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Review article

From allergen genes to new forms of allergy diagnosis and treatment

Type I allergy represents an important health problem that affects more than 25% of the population in industrialized countries. Specific immunotherapy is one of the few causative treatment approaches for type I allergy and is currently performed with crude allergen extracts, which consist of a mixture of allergenic and nonallergenic components, are difficult to standardize and cannot be applied according to the patient's reactivity profile. With the introduction of molecular biological techniques into allergy research, a large panel of individual recombinant allergens has become available. Recombinant allergens can be used for improved diagnosis of allergy to determine the patient's sensitization profile, which is a prerequisite to select the allergens for patient-tailored immunotherapy. They allow the elucidation of the properties of allergens and of the mechanisms of allergy as well as of the mechanisms of immunotherapy. Moreover, recombinant allergens allow the development of hypoallergenic allergen derivatives with reduced allergenic activity and retained immunogenicity. First immunotherapy trials with hypoallergenic allergen derivatives have shown that this treatment might improve immunotherapy in the near future. This review summarizes the results, which were obtained with recombinant allergens and hypoallergenic allergen derivatives. The experiences from the *in vitro* and *in vivo* evaluation of the hypoallergenic derivatives and from clinical studies as well as the contribution of hypoallergenic derivatives to develop new treatment strategies and possibly prophylactic vaccination strategies are discussed.

S. Vrtala

Division of Immunopathology, Department for Pathophysiology, Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria

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Susanne Vrtala
Division of Immunopathology
Department for Pathophysiology
Center for Physiology and Pathophysiology
Medical University of Vienna
Vienna
Austria

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Type I allergy that represents a major health problem worldwide affects more than 25% of the population (1). Allergic patients are characterized by the increased production of immunoglobulin E (IgE) antibodies against *per se* harmless antigens from different sources (e.g. pollen, mites, animal dander and food) (2). The immediate symptoms of type I allergy (e.g. allergic rhinoconjunctivitis, asthma, atopic dermatitis and anaphylactic shock) are caused by allergen-induced cross-linking of effector cell-bound IgE antibodies and the release of inflammatory mediators (e.g. histamine and leukotrienes) (3–5).

Specific immunotherapy has been introduced by Noon in 1911 and is still one of the few causative treatment approaches for type I allergy (6). This treatment is based on the administration of increasing doses of allergens to allergic patients to induce a state of unresponsiveness (6). Several studies have shown the effectiveness of specific immunotherapy for the treatment of allergic disorders and there are several hypotheses regarding the mechanisms underlying immunotherapy. These hypotheses include the modulation of the allergen-specific immune response from a T helper 2 (Th2) response towards a preferential Th1 response (7–9), the induction of allergen-specific T-cell tolerance by the generation of allergen-specific interleukin-10 (IL-10) secreting T-regulatory cells, which suppress allergen-induced proliferation of Th1 and Th2 cells as well as their cytokine production (10–12) and the induction of allergen-specific IgG antibodies that antagonize IgE-mediated effects (13–15). Major disadvantages of immunotherapy are that the treatment is performed with allergen extracts, which consist of a mixture of allergenic and nonallergenic components, are difficult to standardize and cannot be applied according to the patient's reactivity profile (6). Certain allergens may not be present in the extract in sufficient amounts to treat patients successfully which are sensitized to these molecules, whereas patients may become sensitized to allergens in the extract against which they were not allergic before the treatment (16, 17). Moreover, because of the administration of active allergens, severe and life-threatening anaphylactic side-effects can occur. Several attempts were made in the past to reduce the rate of side-effects, including the adsorption of allergen extracts to adjuvants to delay the systemic release of allergens from the site of application (18) and the chemical modification

of allergen extracts to reduce allergenic activity (19–22). However, the lack of single, standardized components did not allow a controlled modification.

