allergen-specific immunotherapy, i.e., the possible induction of severe and life-threatening anaphylactic side effects.

Before we discuss the strategies which are currently used to convert allergens into derivatives with reduced allergenic activity [20], we need to discuss the importance of selecting the relevant allergens for this procedure.

2. Selection of the most relevant allergen sources and allergens

A systematic approach towards the development of allergy vaccines involves several steps. First, it is necessary to identify the most relevant allergen sources for which vaccines need to be developed (Fig. 1). We would like to illustrate this process using spring pollinosis in Middle and Northern Europe as a concrete example.

People showing allergic symptoms in early spring suffer from allergy to birch, alder, hazel, hornbeam, and oak and are frequently sensitized to plant food. Molecular characterization of the relevant allergens has revealed a high sequence homology between allergens of these species [21,22]. Therefore, the wide range of sensitization observed is due to a crossreactivity of serum IgE antibodies from allergic patients to homologous allergens [23]. Crossreactivity could also be observed at the cellular level, which showed that T-cell clones specific for one particular allergen proliferated with homologous allergens [24,25].

Thus, the key step for the production of recombinant allergens and hypoallergenic derivatives is the identification of the pivotal allergen sources and allergens which contain most of the important B cell and T cell epitopes and represent the originally sensitizing agents within a cross-reacting group of allergen sources.

RAST inhibition experiments indicated that birch pollen represents the originally sensitizing agent within tree pollen allergy [26] and additionally showed that all immunological determinants in apple, carrot, and celery were also present in birch pollen but not vice versa [27]. Using recombinant allergens for IgE inhibition studies, it could be demonstrated that preincubation of allergic patients' sera with the recombinant birch pollen allergens rBet v 1 [28] and the pan-allergen profilin (rBet v 2) [29] almost completely inhibited patients' IgE binding to tree pollen extracts of the order *Fagales* and to plant food allergens [18]. These studies identified birch pollen as the original sensitizing agent, and thus the most relevant allergen source, for tree pollen allergy and several forms of plant food allergy. The finding that most tree pollen-specific IgE was directed against rBet v 1 [16] identified Bet v 1 as the most relevant birch pollen allergen.

In this context, it could be demonstrated that immunotherapy with a birch pollen extract efficiently reduced symptoms of related tree pollen (e.g., alder and hazel) and plant food (e.g., apple) allergies [30–32].

Once the pivotal allergens have been identified, cDNAs coding for these allergens can be isolated from cDNA expression libraries with serum IgE antibodies from allergic patients using immunoscreening technology [33]. Recombinant allergens, equalling the natural ones regarding biochemical, biological, immunological, and structural properties [13,14], are produced in

