Recombinant allergens for specific immunotherapy

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Recombinant DNA technology provides the means for producing allergens that are equivalent to their natural counterparts and also genetically engineered variants with reduced IgE-binding activity. The proteins are produced as chemically defined molecules with consistent structural and immunologic properties. Several hundred allergens have been cloned and expressed as recombinant proteins, and these provide the means for making a very detailed diagnosis of a patient's sensitization profile. Clinical development programs are now in progress to assess the suitability of recombinant allergens for both subcutaneous and sublingual immunotherapy. Recombinant hypoallergenic variants, which are developed with the aim of increasing the doses that can be administered while at the same time reducing the risks for therapy-associated side effects, are also in clinical trials for subcutaneous immunotherapy. Grass and birch pollen preparations have been shown to be clinically effective, and studies with various other allergens are in progress. Personalized or patient-tailored immunotherapy is still a very distant prospect, but the first recombinant products based on single allergens or defined mixtures could reach the market within the next 5 years.
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Abbreviations used
DBPC: Double-blind, placebo-controlled
SMS: Symptom-medication score

The first allergens to be cloned with recombinant DNA technology were Dol m 5 from the white-faced hornet *(Dolichovespula maculata)*,[1] Bet v 1 from birch pollen *(Betula verrucosa)*,[2,3] and Der p 1 from the house dust mite *Dermatophagoides pteronyssinus*.[4] Now some 20 years later, several hundred have been cloned and expressed in various systems, including bacteria, yeasts, insect viruses, and plants.

One of the main advantages of recombinant proteins is that they can be fully characterized in terms of their physical, chemical, and immunologic properties and presented as chemically defined entities (Table I), with all batches stemming from 1 master cell bank (Fig 1). Preparations for specific immunotherapy and diagnosis can then be formulated with consistent high pharmaceutical quality to meet more stringent specifications than can normally be achieved with products based on extracts of natural source materials. In the latter case the relative concentrations of various allergens are dictated by the source material, except in those instances in which some postextraction purification is undertaken. In practice, it is usually only realistic to define the activity of an allergen extract in terms of its total IgE-binding activity and the concentration of 1 major allergen. Recombinant products, on the other hand, can be defined with respect to the concentration and activity of each component and the optimal dose for the required application. In addition, recombinant preparations contain only allergens and none of the nonallergenic proteins and polysaccharides present in extracts of natural source materials. Some of the difficulties posed by working with natural source materials, such as the need to demonstrate the lack of contamination of pollen preparations with foreign pollens or pesticides, can be avoided.[5] Recombinant forms of animal allergens might very well find greater acceptance than extracts of natural tissue, thus increasing the practice of immunotherapy for cat allergy, for example. Preparations derived from raw materials that are difficult to collect (eg, yellow jacket and hornet venoms) could be replaced by recombinant products.

The availability of high-quality recombinant allergens is providing new opportunities to obtain a detailed understanding of the nature of sensitization and the cross-reactivity between different allergens, thereby allowing more informed choices to be made regarding strategies for allergen-specific immunotherapy. The use of recombinant DNA technology does not stop with allergens *per se*; it also provides the means to genetically engineer allergen variants embodying features such as reduced IgE reactivity or enhanced immunogenicity. This is attractive from the point of view of enhancing the safety of immunotherapy and facilitating administration of higher doses.

In some instances allergenic source materials contain just 1 major or dominant allergen, such as Fel d 1 from cat *(Felis domesticus)* and Bet v 1 from birch pollen. However, in most cases several allergens are involved. For example, 11 different allergens have been characterized and cloned from sweet grasses, including timothy grass *(Phleum pratense)* and rye grass *(Lolium perenne)*,[6] and for the house dust mites *D pteronyssinus* and *Dermatophagoides farinae*, the number is in excess of 20.[7] Efforts to develop therapeutic preparations are being focused on those major allergens that account for the larger part of the IgE reactivity to the particular source material. If this approach does not achieve consistent clinical benefit, then recombinant products would be placed at a disadvantage, unless of course customized or personalized products can be developed. The first products to reach the market will be for allergies to grass pollen, tree pollen (birch), and house dust mite, with short ragweed *(Ambrosia artemisiifolia)*, wall pellitory (*Parietaria judaica* or *Parietaria officinalis*), Japanese cedar *(Cryptomeria japonica)*, and cat *(F domesticus)* likely