The fast development of molecular biological techniques allowed in the late 1980s the cloning of the first allergen (23). When the first recombinant allergens were produced (24, 25), many concerns were raised regarding the applicability of recombinant allergens for diagnosis and therapy of allergic patients. Major concerns were whether recombinant allergens mimic the properties of natural allergens, whether a limited panel of recombinant allergens can be defined, which resembles the IgE-epitope spectrum of natural extracts and whether they can be used for immunotherapy. Allergy diagnosis with recombinant allergens requires that panels of the most important recombinant allergens from different allergen sources have to be produced, which contain the relevant IgE epitopes of the natural extracts. Improved immunotherapy requires the modification of allergens into derivatives with reduced allergenic activity.

This review summarizes the results which were obtained since the establishment of recombinant allergy technology. It was shown that recombinant allergens can indeed be used for improved diagnosis of allergy to determine the patient's sensitization profile, which is a prerequisite to select the allergens for patient-tailored immunotherapy. Moreover, recombinant allergens may improve the diagnostic selection of patients for immunotherapy with allergen extracts. Recombinant allergens allowed for the first time the elucidation of the properties of allergens and of the mechanisms of allergy as well as of the mechanisms of immunotherapy. Additionally, this review gives an overview over strategies that have been applied to modify recombinant allergens into hypoallergenic derivatives for improved immunotherapy, the experiences which were obtained from the *in vitro* and *in vivo* evaluation of the hypoallergenic derivatives and from clinical studies as well as their contribution to understand the mechanisms of allergy.

Results and discussion

Recombinant allergens can replace natural allergen extracts

To replace allergen extracts with recombinant allergens, it has to be shown that recombinant allergens equal their natural counterparts and that a panel of allergens can be identified which contain most of the important B-cell and T-cell epitopes of the natural allergen source.

Grass pollen represents one of the most important allergen sources against which more than 40% of allergic patients are sensitized and in Central Europe, the grass species *Phleum pratense* (timothy grass) was shown to be prevalent (26). Screening of a *Phleum pratense* cDNA library with serum IgE from grass pollen allergic patients allowed the isolation of the most important grass pollen allergens [i.e. Phl p 1 (27), Phl p 2 (28), Phl p 5 (29), Phl p 6 (30), Phl p 7 (31) and timothy grass profilin (32, 33)]. Phl p 1 and Phl p 5 represent the most important grass pollen allergens with IgE-binding frequencies of more than 80% (27, 29) and inhibition studies showed that recombinant Phl p 5 contained the most relevant IgE-epitopes of natural Phl p 5 isoforms (29). This study and others indicated that recombinant allergens equal their natural counterparts regarding biochemical, immunological and structural properties (29, 34). It was shown that a panel of only four timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5 and profilin) was sufficient to diagnose grass pollen allergy in almost all grass pollen allergic patients and that a high percentage of grass pollen-specific IgE was directed against those four recombinant allergens (25, 35, 36). Recent clinical trials were performed with a panel of five recombinant grass pollen allergens [Phl p 1, Phl p 2, Phl p 5a, Phl p 5b (37), Phl p 6 (38)] and showed that this treatment led to a significant reduction in the symptoms and to the induction of strong allergen-specific IgG responses (39).

Similar results were obtained for house dust mite (HDM) allergy. House dust mites are recognized as one of the most common causes of allergy worldwide, against which more than 50% of allergic patients are sensitized (40). In contrast to birch pollen and grass pollen, HDM contain a much more complex allergen repertoire. So far, 21 mite allergens have been identified but little is known about the importance of most of these allergens (41) and it is supposed that some important allergens have not been identified by now (42). The reason for this is that some allergens can only be discovered by molecular biological techniques because they are not present in natural allergen extracts. Also in the case of mite allergy, two-dimensional IgE inhibition experiments indicated that recombinant mite allergens contain the relevant IgE epitopes of the natural isoallergens and that a panel of a few mite allergens binds a high percentage of mite specific IgE antibodies (43). Figure 1 shows that preadsorption of sera from mite allergic patients with different recombinant mite allergens (Der p 2, Der p 5, Der p 7 and Der p 8) led to a complete inhibition of IgE reactivity to the respective natural isoallergens, indicating that recombinant mite allergens contain the major IgE epitopes of their naturally occurring isoallergens (Fig. 1). These results from different allergen sources show that recombinant allergens can replace natural allergens for diagnosis of allergic patients and that a limited number of allergens can be defined, which are sufficient for diagnosis.

