

The current state of recombinant allergens for immunotherapy

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Purpose of review

Subcutaneous immunotherapy is a well documented treatment of allergic rhinitis and asthma. The majority of the disadvantages of the treatment are related to the poor quality of the natural allergen extracts which can contain varying amounts of individual allergens including allergens to which the patient may not be sensitized. Recombinant allergens offer a possibility to use well defined molecules with consistent pharmaceutical quality defined in mass units. The proof of concept of the clinical efficacy of recombinant allergens is based on two studies published as full articles.

Recent findings

One study applied a mixture of five *Phleum pratense* major allergens in a maximum dose of 40mcg protein. The clinical efficacy showed a significant efficacy with 40% reduction in disease severity. The second study compared a commercial birch extract with both recombinant Bet v 1 and purified Bet v 1 in dosages of 15mcg allergen. The clinical effect was 60% additional efficacy. Systemic side effects occurred more frequently with grass allergens. A third study used hypoallergenic fragments and a trimer of Bet v 1. The study did not show efficacy and a rather high frequency of systemic side effects.

Summary

The advantages of using recombinant allergens for immunotherapy are obvious but more studies on a large scale are needed before the overall value in terms of efficacy and safety can be assessed. Clinical trials are also necessary for new combined vaccines based on recombinant allergens that in experimental studies have shown greatly enhanced immunogenicity and low allergen-specific reactivity.

Keywords

allergen immunotherapy, hypoallergenic allergen derivatives, recombinant allergen vaccines, recombinant allergens, subcutaneous immunotherapy

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Introduction

Allergen-specific immunotherapy is based on the administration of allergenic extracts containing natural allergens. These extracts are complex mixtures of allergens, and not all of them have allergenic characteristics. The production of recombinant allergens allows the use of well defined allergen extracts with consistent pharmaceutical quality. Nonallergenic proteins will be excluded, whereas inclusion of defined mass units of major allergens will be possible [1]. During the last decade the potential advantages of using genetic engineering in improving both diagnosis and specific treatment has been advocated [2,3,4^{*}]. Animal experiments indicate that it is possible to convert important allergens into hypoallergenic derivatives which have the potential to be well tolerated immunotherapeutic agents as they exhibit decreased IgE-binding capacity, contain T-cell epitopes and induce IgG antibodies [5,6]. Whereas diagnostic tests based on the major allergens of the most common allergen sources are now available, immunotherapy is still based on aller-

genic extracts. Because of the progress made in the field of molecular allergen characterization new allergy vaccines based on recombinant allergens, recombinant hypoallergenic allergen derivatives and allergen-derived T-cell peptides have entered clinical trials. These trials are the only way to demonstrate that recombinant allergens and hypoallergenic derivatives can be considered for immunotherapy in allergic patients in terms of both efficacy and safety. Double-blind, placebo-controlled, randomized studies (optimally in comparative studies including natural extracts) and the use of appropriate statistical methods are essential. This review validates clinical studies with recombinant wild-type allergens as well as with recombinant hypoallergenic derivatives.

Strategies for improving efficacy of allergen-specific immunotherapy

Research for candidate molecules for immunotherapy has been intense during the last decade. Since the mid nineties a lot of scientific work has focused on engineering

allergenic molecules with reduced IgE reactivity in order to improve the safety of subcutaneous immunotherapy. As the mechanisms underlying successful immunotherapy is only partly understood, several approaches have been investigated.

With the availability of the first allergen sequences, a new strategy for immunotherapy was developed based on experimental results showing that tolerance against a complete allergen can be induced by using allergen-derived peptides which are recognized by allergen-specific T cells: this was demonstrated with T-cell epitopes derived from Fel d 1 [7], from Der p 1 [8], from Cry j 1 and Cry j 2 [9]. Clinical trials evaluated this concept in the beginning of the 1990s for the treatment of cat allergy [10,11]. Adverse events, primarily late onset of rhinitis, asthma and pruritis occurred regularly related to the dose of peptide used [12,13]. These side effects were not IgE-mediated. Further investigations are going on to evaluate the effect of T-cell peptides on IgE-mediated immediate allergic symptoms [14]. Immunotherapy with short and long synthetic peptides of the major bee venom allergen were also investigated; Mueller *et al.* [15] used a mixture of three short peptides of Api m 1 in five patients and have demonstrated that three patients became tolerant to realistic provocation tests.