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TABLE I. Advantages and disadvantages of proteins produced with recombinant DNA technology

Advantages
Molecules with defined amino acid sequence
Preparations of consistent pharmaceutical quality
All batches of one allergen derive from the same master cell bank
Avoidance of possible contamination and the risk of infectious agents
Dosage in mass units in respect of all components: absolute standardization
Inclusion of only the relevant proteins
Optimization of the dosage of all components of a preparation
Possibility to tailor preparations to a patient's sensitization profile
Precise monitoring and investigation of mechanisms underlying treatment
Option to create genetically engineered variants (eg, with reduced IgE reactivity)
Disadvantages
Each allergen has to be developed by using a specific approach.
For those allergens occurring in many isoforms, there is a need to choose the most relevant.
It might be necessary to include >1 isoallergen in cases of limited identity.
There are high development costs in relation to limited market potential.

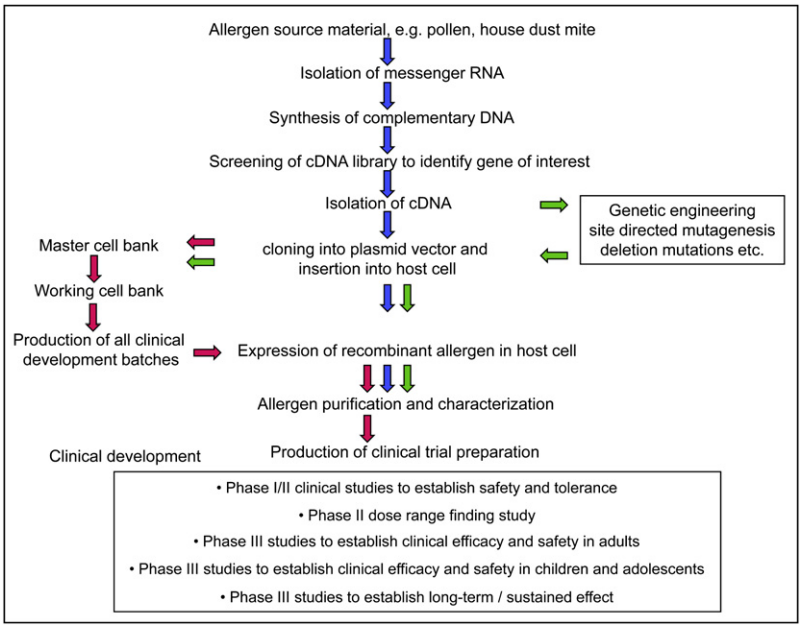

FIG 1. Development of recombinant allergen products for specific immunotherapy. *Blue arrows*, Cloning, expression, and characterization of recombinant allergen. *Green arrows*, Development and characterization of hypoallergenic variants. *Red arrows*, Production of clinical trial preparations and future products all stemming from a single master cell bank.

to follow. The complete development program for such preparations is depicted in Fig 1.

WILD-TYPE RECOMBINANT ALLERGENS

The term wild-type recombinant allergen is often used to describe the equivalent of the natural unmodified allergen in respect to both structural and immunologic characteristics. In practice, it might not always be possible to achieve the desired result with a prokaryotic bacterial expression system, such as *Escherichia coli*, and indeed, different production conditions can have a strong influence on the characteristics of the recombinant allergen, as shown recently for an isoform of the birch pollen Bet v 1.[8] It might be necessary to accept some structural differences or alternatively to undertake modifications to achieve satisfactory expression of the recombinant protein or to switch to eukaryotic systems, such as the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, or baculovirus. Furthermore, bacterial expression systems lack the ability to perform protein glycosylation, and therefore allergens that normally occur as glycoproteins are expressed devoid of carbohydrate moieties. In the case of the glycoprotein Phl p 1, the group 1 allergen of the grass *P pratense*, expression without the carbohydrate component appears not to have any appreciable effect on IgE antibody binding or T-cell reactivity[9,10] or on the ability to induce allergen-specific IgG1 and IgG4 responses.[11] On the other hand, whereas approximately 50% of sera from subjects with grass pollen allergy showed IgE reactivity toward natural glycosylated Phl p 13, only 21% reacted with the