Recombinant allergens may improve diagnosis of allergic patients

Natural allergen extracts only allow determining whether a patient is sensitized to a certain allergen source but not to which allergens he is sensitized. Recombinant allergens allow for the first time to determine the exact sensitization profile of a certain individual (24, 25, 44–46). This serves as

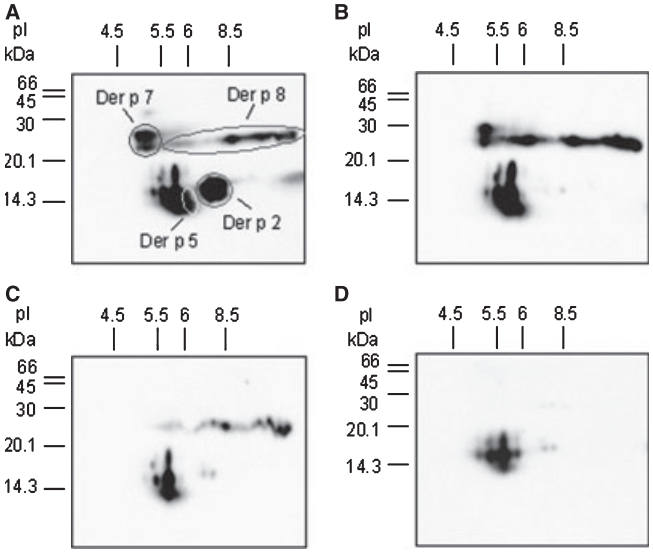

Figure 1. Sequential two-dimensional (2D) IgE inhibition. Serum from a mite allergic patient was preadsorbed with bovine serum albumin (BSA) (A), rDer p 2 (B), rDer p 2 + rDer p 5 + rDer p 7 (C) and rDer p 2 + rDer p 5 + rDer p 7 + rDer p 8 (D). Blots containing 2D-separated *Dermatophagoides pteronyssinus* extracts were incubated with the preadsorbed serum and bound IgE antibodies were detected with ^{125}I – labelled anti-human IgE antibodies.

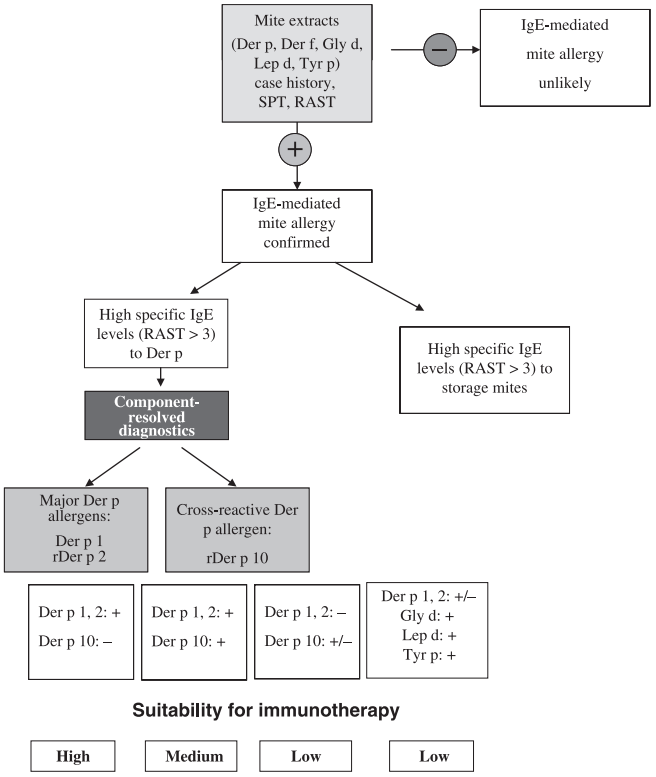

Figure 2. A possible decision tree for immunotherapy with house dust mite (HDM) extracts. IgE-mediated allergy to HDM should be confirmed using HDM allergen extracts. Patients with strong reactivity to HDM allergens can further be analysed with purified HDM allergens (Der p 1, Der p 2 and Der p 10) to determine their suitability for HDM immunotherapy.