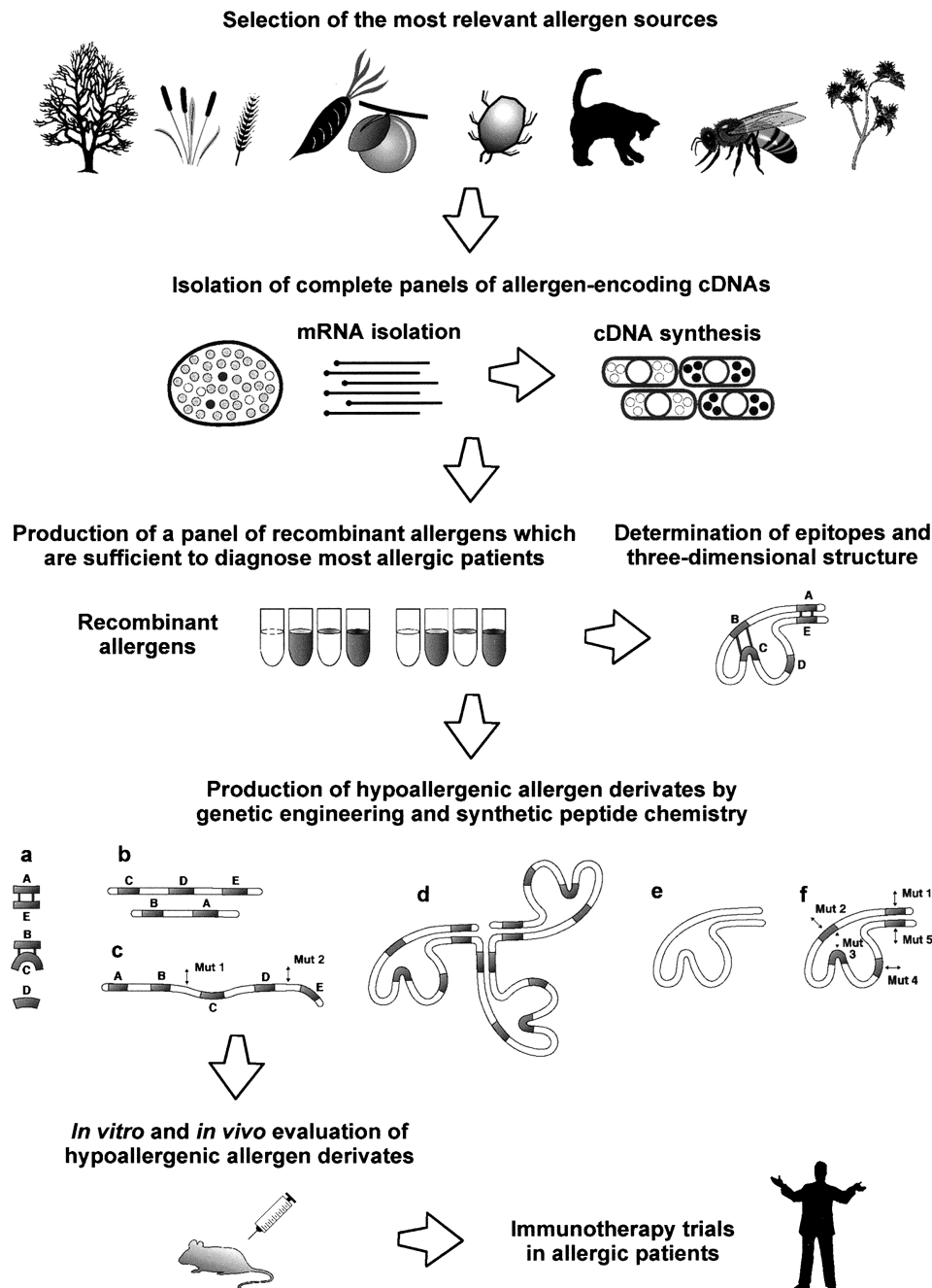


Fig. 1. Suggested protocol for the production of hypoallergenic allergy vaccines. In a first step, allergen sources which contain the important B cell and T cell epitopes and represent the originally sensitizing agents are identified. The allergen-encoding cDNAs are isolated, which allow us to produce recombinant allergens covering the epitope spectrum of the allergen source. These cDNAs then serve as templates for the production of allergen derivatives with reduced allergenicity for specific immunotherapy. In vitro and in vivo evaluation strategies are then applied to identify the most suitable vaccines (i.e., reduced allergenicity, retained immunogenicity), which can then be tested in immunotherapy trials in allergic patients.

suitable expression systems and can be used for component-resolved diagnosis (CRD) to select the patients who are sensitized against these allergens for component-resolved immunotherapy (CRIT) [11] (Fig. 1). In this context, it is noteworthy that it could be shown that a few recombinant allergens are sufficient to diagnose most tree and grass pollen allergic patients [34,35].

Additionally, recombinant allergens can be used to analyze allergenic proteins in detail, e.g., to determine their three-dimensional structure and their B- and T-cell epitopes (Fig. 1).