Other approaches conjugating allergens to immunostimulatory sequences, either DNA oligonucleotides containing CpG motifs [16] or genes encoding bacterial surface proteins [17], have also been proposed. A double-blind immunotherapy study performed with a conjugate of the major ragweed pollen allergen, Amb a 1, and an immunostimulatory oligonucleotide resulted in a decrease of symptoms after nasal provocation tests in the actively treated group; however, after the second year of treatment the number of patients was very limited in each group [18]; a phase III study was started but was stopped prematurely.

Recombinant technology has allowed the obtaining of hypoallergenic variants with an altered tertiary structure obtained by making mutations and deletions in allergens around B-cell epitopes (IgE-binding) areas as shown for Der f 2 [19] for Bet v 1 [20,21], Lol p 5 [22], Hev b 6 [23], Par j 1 [24], Ole e 1 [25]. Reduced IgE capacity can also be obtained by fragmentation, by oligomerization, or by fusion of allergen variants [26]. All these molecules possess most T-cell epitopes of the wild-type allergen, thus being able to target the broad T-cell repertoire of allergic patients while exhibiting reduced IgE reactivity. So they should not induce IgE-mediated side effects when administered to allergic patients. Moreover they should permit increase of the doses injected compared with wild-type molecules, and by the way eventually induce a pronounced Th1 response.

Most of these molecules were studied extensively as to their IgE-binding capacity *in vitro* and *in vivo*, their potential to stimulate T-cell lines either in animal models or in patients sensitized to the allergen extract, and their ability to induce production of IgG. For several hypoallergenic molecules skin prick tests evaluated the reduced in-vivo allergic activity in comparison with the natural extract. 100 to 1000-fold less reactivity was observed for some hypoallergenic molecules (Bet v 1 trimer and Bet v 1 fragment, hybrid protein from *Parietaria judaica* pollen) when tested in several dozen patients [27–29].

Clinical trials already published are up to now limited to recombinant wild-type allergens, defined as a recombinant molecule resembling the structural and immunological properties of the corresponding natural allergen and to hypoallergenic derivatives. Two approaches are obvious; in monosensitized patients, in birch pollinosis f.i., with a dominant major allergen (Bet v 1), the use of the disease eliciting allergen alone is applicable. In grass pollinosis with several important allergens, the use of a cocktail of recombinant allergens is another approach. The latter should demonstrate that a limited spectrum of recombinant allergens is useful for immunotherapy in patients with multiple sensitivities [3].

Clinical trials

Only two studies (published in full length in peer-reviewed journals) have been performed with wild-type recombinant allergens, one with a cocktail of five recombinant allergens from grasses [30^{••}], and one with rBet v 1 [31^{••}], and another single study performed with hypoallergenic derivatives of Bet v 1, including two groups treated with either a trimer or a mix of two fragments of Bet v 1 [32^{••}]. A study [33] using a folding variant of Bet v 1 was published in part.

Clinical efficacy can be estimated by the clinical symptoms as the primary outcome. As the total burden for the patients is a combination of symptoms and the need for rescue drugs, medication need also be taken into account in the primary outcome to give a complete picture of the disease-reducing capacity of immunotherapy. Either symptom score and medication score could be handled individually and then combined to a common score or they could be scored globally as a total symptom score. According to the World Allergy Organization recommendations [34] the magnitude of efficacy should be established as the per cent reduction of the global clinical scores in the active vs. placebo group. Additional efficacy inferior to the one established by antihistamines is not considered acceptable, and consequently the minimal clinically relevant efficacy should be at least 20% higher than placebo, and be statistically significant.

Skin tests, conjunctival and nasal provocation tests are used in some studies as secondary end-points. The activity of an allergenic extract used for immunotherapy may be evaluated by clinical and biological markers: modification of cutaneous tests, modification of specific IgG (especially IgG1 and IgG4), evolution of specific IgE. Cytokine production was evaluated in some studies.

Recombinant grass allergen immunotherapy

The first study using recombinant allergens for subcutaneous immunotherapy was performed by Jutel *et al.* [30^{••}]. The study included grass pollen allergic rhinitic adults (not excluding mild asthma). A total of 64 participants were enrolled and 62 randomized to treatment, the 'intention-to-treat' (ITT) population. The 'per-protocol' population consisted of 29 actively treated and 28 placebo-treated patients. Treatment was continued for approximately 20 months and efficacy evaluated during the second grass pollen season (defined as the 42-day period encompassing the main pollen exposure). The immunotherapy preparation used was a mixture of five *Phleum pratense*-derived allergens given in a maximum dose of 40 mcg total protein (10mcg Phl p 1, 5mcg Phl p 2, 10mcg Phl p 5a, 10 mcg Phl p 5b, 5mcg Phl p 6).