a prerequisite to select those allergens against which a patient is sensitized for a component-resolved specific immunotherapy (47, 48). Moreover, recombinant allergens allow the development of new diagnostic tests for an improved selection of patients for immunotherapy with allergen extracts (46). Figure 2 shows a possible decision tree for the selection of patients for immunotherapy with HDM (*Dermatophagoides pteronyssinus*) extracts. In the case of HDM allergy, diagnosis with Der p 1 and Der p 2 would discriminate patients with IgE-reactivity to these allergens from those which show no reactivity to Der p 1 and Der p 2. As therapeutic extracts are only standardized for Der p 1 and Der p 2 and some other important allergens are only present in small amounts in these extracts, only patients who are sensitized to Der p 1 or Der p 2 should receive immunotherapy with mite extracts, whereas patients without sensitization to Der p 1 and Der p 2 will presumably not benefit from therapy with mite extracts. The incorporation of the cross-reactive mite allergen, tropomyosin (Der p 10), into a diagnostic test would allow identifying patients with broad cross-reactivities, who may be less suitable for immunotherapy (Fig. 2). Diagnostic tests based on recombinant allergens may also be used for the immunological monitoring of patients undergoing immunotherapy to detect novel IgE reactivities to allergens, which may possibly be induced during therapy. In this context, it has been speculated that immunotherapy with mite extracts can induce food allergy to sea foods, because of the induction of novel IgE responses to the mite tropomyosin, Der p 10, which represents a highly cross-reactive allergen found in a variety of sea foods (49).

The fact that some allergens can only be identified by molecular biological techniques but are not present in natural extracts, further demonstrates the superiority of recombinant allergens for diagnosis of allergy because individuals with IgE-reactivity to allergens not present in the extracts will not be diagnosed as allergic patients with natural allergen extracts.

Recombinant allergens allow studying the properties of allergens and the mechanisms of allergy and immunotherapy

Recombinant allergens allow for the first time to study the properties of allergens. It was shown that major allergens are released in large amounts very rapidly from hydrated pollen, whereas proteins with lower IgE-binding frequencies and nonallergenic proteins retain in the pollen grains (50). Other studies postulated that some functional properties of proteins such as enzyme activity may contribute to the allergenicity although several allergens have been identified without enzymatic activity (51). Immunogold electron microscopy localized recombinant

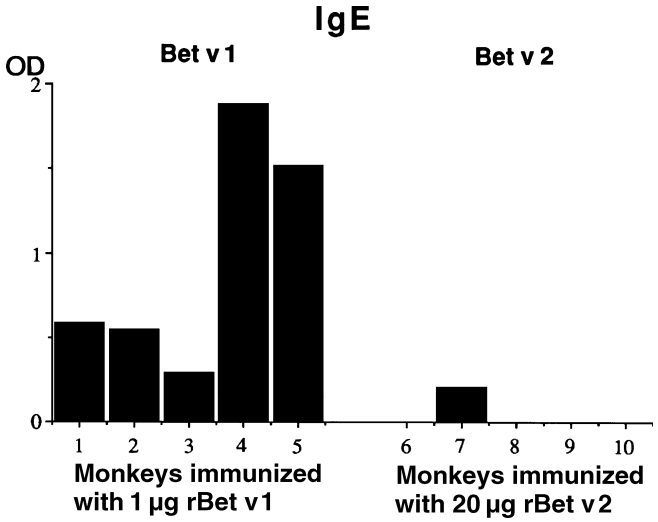

Figure 3. Immunization of rhesus monkeys with Bet v 1 and Bet v 2. Groups of five monkeys were immunized with the birch pollen allergens, rBet v 1 and rBet v 2, and induced IgE antibodies were determined by ELISA. The optical density values (OD 405 nm) displayed on the *y*-axis correspond to the level of IgE antibodies in the monkey sera.