However, since recombinant allergens equal their natural counterparts, they bear the danger of inducing anaphylactic side effects when used for immunotherapy.

Therefore, allergen derivatives with reduced allergenic activity are developed and evaluated for safer forms of specific immunotherapy (Fig. 1). For the production of hypoallergenic allergen derivatives, recombinant DNA technology as well as synthetic peptide chemistry can be employed, according to the properties of the given allergens. The following strategies for the production of hypoallergenic derivatives will be discussed: (i) Production of recombinant allergen fragments, (ii) Insertion of mutations by site-directed mutagenesis, (iii) Production of peptides representing B-cell or T-cell epitopes, and (iv) Chemical modification of allergens.

3. Strategies for converting allergens into hypoallergenic derivatives

3.1. Recombinant allergen fragments

3.1.1. Background

Allergens represent low molecular weight proteins containing several different IgE epitopes mainly of proteinaceous nature. Two types of IgE epitopes have been defined. IgE epitopes can be represented by a stretch of amino acids in a row (continuous epitopes) or can be composed of at least two different areas of the molecule brought together by folding of the protein (discontinuous or conformational epitopes). Since discontinuous epitopes are dependent on the correct conformation of the allergen, disruption of the three-dimensional structure may reduce the IgE binding capacity and thus the allergenic activity of the given allergen. This can be obtained by expressing recombinant allergen fragments which contain the relevant T-cell epitopes in heterologous host organisms [36,37].

3.1.2. Method

Cloning. Recombinant fragments of allergens with discontinuous IgE-epitopes should be generated to maintain the relevant T-cell epitopes and to obtain rather large fragments which retain their immunogenic properties. cDNAs coding for fragments are obtained by PCR amplification using the cDNA of the wild type allergen as template and oligonucleotides, containing restriction sites, as primers. *NdeI* sites or *NcoI* sites are incorporated into the N-terminal primers to allow subcloning into the *NdeI* site or the *NcoI* site of an appropriate expression plasmid (e.g., pET vectors, Novagen) where the ATG of these sites serves as start codon for the expression of a recombinant protein. Since PCR products can only be cut very inefficiently with certain restriction enzymes (e.g., *NdeI*, *NcoI*), it is advised to incorporate additional *EcoRI* sites into the N-terminal primers to improve subcloning efficiency. In general, care should be taken that the selected restriction sites for the primers are not present in the template DNA.

PCR products are then cut with *EcoRI* and the restriction enzyme selected for the C-terminal primer, purified by agarose gel electrophoresis or with nick columns, and subcloned into the corresponding restriction sites of the vector. We recommend to use vectors from the pET series, which contain signal sequences for the expression of recombinant proteins as non-fusion proteins and make use of the bacteriophage T7 RNA polymerase which allows high-level expression of the foreign protein. The constructs are transformed into an *Escherichia coli* strain and inserts are excised with *NdeI* or *NcoI* and the enzyme of the C-terminal primer and subcloned into the corresponding sites of the expression plasmid. After transformation, positive clones can be identified either by mini-prep analysis, by PCR amplification or by immunoscreening with antibodies which recognize the fragments. DNA from positive clones can be isolated by a plasmid maxi-prep procedure and the cDNA inserts should be sequenced to identify possible PCR mismatches. cDNAs cloned into pET vectors and transformed into *E. coli* BL 21 (DE3) (Novagen) can be expressed in liquid culture upon induction with isopropyl- β -thiogalactopyranoside (IPTG).

Purification. For the purification of recombinant allergen fragments, two different methods can be employed. The proteins can either be purified by conventional chromatographic methods (e.g., ion exchange, gel filtration) or it is also possible to produce recombinant proteins containing tags (e.g., His-tags), which can be purified by single-step affinity purification. For this purpose, vectors are available which allow the production of recombinant proteins fused to an N-terminal or C-terminal His-tag, but it is also possible to insert His-tags, containing six consecutive histidine residues, into one of the primers used for PCR amplification of the allergen fragments. The His-tag sequence binds to divalent cations (e.g., Ni^{2+}), which allows us to purify his-tagged proteins by affinity chromatography with nickel-nitrilotriacetic acid (Ni-NTA) matrices (Qiagen, Hilden, Germany).