The clinical efficacy is evaluated as both individual scores and a combined score. On the basis of the ITT population the symptom score did not reach statistical significance. However, based on the per-protocol population, the median difference between actively treated and placebo was 37% for symptom score and 37% for medication score. The combined score showed a 39% ($P = 0.44$) reduction in disease severity for actively treated patients compared with placebo patients. As a secondary outcome a rhinitis quality-of-life questionnaire [35] was used, and differences exceeding 0.5 score were observed for the domains 'total', 'activities', 'nonhay fever symptoms', 'practical problems', 'nasal symptoms', 'eye symptoms' and 'emotional fields' in the ITT population.

Active treatment induced a statistical significant increase in IgG1 and IgG4 *P. pratense*-specific antibodies. Specific *P. pratense* IgE levels decrease significantly in actively treated patients with no change in the placebo group. No patients developed IgE antibodies to Phl p 5a/b though four actively treated patients showed no IgE response to this allergen before immunotherapy.

Systemic side effects were observed in 7 out of 29 actively treated (24%) patients. These include two cases of generalized urticaria (one with additional dyspnea), two cases of local urticaria, one case of rhinoconjunctivitis and one with asthma.

In the design of this study it was planned to include 80 patients in order to ensure a sufficient power of the study.

Only 62 patients were included in the ITT population, and although the data from the per-protocol population (57 patients) just managed to show statistically clinical efficacy ($P = 0.44$), the magnitude of the additional efficacy was as high as 39%. The value of the treatment is reinforced by the excellent improvement in quality of life. The study also shows that one important disadvantage of recombinant wild-type allergens is that these molecules are as allergenic as the corresponding natural allergens and may induce IgE-mediated side effects during immunotherapy, the percentage of systemic reactions (24%) being even higher than that published for natural allergens [36]. The high frequency of systemic side effects may be related to the rather high dose of allergens injected (40mcg rPhl p) [37].

Recombinant birch allergen immunotherapy

The second study using wild-type recombinant allergen compared treatments performed with molecular compounds (natural and recombinant Bet v 1) with treatments performed with a commercial birch pollen extract and placebo [31^{••}]. It showed for the first time that the efficacy of immunotherapy with a molecular allergen was at least equal to treatment with allergenic extracts containing natural allergens. A total of 165 participants were screened and 147 randomized to treatment after a baseline season (randomization was based on symptom severity and degree of birch sensitization). The ITT population (134 adults suffering from birch pollen-induced rhinitis and possible mild asthma) was randomized into four groups receiving either placebo, or a commercial birch pollen extract, or rBet v 1, or purified natural Bet v 1. The per-protocol population consisted of 33 first year patients and 29 second year placebo-treated patients, 28 first year and 25 second year patients receiving birch pollen extract, 27 first year and 25 second year patients treated with rBet v 1 and 24 first year and 24 second year patients treated with nBet v 1. Treatment was continued for 2 years and efficacy evaluated during both birch pollen seasons (defined as the period between the first and the last day with at least 40 grains/m³/24 h). After a build-up phase, the immunotherapy schedule included monthly injections of 15mcg Bet v 1 allergen or placebo.

The clinical efficacy evaluated by individual daily symptom score and rescue medication score showed that the mean difference between actively treated and placebo patients was 48% for birch pollen extract, 49% for rBet v 1 and 58% for nBet v 1 with respect to symptom score and 67% for birch pollen extract, 64% for rBet v 1 and 64% for nBet v 1 for medication score during the first season after 6 months of treatment. Combining symptom and medication scores (equivalent weight) indicates a 59% reduction in disease severity in patients treated with birch pollen extract, 57% for rBet v 1 and 57% for nBet v 1 compared with placebo. Statistical analysis indicates no

difference in symptom and rescue medication reduction between the three actively treated groups. Identical results were obtained during the second birch pollen season.

Active treatment induced a statistically significant increase in IgG1, IgG2 and IgG4 Bet v 1-specific antibodies of identical value in the three actively treated groups. Three patients in the birch pollen extract group developed de-novo IgE antibodies to Bet v 2. No new sensitizations to Bet v 2 were observed in the r and n Bet v 1-treated groups. Skin reactivity (as estimated by SPT weal diameter) decreased significantly ($P < 0.001$) after 1 and 2 years of treatment in actively treated patients. The decrease of prick tests to rBet v 1 and nBet v 1 was significantly higher in the rBet v 1-treated patients compared with patients who had received birch extracts [31^{••}]. A negative correlation was found in the rBet v 1-treated patients between the decreases of skin tests and the fold increases of the allergen-specific IgG levels measured by ImmunoCAP (G. Pauli, *et al.*, manuscript in preparation). Concerning specific IgE, measured at six time points, the postseasonal pollen peak was blunted in the three actively treated groups (G. Pauli, *et al.*, manuscript in preparation).