nonglycosylated recombinant molecule.[12] It also proved to be very difficult to produce recombinant Phl p 1 in a soluble monomeric form, but a single amino acid substitution of cysteine for alanine in the C-terminal region was sufficient to overcome these problems without effecting immunologic activity.[9] A further point concerns differences in codon use between *E coli* and the protein source, which can have a large influence on recombinant protein yields. When the genes are engineered by introducing point mutations to substitute those codons most frequently used in *E coli*, then yields can often be increased several fold without any affect on the primary amino acid sequence.[13]

RECOMBINANT HYPOALLERGENIC VARIANTS

The magnitude of the dose administered in allergen-specific immunotherapy is apparently important in ensuring the success of treatment. Although low allergen doses favor a T_H2 cytokine response and a switch to IgE, high allergen doses favor induction of regulatory T cells and modification or downregulation of the T_H2 phenotype.[14] However, the higher the dose administered, the greater the risk for inducing undesirable side effects. Subcutaneous immunotherapy with hypoallergenic variants offers a way around this problem and also makes it possible to achieve a maximum dose with relatively few injections. Although such preparations based on chemically modified allergen extracts (allergoids) are widely used in Europe, they have previously failed to gain acceptance in the United States. Recombinant DNA technology provides the opportunity to develop this concept one step further and to produce precisely defined variants that retain their T-cell reactivity but show reduced IgE reactivity.

IgE-binding reactivity is very dependent on the 3-dimensional structure of an allergen, and several strategies have been devised to create candidate molecules with modified conformation and reduced IgE reactivity (Table II).[15,16] The design of hypoallergenic variants has been very much an empiric exercise, and theoretic considerations alone often fail to lead to the anticipated reduction in reactivity. In this regard it will be interesting to see whether the concept of *in silico* mutation and screening recently applied to the design of the birch pollen Bet v 1 and the grass pollen Phl p 5b allergens will find general application.[17] However, the design process does not stop with consideration of the immunologic properties alone because factors such as yield, stability, and aggregation state also have to be taken into account. Reduced IgE reactivity can be demonstrated by using *in vitro* techniques, including immunoinhibition assays, basophil activation, and basophil mediator release,[18-20] and *in vivo* methods, including skin testing and nasal provocation.[21] The antigenicity can be demonstrated by the ability of variants to activate allergen-specific T cells[19,22] and immunogenicity by the induction of allergen-specific IgG antibody responses.[23,24]

The specification for a variant must ensure that it exhibits reduced IgE antibody binding characteristics in the very large majority of potential recipients, thus ensuring that one accounts for all epitopes. It is important to investigate individual sera rather than pooled sera so as to avoid the risk of missing specific reactivities that might occur infrequently. Furthermore, a panel of tests should be used, including basophil activation and both solid- and liquid-phase immunoassays, to ensure that test conditions do not influence the outcome. A very important consideration is the magnitude of the reduction in IgE reactivity. The main objectives are to improve safety and ensure the safe administration of high doses of the protein, and with this in mind, we have previously suggested that a minimum 10-fold reduction in IgE reactivity seems appropriate, particularly when considering aggressive allergens.[16] The literature now contains numerous publications describing variants with reduced IgE reactivity; however, when the criteria and considerations described here are applied, a very substantial number of these would fail to make the grade. The major criticisms concern the small numbers of subjects in the screening processes and large variations in the degree of hypoallergenicity within the groups tested.[16]

TABLE II. Methods to produce genetically engineered variants with reduced IgE reactivity

Site-directed mutagenesis: substituting one amino acid with another
Deletion mutations: removal of single or multiple amino acids
Fragmentation
Oligomerization
Molecular shuffling and hybridization