Regardless of the method used for purification, as the first step it is important to determine whether the recombinant protein is soluble or located in inclusion bodies. If a recombinant protein can be produced as soluble protein in *E. coli*, it can be purified directly under native conditions. However, many proteins form inclusion bodies when they are expressed at high levels in bacteria. These insoluble aggregates have to be solubilized using strong denaturants, such as 8 M urea or 6 M GuHCl. The proteins can be purified under denaturing conditions and afterwards be refolded in solution; however, for many proteins the refolding procedure has been shown to be more successful while the proteins are immobilized on the Ni-NTA matrix. The purified proteins can be lyophilized and stored at -20°C , but it should be considered that insoluble proteins often form aggregates after lyophilization.

3.1.3. Mode of administration

Hypoallergenic allergen fragments may be administered in different ways. Fragments may be coupled to Al(OH)₃ as adjuvant and patients are treated with this conjugate using a classical specific immunotherapy (SIT) scheme. High doses of the derivatives may be administered due to their hypoallergenic nature, which will be accompanied by high titers of blocking IgG antibodies. In this context, animal models have demonstrated that hypoallergenic rBet v 1 fragments induce IgG antibodies which inhibit patients' IgE binding to the Bet v 1 wild-type [38]. Alternatively, fragments may be used as soluble T-cell containing peptides to induce systemic tolerance. Mouse models showed that not only short peptides but also long peptides induce specific T cell tolerance to the native allergen [39] and that mucosal tolerance can be induced with hypoallergenic rBet v 1 fragments by mucosal administration of the soluble antigen [40].

3.2. Recombinant allergen mutants

3.2.1. Background

Using site-directed mutagenesis, point mutations can be inserted into recombinant allergens which may reduce the IgE binding capacity of the allergen in two different ways. First, point mutations outside the IgE binding epitopes may lead to a change of the three-dimensional structure of the allergen, leading to the destruction of discontinuous IgE epitopes. This can also be achieved by destroying cysteine residues and thus disulfide bonds by *in vitro* mutagenesis [41,42]. Second, point mutations or deletions of several amino acids within the IgE epitope may reduce the IgE binding capacity by abolishing the antigen–antibody interaction [43,44]. The second strategy rather leads to an alteration of IgE epitopes than of the three-dimensional structure of the allergen and represents therefore a possible way to convert allergens which contain preferentially continuous IgE binding sites into hypoallergenic derivatives.

3.2.2. Method

Regardless of whether point mutations are introduced within or outside IgE-binding sites, care should be taken to preserve T-cell epitopes and immunogenicity. Oligonucleotides, containing the appropriate point mutation, have to be constructed and the site-directed mutagenesis can be performed using commercially available kits according to manufacturer's instructions. These kits bear the advantage that the mutagenesis can be performed directly in the desired vector (e. g. expression plasmid of the pET series). The mutagenized allergen can then be expressed and purified as described for hypoallergenic allergen fragments.

3.2.3. Mode of administration

Mutagenized allergens have generally a comparable size and thus a similar immunogenicity as their natural counterparts. Therefore, they may be used as vaccines conjugated to an adjuvant (e.g., Al(OH)₃) with the aim of inducing blocking IgG antibodies which inhibit IgE binding to the corresponding natural allergen. Mutants of the *Brassica* pollen allergen, Bra r 1, induced IgG antibodies in a mouse model which recognized native Bra r 1 [45]. On the other hand, intranasal administration of a soluble, mutagenized house dust mite allergen, Der p 2, has significantly reduced immediate bronchoconstriction provoked by the wildtype allergen [46].