grass pollen allergens in different compartments of the pollen. Whereas Phl p 6 was localized on the polysaccharide-containing wall-precursor bodies (P-particles), groups 1 and 2 allergens were found in cytoplasmic debris (30, 52, 53). Despite the different localization, P-particles as well as debris particles can act as small respirable allergen carriers, which are expelled from hydrated grass pollen grains (52, 53). This may explain the frequent occurrence of asthma attacks seen after heavy rainfalls (54). The immunogold electron microscopical localization of allergens in mite bodies revealed that most allergens are located in the gut of the mites and in the faecal pellet assuming that patients become sensitized via the faecal pellet (40, 55). Epidemiological studies showed that farm exposure protects against grass specific IgE responses but that it has no effect on mite responses (56). Moreover, it was shown that allergens which bind high levels of IgE in humans induce considerably higher levels of IgE and IgG1 antibodies in mice and monkeys than allergens with little or no allergenic activity in humans (57, 58). Figure 3 shows that the major birch pollen allergen, Bet v 1, induced considerably higher titres of specific IgE antibodies in monkeys already at low doses (1 µg) compared with the minor allergen, Bet v 2, which induced very low IgE responses only at high doses (20 µg). Recombinant allergens also allowed the determination of the structures of a series of important allergens, which revealed that different allergens display different conformations, indicating that there is no common fold that would predetermine the allergenicity of a given protein (59, 60).

Hypoallergenic allergen derivatives may improve immunotherapy of allergic patients

As recombinant allergens equal their natural counterparts, they bear the danger of inducing anaphylactic side-effects when used for immunotherapy. Therefore, several strategies have been applied to modify allergens into hypoallergenic variants, based on the knowledge obtained through the molecular, immunological and structural characterization of allergens (61). Recombinant DNA technology and synthetic peptide chemistry can be used for the production of hypoallergenic allergen derivatives for improved immunotherapy. The following strategies were applied to construct hypoallergenic derivatives: Recombinant allergen fragments were produced to destroy the three-dimensional structure of the allergen and thus conformational IgE epitopes (62–64). Oligomers (65) of allergens as well as hybrids of different allergens (66) were constructed to reduce the allergenic activity presumably through microaggregation but to retain the immunogenicity of the protein and deletion variants were produced (38, 67) to remove the IgE-reactive regions. Point mutations were inserted into recombinant allergens by site-directed mutagenesis (68–71), either outside the IgE epitopes to destroy the three-dimensional structure and thus conformational IgE epitopes or within the IgE epitopes to alter the IgE epitopes directly. Allergens were modified by conjugation to immunostimulatory sequences (ISS-ODN) to reduce the allergenic activity and to induce Th1-biased immune responses (72, 73). By synthetic peptide chemistry, T-cell epitope containing peptides were produced to induce tolerance or anergy in allergen-specific T-cell (74, 75) and B-cell epitope containing peptides were constructed with the aim of inducing blocking IgG antibodies (76, 77).

In vitro and *in vivo* evaluation of hypoallergenic allergen derivatives for improved immunotherapy

Before hypoallergenic derivatives can be used for immunotherapy of allergic patients, a thorough *in vitro* and *in vivo* evaluation is required to select those derivatives suitable for immunotherapy. This process is exemplified for hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, which were the first recombinant hypoallergenic allergen derivatives tested in immunotherapy trials in patients (15).

Bet v 1, the major birch pollen allergen, was selected as model system because radioallergosorbent test inhibition experiments showed that birch pollen represents the originally sensitizing agent within tree pollen allergy and that all immunological determinants in apple, carrot and celery were present in birch pollen but not vice versa (78, 79). Bet v 1 represents one of the most common allergens which is recognized by more than 90% of tree pollen allergic individuals and almost 60% of them are