Systemic side effects were observed almost equally in all actively treated (most mild reactions which were almost as common in the placebo group). One patient in the nBet v 1 group developed anaphylaxis with urticaria and hypotension. Local reactions at the injection site occurred more frequently in the rBet v 1 group.

The study not only confirms the proof of concept of the clinical efficacy of recombinant allergens, but also shows (for the birch system) that recombinant allergens are as clinically effective as purified allergen and the crude allergen extract. The magnitude in the order of a 60% additional efficacy is great; it is observed after 1 year of treatment and is consistent during the second season. The clinical results were obtained with a rather modest dose of Bet v 1 (15mcg), which may explain the low frequency of severe systemic reactions.

Recombinant birch allergen derivatives immunotherapy: fragments and trimer

The second study using recombinant birch pollen allergen compared treatments performed with molecular modified derivatives of recombinant Bet v 1 and placebo [32^{••}]. A total of 124 participants were randomized to treatment; the ITT population suffered from birch pollen-induced rhinitis and possible mild asthma. Patients were randomized into three groups receiving placebo, rBet v 1a trimer (created by expressing three copies of the gene in sequence) or Bet v 1 fragments (two recombinant peptides that together represent the whole sequence of Bet v 1).

The ITT population consisted of 49 placebo-treated patients, 37 treated with rBet v 1 trimer and 38 treated with Bet v 1 fragments. The per-protocol population was reduced to 68% of the ITT population and consisted of 37 placebo-treated patients, 28 treated with rBet v 1 trimer and 19 with Bet v 1 fragments (only half of the randomized patients). Treatment consisted of 4–5 months' preseasonal injections and efficacy evaluated during an 8-week period encompassing the whole birch pollen season. After a build-up phase the immunotherapy schedule included monthly injections of 80mcg protein.

The clinical efficacy was evaluated by a combined symptom-medication score. On the basis of the per-protocol population, there was no difference between the trimer group and the placebo group and the mean difference between the fragments and the placebo groups was 28% (NS), during the birch pollen season.

Active treatment induced a statistically significant increase in IgG1 and IgG4 Bet v 1-specific antibodies in the two actively treated groups. No significant difference in skin sensitivity and nasal challenge test was observed between the three groups 12 months after initiating immunotherapy.

Systemic side effects related to injections were observed in both actively treated groups. Generalized urticaria was observed in 21% of the fragments-treated patients and 16% of the trimer group. One patient in the fragments group developed anaphylaxis with hypotension requiring epinephrine, one generalized urticaria with severe gastro-intestinal disorders. Secondary systemic reactions occurred equally during the build-up phase or the maintenance phase; only five adverse general reactions out of 28 appeared within 30 min.

Hypoallergenic derivatives of Bet v 1 [32^{••}] that exhibited reduced allergen activity both *in vitro* and *in vivo* appeared good candidates to investigate the potential of a safer (and effective) immunotherapy; however, at that time the potential efficacy of rBet v 1 had not been documented. A major problem in the study is the high number of drop-outs (in one of the centers half of the patients were lost during the study). Power calculation is not presented and the low number of participants in the actively treated groups is a major obstruction for demonstrating clinical efficacy. The symptom score is reduced in both the fragments and the trimer groups (58% for fragments and 44% for trimer) but it seems that this is caused predominantly by increasing the intake of rescue medication (almost 50% more than placebo in both the fragments group and the trimer group). The lack of efficacy of these molecules based on the primary outcome – the combined symptom-medication score could not be explained by a very low pollen count in one of the

centers, because when looking at data from the other two centers, the magnitude of efficacy was even poorer. Looking at the immunogenicity based on the increase in IgG1 and IgG4, both the fragments and the trimer were able to stimulate the immune system. The clinical results from this study clearly indicate that induction of IgG antibodies alone is not responsible for the clinical efficacy. This may be related to not differentiating between immunoreactive IgG4 (as estimated by ELISA) and functional inhibitory IgG4 antibodies (inhibition *in vitro* of IgE-facilitated allergen presentation) [38].