3.3. T-cell or B-cell epitope containing peptides

3.3.1. Background

The production of peptides containing T-cell or B-cell epitopes is a generally applicable strategy for the generation of hypoallergenic allergen derivatives. The only prerequisite is the knowledge of the epitope regions, which can be determined by experimental epitope mapping. Whereas T-cell epitope containing peptides aim to induce tolerance or anergy in allergen-specific T-cells [47], IgE-epitope-derived synthetic peptides aim at inducing blocking IgG antibodies [48].

3.3.2. Method

Peptides should be synthesized, which are between 25 and 35 amino acids long. Shorter peptides bear the danger of not being immunogenic even when they are coupled to carriers, whereas peptides longer than 35 amino acids may become IgE reactive and allergenic and are difficult to synthesize.

Peptides are synthesized using a peptide synthesizer, their identity must afterwards be checked by mass spectrometry and they must be purified to >90% purity by HPLC. Due to the short length of the peptides, they may be coupled to carriers to improve their immunogenicity. KLH (Keyhole limpet hemocyanin) (Pierce, Rockford, IL) can easily be conjugated to peptides using an Inject Maleimide Activated Immunogen Conjugation Kit (Pierce, Rockford, IL). For this purpose, it is advantageous to add an additional cysteine residue to the N- or C-terminus of the peptides to facilitate coupling to KLH.

3.3.3. Mode of administration

B-cell epitope containing peptides are coupled to an adjuvant (e.g. Al(OH)₃) and used for vaccination to induce blocking IgG antibodies [48]. T-cell epitope containing peptides, however, may be used as soluble peptides to induce T cell tolerance to the corresponding natural allergens. Using mouse models it could be shown that subcutaneous injections as well as oral administration of T-cell epitope containing peptides

induced tolerance in T-cell responses against their wildtype allergens [49–51].

3.4. Chemical modification of allergens

3.4.1. Background

Chemical modification of allergen extracts and allergens to reduce the allergenic activity has already been a research goal for many years. Polymerization of allergens by aldehyde treatment as well as coupling of methoxypolyethylene glycol (mPEG) to allergens yielded allergoids that retained immunogenicity, but had a reduced allergenic activity [52–55]. Plasmid gene vaccination studies have led to the discovery of immunostimulatory DNA sequences (ISS-ODN or cytosine–phosphate–guanine (CpG) motifs) in the bacterial vectors with adjuvant activity [56,57]. ISS-ODNs were first described in Freund's adjuvant, which has been used as adjuvant to improve immune responses since 1937 [58].

3.4.2. Method

When ISS-ODN is mixed with an allergen, it serves as a potent adjuvant which induces strong Th1 responses against the respective allergen [59]. However, it could be shown that physical conjugates of allergen and ISS-ODN are more immunogenic and less allergenic than allergens mixed with ISS-ODN [60,61]. The optimal conjugation ratio between allergen and ISS-ODN remains to be determined, since it has been shown that increases in the ISS-ODN–allergen ratios lead to decreases in the allergenicity but that higher ISS-ODN–allergen ratios have reduced immunogenicity [60]. The reduced allergenicity of the conjugates may result from a hiding of IgE epitopes, a principle which has also been proposed as the basis for the reduced allergenicity of allergoids (e.g., glutaraldehyde-polymerized allergens, PEG-conjugated allergens). Initial experiments with ISS-ODN conjugates were performed with the natural short ragweed allergen, Amb a 1 [62,63], but research will be facilitated by using purified recombinant allergens. Recombinant allergens are available in large amounts and offer the opportunity to introduce site-directed modifications, e.g., to allow better coupling of ISS-ODN to the allergen or to reduce the allergenicity of the allergen.