The only clinical studies undertaken with such variants thus far have been with preparations of the birch pollen allergen Bet v 1 in the form of 2 recombinant fragments: a trimer and a folding variant. Various promising grass pollen allergen variants have also been developed,[18,20,22] and it is to be hoped that at least some of these will reach the clinic. Recombinant variants of 3 peanut allergens (Ara h 1, 2, and 3) encapsulated in killed *E coli* are being tested in a phase I clinical study (Table III). Severe food allergies to fish and fruits are caused by the single major allergens parvalbumin and lipid transfer protein, respectively. The development of hypoallergenic variants, including phase I and II clinical studies, is being made possible through financial support from a European Union 7th-Framework grant.[25] Hypoallergenic variants of the cat allergen Fel d 1 have also been developed, and one of these that retains T-cell reactivity has been tested in a murine model of cat allergy.[26,27] Fel d 1–sensitized mice were treated by means of subcutaneous injection of either the variant or the recombinant unmodified protein. Both treatment protocols induced allergen-specific IgG antibodies that could block IgE binding to the allergen. The variant showed tendencies to reduce airway hyperreactivity and allergen-specific skin test responses.

Hypoallergenic variants are well suited for subcutaneous application, but it has been suggested that it is preferable to use wild-type recombinant allergens for sublingual application.[28] Oral Langerhans cells exhibit constitutively high expression of IgE receptors, and it is thought that these facilitate capture of wild-type allergens and promote a regulatory T-cell response.[29,30] Nevertheless, allergoids produced by means of protein modification with potassium cyanate have been shown to be clinically efficacious when administered in tablet form.[31,32]

HOW MANY ALLERGENS AND WHICH ONES?

In a few cases there is a real prospect that a single allergen or allergen derivative will suffice to achieve a substantial improvement in clinical symptoms, such as cat (Fel d 1) and birch (Bet v 1), the rationale being that 1 major allergen dominates and accounts for a large proportion of the specific IgE. A double-blind, placebo-controlled (DBPC) study comparing natural and recombinant Bet v 1 with birch pollen has confirmed this view.[33] In cases in which several allergens are identified in a single source material, those associated with the larger part of

TABLE III. Registered clinical studies with recombinant allergen preparations

Allergen source	Interventions	Study design	Reference*
Birch pollen	Bet v 1 trimer Bet v 1 fragments Placebo	SCIT DBPC	Niederberger et al[42] Purohit et al[43]
Grass pollen	Phl p 1, 2, 5a, 5b, and 6 Placebo	SCIT DBPC	Jutel et al[11]
Birch pollen	Bet v 1 folding variant Pollen extract	SCIT Open controlled	NCT00266526
Birch pollen	Bet v 1 nBet v 1 Birch pollen Placebo	SCIT DBPC	NCT00410930 Pauli et al[33]
Birch pollen	Bet v 1 folding variant Placebo	SCIT DBPC	NCT00309062
Birch pollen	Bet v 1 folding variant Placebo	SCIT DBPC	NCT00554983
Birch pollen	Bet v 1 folding variant Placebo	SCIT Immunologic and histologic evaluation	NCT00841516
Grass pollen	Phl p 1, 2, 5a, 5b, and 6 Placebo	SCIT Dose-response study	NCT00666341
Grass pollen	Phl p 1, 2, 5a, 5b, and 6 Placebo	SCIT DBPC	NCT00309036
Grass pollen	Phl p 1, 2, 5a, 5b, and 6 Placebo	SCIT DBPC	NCT00671268
Birch pollen	Bet v 1 Placebo	SLIT tablet Safety and tolerability Dose 12.5 to 100 μg	NCT00889460 Winther et al[47]
Birch pollen	Bet v 1 Placebo	SLIT tablet Safety and tolerability Dose 50 to 300 μg	NCT00396149
Birch pollen	Bet v 1 Placebo	SLIT tablet DBPC	NCT00901914
Cat	Fel d 1–MAT Placebo	Intra-lymph node	Senti et al[50]
Peanut	Modified Ara h 1, 2, and 3 encapsulated in *E coli*	Rectal	NCT00850668

SCIT, Subcutaneous immunotherapy; *SLIT*, sublingual immunotherapy.
*NCT numbers: studies listed at http://www.clinicaltrials.gov.