sensitized exclusively to Bet v 1 (80, 81). In addition, Bet v 1 possesses conformational IgE epitopes and shares epitopes with homologous proteins present in the pollen of trees of the order *Fagales* and in plant-derived food (82). By genetic engineering, two Bet v 1 fragments, comprising aminoacids (aa) 1–74 and aa 75–160, as well as Bet v 1 oligomers were produced as recombinant proteins in *Escherichia coli* (62, 65, 83, 84). The Bet v 1 derivatives induced strong lymphoproliferative responses in peripheral blood mononuclear cells of birch pollen allergic patients and contained the relevant Bet v 1-specific T-cell epitopes (62, 65, 85). While the Bet v 1 fragments have lost their IgE binding capacity because of a loss of their native-like structure and therefore of conformational IgE epitopes, Bet v 1 oligomers retained their IgE-binding capacity when compared with the recombinant Bet v 1 wildtype. Nevertheless, Bet v 1 fragments as well as oligomers have a markedly reduced capacity to induce histamine release of basophils from birch pollen allergic patients and to induce immediate type skin reactions (62, 65).

It has been demonstrated that immunotherapy induces strong allergen-specific IgG responses and that protective IgG antibodies are important for the success of allergen-specific immunotherapy (13, 86, 87). Therefore, it is important to evaluate hypoallergenic allergen derivatives further regarding their immunogenicity. Whereas most of the derivatives which have been produced so far showed reduced allergenic activity, some of them also showed reduced immunogenicity and several were not tested for their immunogenicity. If allergens are conjugated to ISS-ODN, the conjugation ratio between allergen and ISS-ODN is important, as it has been shown that increases in the ISS-ODN – allergen ratios lead to decreases in the allergenicity but that higher ratios also have reduced immunogenicity (88). In the case of Phl p 6, a major grass pollen allergen, two fragments were produced by genetic engineering, which exhibited greatly reduced allergenic activity. Figure 4 shows the lack of skin reactivity of the C-terminal fragment in one representative skin test of a grass pollen allergic patient. However, only the C-terminal fragment induced IgG antibodies in mice whereas the N-terminal fragment has lost its immunogenicity (38). These results show the importance of a thorough *in vitro* and *in vivo* evaluation of the hypoallergenic derivatives to select the best vaccines for immunotherapy.

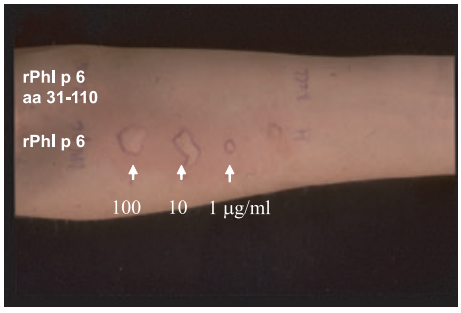

Figure 4. Lack of skin reactivity of the C-terminal fragment of Phl p 6 (rPhl p 6 aa 31–110). A grass pollen allergic patient was skin prick tested on the forearm with various concentrations (100, 10 and 1 µg/ml) of rPhl p 6 and rPhl p 6 aa 31–110. Wheal areas were surrounded.

In the case of the rBet v 1 derivatives (fragments and trimer), immunization of animals (mice and rabbits) showed that the hypoallergenic derivatives induced IgG antibodies, which recognized the wild-type allergen and cross-reacted with homologous allergens (65, 85). Moreover, IgG antibodies induced with rBet v 1 derivatives inhibited patients' IgE binding to rBet v 1 wildtype and to natural extracts from birch and related tree pollen (85, 89).

The reduced allergenic activity and retained immunogenicity of the rBet v 1 derivatives showed their potential as safe and effective vaccine for improved immunotherapy of patients allergic to birch pollen and related species (tree pollen, plant-derived food).

Encouraged by the results obtained in animals, the hypoallergenic derivatives were tested in clinical studies in birch pollen allergic patients. In a Swedish and a French study with 60 birch pollen allergic patients (90–92), skin prick and intradermal tests with hypoallergenic Bet v 1 fragments and Bet v 1 oligomers were performed. The recombinant Bet v 1 derivatives exhibited a more than 100-fold reduced allergenic activity compared with Bet v 1 wildtype and no late phase reactions were observed. The ability of the rBet v 1 derivatives (trimer, fragments) to induce late phase reactions and eosinophil activation was studied in a skin blister model, which showed that the hypoallergenic rBet v 1 derivatives induced less activation of eosinophils and lower release of eosinophil cationic protein and granulocyte macrophage colony-stimulating factor (GM-CSF) compared with Bet v 1 wildtype (93).