3.4.3. Mode of administration

ISS-ODN serves as adjuvant for the induction of immune responses. ISS-ODN conjugates are therefore used as vaccines to induce Th1-biased immune responses. In this context, it could be shown that an Amb a 1-ISS conjugate induced a Th1 response in mice [62,64], whereas Amb a 1 induced a Th2 response [62]. Immunization of rabbits and cynomolgus monkeys with Amb a 1-ISS conjugates induced anti-Amb a 1 IgG antibodies [62].

4. In vitro and in vivo evaluation of hypoallergenic allergen derivatives

To identify those hypoallergenic derivatives which are best suited for immunotherapy, they have to be evaluated thoroughly in vitro and in vivo. In vitro assays for the evaluation of hypoallergenic derivatives comprise the comparison of the derivatives with the corresponding wild-type allergens regarding their IgE binding capacity, induction of basophil histamine release, and T cell proliferation. Suitable allergen derivatives are characterized by a reduced capacity to release histamine from patients' basophils and by the preservation of relevant T-cell epitopes. Although a reduced IgE-binding capacity seems to be desirable, hypoallergenic allergen derivatives have also been identified with B cell epitopes comparable to that of the wild-type allergen [65]. Those derivatives with reduced allergenic activity in vitro have to be further evaluated in vivo.

The in vivo evaluation of allergen derivatives should comprise animal studies and provocation tests in allergic patients. Appropriate animal models are required depending on the desired mode of action of the hypoallergenic derivatives and thus on the mode of administration. Treatment of animals should take place under similar conditions as immunotherapy of allergic patients regarding the choice of the adjuvant, the frequency of treatment, and the doses of the hypoallergenic derivative to obtain results applicable to the treatment of patients.

Immunization of animals (e.g., mice) with allergen derivatives coupled to adjuvants should indicate whether the hypoallergenic derivatives can induce Th1-like responses accompanied by the induction of blocking antibodies, which recognize the wildtype allergen as well as cross-reacting allergens present in related and unrelated species and inhibit patients' IgE binding to these allergens. Treatment of animals with soluble T-cell based epitopes should demonstrate that the T-cell peptides can induce T-cell tolerance against the respective wildtype allergen.

Hypoallergenic allergen derivatives which have been shown to be suited in animal models should also be tested in a representative number of patients in provocation tests, to test the allergenic activity of the derivatives and to select the best variants for immunotherapy. In the case of Bet v 1, two fragments, which have been shown to have a reduced allergenic activity in vitro [36] and to induce blocking antibodies in mice and rabbits [38], exhibited a more than 100-fold reduced allergenic activity compared to rBet v 1 wildtype in skin prick and intradermal tests [66,67]. Moreover, these hypoallergenic derivatives induced less activation of eosinophils in a skin blister model in patients [68], which therefore may be considered as an additional in vivo evaluation method to study the induction of late phase reactions.

Peptides, containing the immunodominant T cell epitopes of the cat allergen, Fel d 1, induced T-cell tolerance to complete Fel d 1, when administered to cat allergic patients, although adverse late reactions occurred [69,70]. Mutants of the major ryegrass pollen allergen, Lol p 5, with reduced IgE binding capacity exhibited a reduced allergenic activity compared to the Lol p 5 wildtype in skin prick tests [43]. Intradermal tests indicated that Amb a 1-ISS conjugates are considerably less allergenic than a crude ragweed extract [71] and an initial immunotherapy trial suggested that they were well tolerated by ragweed allergic patients [72].

5. Concluding remarks

Hypoallergenic allergen derivatives which were identified as the best candidate vaccines by a series of evaluation procedures (Fig. 1) can be further evaluated regarding their safety and efficacy in clinical immunotherapy trials.

Specific immunotherapy with hypoallergenic allergen derivatives can be tailored according to the patient's sensitivity profile, so that therapy-induced de novo sensitization to originally not recognized allergens does not occur [73]. It is expected that a reduced rate of anaphylactic side effects will be observed due to the reduced allergenic activity of the derivatives. Furthermore, such derivatives may represent candidate molecules for prophylactic vaccination against allergy [74].

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