In spite of the anticipation that hyporeactive derivatives of Bet v 1 should be less allergenic (as documented by skin and provocation testing) [27,28,39] the frequency of systemic side effects is considerably higher than in the wild-type recombinant studies. The comparisons of the rate of systemic reactions by sensitivity to Bet v 1 (evaluated by skin prick tests with different dilutions of Bet v 1 and measurement of specific IgE to Bet v 1 at inclusion in the trial) indicated that patients who experienced systemic side effects were not significantly more sensitive to Bet v 1 than those without systemic side effects. Kruskal–Wallis' test (results not published) showed no statistically significant difference in IgE variations, nor in changes in IgG1 and IgG4. No statistically significant difference in occurrence of allergen-specific IgE to either trimer or fragments was observed between the group with and without side effects. This may indicate (together with the observation that most of the side effects appeared later than 1 h after injection) that these side effects were not IgE-mediated.

Recombinant birch allergen derivatives immunotherapy: folding variant rBet v 1-FV

Two studies have been performed with the folding variant of recombinant Bet v 1; the first one was an open comparative trial with a native birch pollen preparation. Fifty-one patients (24 rBet v 1-FV and 27 comparator) started immunotherapy, receiving one course of pre-seasonal injections during the 5 months prior to the pollen season. The median of the symptom-medication score (primary end-point) was 5.90 for the Bet v 1-FV group and 12.48 for the comparator group during the 3 weeks encompassing the pollen season; the number and nature of the adverse events were comparable in both groups [33]. A phase III clinical trial including 228 patients with rhinoconjunctivitis with or without asthma (116 treated actively and 112 placebo) showed, after an 18-month perennial treatment, a significant reduction in the median symptom medication score per day in the active group compared with placebo. Significant increases in both IgG1 and IgG4 birch-specific antibodies were observed in the Bet v 1-FV-treated group, whereas no increases were observed in the placebo group [40]. However, the

full results of this pivotal study have not been published yet.

Future perspectives

The proof of the concept is given by the clinical efficacy of immunotherapy trials performed with recombinant allergens. As a matter of fact this was to be anticipated for the birch system as Bet v 1 is a very dominating allergen responsible for the majority of the birch pollen allergen activity [41]. The study of the mixture of five different grass pollen allergens indicate that this may be essential for obtaining clinical efficacy in grass pollen allergic patients. To further develop recombinant allergens for immunotherapy, we need to have more studies with complex allergen systems such as house dust mites which represent one of the most important allergen sources worldwide. The relative importance of the 23 mite allergens described so far has to be established in order to select the panel of allergens necessary for immunotherapy of mite allergic patients in different populations [3]. To optimize efficacy and safety of recombinant allergen-based vaccines, dose-finding studies will be needed to determine the most convenient and well tolerated doses.

A new approach proposed by different researchers is the utilization of the hapten-carrier concept for the design of new vaccines with reduced or no IgE reactivity. Carrier molecules such as virus-like particles derived from the coat protein of the bacteriophagous Q beta have shown to be highly immunogenic in humans [42]; in a recent study vaccination with this carrier combined to Feld 1 in a mouse model resulted in strongly reduced immediate type I allergic responses and shutdown of memory IgE responses after subsequent allergen challenges [43^{••}]. Another way proposed by the Vienna group is to select peptides derived from or close to the IgE-binding sites of the allergen [44,45[•]] with low or no allergenic activity and with low or no activation of T-cell responses; fusion to a carrier molecule (a rhinovirus capsid protein) increases the immunogenicity of the vaccine [46^{••}]. These new proposed technologies could be applied to every allergen in the future. Protocols with a reduced number of injections per year would be sufficient to induce long-lasting protection against allergic reactions. It, however, remains necessary to prove the clinical efficacy and the lack of any side effects of these new molecules by solid immunotherapy trials.

Conclusion

The clinical trials performed with wild-type recombinant allergens have demonstrated both the clinical efficacy and the immunogenic power of genetically engineered allergens. The study with hypoallergenic recombinant allergenic molecules (oligomer and fragments) shows

interesting perspectives, but the final form has not been reached as no clinical efficacy has been achieved. The occurrence of side effects points out the difficulty in determining the maximum tolerated doses of molecules thought to be hypoallergenic. This, contrary to many hopeful ideas, cannot be predicted by previous in-vitro studies or by cutaneous tests performed in sensitized patients. Even if the contents of the extracts using modified hypoallergenic proteins are accurately known, it remains necessary to perform clinical studies assessing clinical responses and adverse reaction rates related to different dosage regimens. The lack of an efficacy marker makes the procedures time-consuming as clinical trials for instance in pollinosis should include 2 years of treatment. This explains in part why expected new vaccines using recombinant allergens are not to become available for routine practice in the very near future.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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