the total specific IgE reactivity might very well be sufficient. Eleven different grass allergens have been cloned and characterized; however, when the prevalence of sensitization and the relative contribution to the total pollen–specific IgE is taken into account, the group 1 and 5 allergens emerge as the strongest candidates for inclusion in a therapeutic vaccine. In excess of 90% of subjects with grass pollen allergy react to group 1 and up to 85% react to group 5 allergens,[6,34] with a large part of a patient's specific IgE often directed against these 2 allergens. These considerations were taken into account by Jutel et al[11] when deciding to investigate a mixture of 5 *P pratense* allergens for the treatment of subjects with grass pollen allergy. Although 10 short ragweed allergens have been identified, it has been proposed that Amb a 1 might be sufficient for ragweed therapy. However, inadequate clinical efficacy of a giant ragweed extract was related to differences in allergenic activity between the short and giant species, suggesting that one recombinant allergen might not be sufficient.[35]

Allergen-specific immunotherapy is currently practiced with whole allergen extracts, and consequently, a patient who is only sensitized to the group 1 allergen of grass, for example, is treated with a preparation containing several additional allergens in various amounts. By the same token, a preparation of recombinant allergens containing allergens to which the patient is not sensitized should not be a problem. It might only be seen as such in the context of the theoretic possibility of inducing new sensitizations, but there is little evidence for this. One of the very few studies to address this subject concerned birch pollen immunotherapy in polysensitized subjects, many of whom showed positive responses to fruits and vegetables.[36] Evidence of new sensitizations was found for 2 subjects after 4 to 5 months' treatment, and after 12 months or more, 17 of 26 subjects were affected, despite the fact that total specific IgE levels were similar to preimmunotherapy levels. Specific measurements suggested that 7 subjects had new sensitizations to one of Bet v 2 and Bet v 4, but specific IgE levels were generally very low. Other allergens were not identified. Such allergens are present in relatively low concentrations in an extract, concentrations that might be expected to favor sensitization rather than induction of tolerance. On the other hand, a DBPC study conducted with a mixture of 5 recombinant grass pollen allergens, including both Phl p 5a and 5b isoallergens, found no evidence for induction of group 5–specific IgE antibodies in 4 subjects with negative test results at the outset of the study but who showed IgE to Phl p 1 and other grass pollen

allergens.[11] These subjects did, however, have strong group 5 allergen-specific IgG1 and IgG4 responses, indicating either pre-existing immunity without class-switching to IgE or induction of a new nonallergic response. The former is particularly interesting because it raises the question as to why subjects would have a T_H2 response to one grass pollen allergen but tolerance to another.

A further factor that might play a role in successful immunotherapy is the so-called bystander effect, which is based on the hypothesis that the immunomodulatory effect induced by treatment with one allergen has a beneficial effect on the response to others, thus providing a possible argument against the necessity to include every allergen to which a patient is sensitized. Firm scientific evidence is still lacking, although studies in mice suggest that antigen specificity is not a requirement for modulation of allergic responses by naturally occurring regulatory T cells.[37] From a clinical standpoint, a bystander effect could explain the apparent ability of specific immunotherapy to prevent new sensitizations.[38]

PROGRESS WITH CLINICAL DEVELOPMENT: SINGLE-ALLERGEN PREPARATIONS

The clinical trials with recombinant allergen preparations that have either been completed or are ongoing are listed in Table III. The first clinical trial of allergen-specific immunotherapy with a recombinant allergen preparation was conducted with derivatives of the Bet v 1 allergen of birch pollen with reduced IgE reactivity. One preparation was prepared by cleaving the Bet v 1 cDNA and expressing 2 allergen fragments separately. These showed random coil conformation and minimal allergenicity. The second preparation was produced by linking 3 copies of the allergen's cDNA in sequence and expressing the construct in *E coli*, resulting in a trimeric form of the protein. IgE reactivity was reduced, as judged based on histamine release and skin testing.[39] The hypoallergenic character of the preparations was confirmed by means of skin prick and intradermal testing,[21,40] but the trimer was seen to be less hypoallergenic than the fragments, as confirmed by basophil activation measured in terms of CD203c expression.[41] The reasons for the hypoallergenic characteristics were not elucidated, but steric hindrance of the IgE-binding sites seems to be a likely explanation.