Immunotherapy trials with hypoallergenic allergen derivatives

During the last few years, first immunotherapy trials were conducted with hypoallergenic allergen derivatives. T-cell epitope containing peptides were administered without adjuvant to induce T-cell tolerance in patients. Although the peptides had no or only low IgE-binding capacity, they induced late phase systemic side-effects (94–96).

The first immunotherapy trials with recombinant hypoallergenic derivatives were performed with the rBet v 1 fragments and rBet v 1 trimer adsorbed to aluminium hydroxide (15). The treatment showed that beneficial immunomodulatory effects, reduction of clinical symptoms and inhibition of IgE memory responses were

associated with the induction of IgG antibodies which inhibited patients' IgE binding to the allergens and allergen-induced basophil degranulation (14, 15, 97).

Future perspectives

The encouraging results obtained in immunotherapy trials with hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, indicate that vaccines based on hypoallergenic allergen derivatives can also be developed for other allergen sources. In contrast to birch pollen allergy, where more than 60% of the patients are exclusively sensitized to Bet v 1, the allergen repertoire of the other most common allergen sources is much more complex. However, a recent immunotherapy trial with recombinant grass pollen allergens indicated that a limited panel of only five allergens is sufficient for a successful treatment of grass pollen allergic patients (39). The allergen repertoire of HDM, which represent one of the most important allergen sources worldwide (98), is even more complex (41). So far, 21 mite allergens have been described but new allergens are still discovered and little is known about the importance of most of the mite allergens. Future work will have to evaluate the importance of the mite allergens and to establish the panel of mite allergens necessary for immunotherapy of mite allergic patients in different populations. Hypoallergenic derivatives of those allergens, which are necessary for immunotherapy, need to be evaluated thoroughly *in vitro* and *in vivo* before first immunotherapy trials can be conducted. Immunotherapy trials with hypoallergenic allergen derivatives will also allow studying extensively the mechanisms underlying immunotherapy. Former studies showed that the fusion of weakly immunogenic allergens to unrelated allergens yielded hybrid molecules with strongly enhanced immunogenicity (99, 100). Therefore, it will be possible to construct hybrid molecules with improved immunogenicity consisting of low immunogenic allergens or hypoallergenic derivatives.

Because of the increasing prevalence of allergic diseases, prophylactic vaccination with hypoallergenic derivatives might be considered. Prophylactic treatment would prevent the onset of allergy and the development of severe symptoms. However, prophylactic treatment would require vaccines with reduced allergenicity. A new approach to construct vaccines for prophylactic treatment is the production of B-cell epitope derived peptides (77). Future studies will have to construct B-cell peptides of the most important allergens and to test them regarding their applicability for prophylactic treatment.

Concluding remarks

When in the early 1990s the first recombinant allergens were produced, many concerns were raised regarding the applicability of recombinant allergens for diagnosis and immunotherapy of type I allergy. Extensive research since that time showed that recombinant allergens can indeed replace natural allergens extracts. It was shown that recombinant allergens equal their natural counterparts regarding biochemical, immunological and structural properties and that a limited number of allergens can be defined, which resembles the epitope spectrum of natural allergen extracts. Recombinant allergens allow the estab-

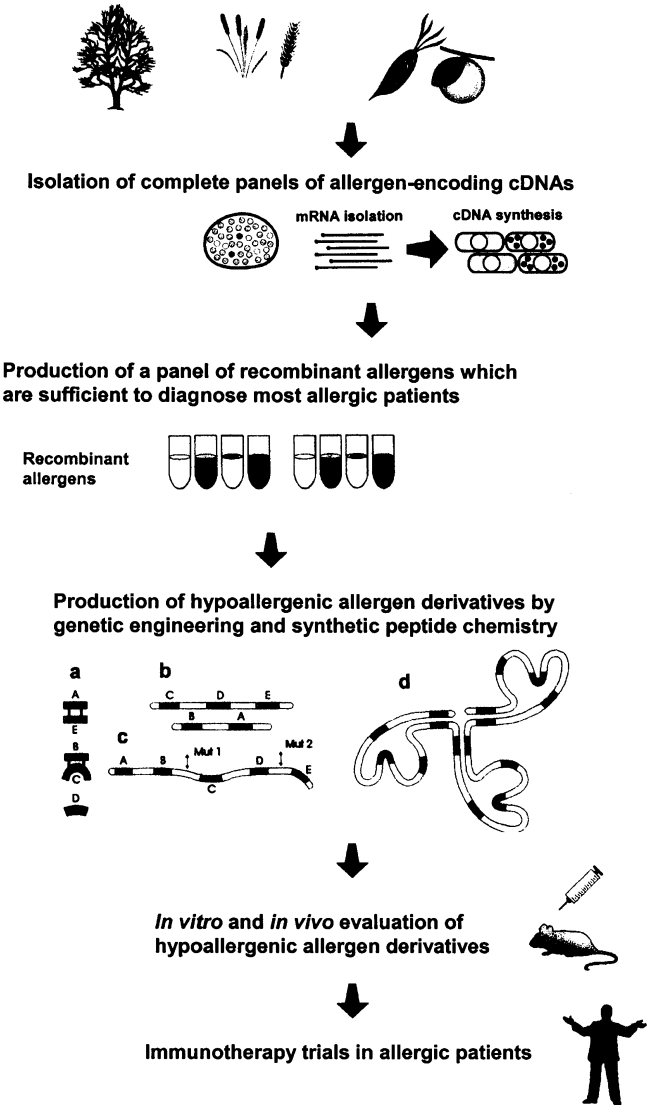

Figure 5. Strategy for the production of hypoallergenic allergy vaccines. First, the allergen sources which represent the originally sensitizing agents have to be identified. From cDNA libraries, the allergen-encoding cDNAs have to be isolated, which allows the production of the panel of recombinant allergens sufficient for diagnosis of allergic patients. The cDNAs further allow the production of hypoallergenic allergen derivatives. *In vitro* and *in vivo* evaluation strategies are then needed to identify those derivatives, which are most suitable for immunotherapy. These vaccines can then be tested in immunotherapy trials in allergic patients.

lishment of new diagnostic concepts and they can be used for component-resolved diagnosis, which represents the basis for patient-tailored immunotherapy. Moreover, they are useful tools to study the mechanism of allergy and of immunotherapy and to elucidate the properties of allergens.

Immunotherapy with recombinant allergens would allow for the first time a patient-tailored treatment. For safer immunotherapy, several recombinant allergens were modified into hypoallergenic derivatives with reduced allergenic activity. Figure 5 shows the strategy for the production and evaluation of hypoallergenic derivatives. Different methods can be applied to produce hypoallergenic derivatives. By synthetic peptide chemistry, T-cell epitope containing peptides or B-cell epitope containing peptides can be constructed (76, 77) (Fig. 5a); recombinant allergen fragments can be produced to destroy the three-dimensional structure of allergens and thus conformational IgE epitopes (62–64) (Fig. 5b); point mutations can be inserted into allergens by site-directed mutagenesis (68–71), either outside the IgE epitopes to destroy the three-dimensional structure and thus conformational IgE epitopes (Fig. 5c); or within the IgE epitopes to alter the IgE epitopes directly. Oligomers of allergens can be constructed to reduce the allergenic activity presumably through microaggregation (65) (Fig. 5d). The hypoallergenic allergen derivatives need to be evaluated thoroughly *in vitro* and *in vivo,* regarding their allergenic activity and their immunogenicity in animal studies, to select the best vaccines for immunotherapy. Immunotherapy trials with hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, confirmed that this treatment might improve immunotherapy in the near future. Finally, hypoallergenic derivatives are useful tools to understand the mechanisms of allergy, and immunotherapy with hypoallergenic derivatives will allow understanding the mechanisms underlying immunotherapy.

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