Proteins were formulated in aluminum hydroxide suspensions at concentrations of 100 μg/mL, and dosage escalation was conducted with a course of 8 injections from 1 to 80 μg of total protein and further injections up until the beginning of the pollen season. A combined symptom-medication score (SMS) did not show differences between active treatment and placebo, despite the fact that both recombinant preparations induced Bet v 1–specific IgG1, IgG2, IgG4, and IgA antibody responses, with the trimer proving to be the stronger immunogen.[42,43] The antibodies inhibited allergen-induced basophil histamine release *in vitro*, and IgG1 antibody titers correlated with improvement in clinical symptoms, as judged based on a 10-point interval scale, and reduction in skin test reactivity to Bet v 1. Specific IgE responses showed a 3-fold increase in the placebo group as a consequence of seasonal pollen exposure, whereas those in the 2 treatment groups were blunted.[42] IL-5– and IL-13–producing cell numbers were significantly reduced during the course of treatment, which is indicative of a suppression of the T_H2 response.[44] There were also trends for decreased numbers of IL-4–producing cells and increased numbers of IL-12–producing cells, but differences were not significant. These results were seen as favoring further development of hypoallergenic derivatives but emphasized the need for more evidence of clinical efficacy.[43]

Two DBPC studies of injection immunotherapy have compared the therapeutic effects of rBet v 1 preparations and birch pollen extracts, and one of these also investigated a purified nBet v 1 and placebo. Pauli et al[33] showed that aluminum hydroxide–adsorbed preparations of a birch pollen extract, nBet v 1 and wild-type rBet v 1, each with the equivalent of 15 μg of Bet v 1 in the maximum maintenance dose, were essentially equivalent in their abilities to reduce daily symptom and rescue medication scores. Rhinoconjunctivitis symptom scores were reduced by 48.0%, 58.3%, and 49.4% in comparison with placebo in subjects treated with extract, nBet v 1, and rBet v 1, respectively, during the first pollen season after 6 months of treatment. Rescue medication scores were reduced by 69.9%, 63.5%, and 64.2%, respectively. The levels of improvement were maintained in the following pollen season after continuation of the treatment. Skin test reactivity to the pollen extract and Bet v 1 alone was reduced in the 3 active treatment groups in comparison with placebo, and Bet v 1–specific IgG1, IgG2, and IgG4 levels were increased.

The second study comparing rBet v 1 and birch pollen extract preparations looked at a hypoallergenic variant of the major allergen that shows a random coil structure clearly distinguishable from the secondary structure of the native molecule, as can be shown by means of circular dichroism spectroscopy. The hypoallergenic properties could be shown by immunoassay inhibition tests, basophil activation, and skin testing, whereas T-cell reactivity was comparable with that of the wild-type molecule.[19] An open, randomized, comparative controlled study compared aluminum hydroxide–adsorbed preparations administered in a dosage escalation protocol before the birch pollen season with injections at weekly intervals. The maximum dose of the recombinant protein was 80 μg, 5-fold higher than the amount of nBet v 1 in the allergen extract. A combined SMS produced median daily scores of 5.90, 12.48, and 14.67 for patient groups treated with the recombinant protein, pollen extract, and antisymptomatic medication, respectively,[45] showing a clear trend in favor of the recombinant preparation. Safety data indicated that the preparations were comparable with respect to the occurrence of adverse events. Improvements in specific nasal tolerance were seen in both study groups, and the induction of strong IgG1 and IgG4 antibody responses confirmed the immunogenic activity of both preparations.

The rBet v 1 variant has since been investigated in a phase III DBPC, and the data were published in an abstract. A study in 226 patients with allergic rhinitis with or without asthma (Global Initiative for Asthma I and II) used a daily combined SMS during the pollen season as the primary end point. This showed a significant and clinically relevant reduction in comparison to placebo after treatment for 18 months. Increases in treatment-specific birch pollen–specific IgG1 and IgG4 antibody responses were observed, and the preparation was shown to be well tolerated, with no untoward adverse reactions.[46]

Recombinant allergen products for sublingual immunotherapy are also in development, and one of the first of these is based on tablet formulations of rBet v 1. Abstracts have been published presenting the first results of 2 phase I studies to investigate the safety and tolerability of doses from 12.5 to 300 μg and showed that doses of up to 50 μg/d for 2 weeks were well tolerated, but doses of 100 μg or more were not.[47] Treatment-related adverse

events included oropharyngeal pruritus, ear pruritus, rhinitis, and pharyngeal edema. A total of 483 adult patients were randomized for treatment in a phase IIb/III follow-up study with tablets containing 12.5, 25.0, and 50 μg Bet v 1 or placebo. Clinical efficacy was judged in terms of an average adjusted symptom score, which showed a significant reduction of approximately 25% over the whole pollen season. Tolerance was reported as very good, particularly for the 2 lower doses.[48]

It is considered realistic to treat allergy to cats with the single major allergen Fel d 1. A recombinant form of the allergen has been modified by mean of fusion with a TAT-derived protein translocation domain and a truncated invariant chain to target the MHC class II pathway (MAT–Fel d 1) with the aim of enhancing immunogenicity.[49] In a first clinical study the construct was administered by using a course of 3 intra–lymph node injections and produced encouraging results.[50] The development of this preparation has continued with phase II clinical studies.

PROGRESS WITH CLINICAL DEVELOPMENT: MULTIPLE-ALLERGEN PREPARATIONS

Grasses belonging to the subfamily Pooideae, the so-called sweet grasses, show very substantial immunologic cross-reactivity, and therefore it is realistic to consider a single grass, such as timothy grass *(P pratense)* as representative.[6,51] Aluminum hydroxide adsorbates of Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, and Phl p 6 were included in one preparation for testing in a DBPC trial of subcutaneous immunotherapy in 62 patients with grass pollen allergy and rhinoconjunctivitis with or without asthma.[11] Subcutaneous injections of increasing doses up to 40 μg were administered at 7-day intervals before the pollen season, with the maximum dose containing 10 μg each of Phl p 1, Phl p 5a, and Phl p 5b and 5 μg each of Phl p 2 and Phl p 6. Maintenance injections were continued until after the subsequent pollen season.

A combined SMS based on diaries documenting the nature and severity of eye, nose, and chest symptoms and the type and dose of any medication was the primary outcome measure to assess efficacy. A per-protocol analysis showed a 39% improvement in the active treatment group relative to the placebo group ($P = .041$). Symptoms alone improved by 37% ($P = .015$), and use of rescue medication decreased by 36.5%. A validated rhinitis quality-of-life questionnaire[52] as a secondary end point showed benefits in the first pollen season and further improvement during the second pollen season, with an overall significant benefit for active treatment over placebo ($P = .024$). Conjunctival provocation testing showed a trend toward increased tolerance in favor of the active treatment. The recombinant preparation was highly immunogenic, inducing a 60-fold peak increase in grass pollen–specific IgG1 levels during the first 12 months and an approximately 4000-fold increase in IgG4 levels by the end of treatment. Specific IgE levels decreased in the active treatment group over the course of the study. Treatment-related local adverse events involved erythema and swelling in the vicinity of the injection site with or without pruritus, and the 7 systemic reactions recorded did not result in any interruption in therapy, leading to the conclusion that the preparation had a favorable safety when compared with findings from other immunotherapy studies.[11] A subsequent dose finding study showed that even a total major allergen dose of 120 μg did not cause problems with safety or tolerance.[45]

The only other study registered at www.clinicaltrials.gov (Table III) and that involves more than 1 allergen from a particular source is a phase I trial with recombinant variants of 3 peanut allergens encapsulated in heat/phenol-killed *E coli*. It is intended that the immunotherapy should be administered rectally. No further information concerning characteristics of the proteins or the progress with this study is available.

PERSONALIZED IMMUNOTHERAPY

Personalized allergen-specific immunotherapy is not new, particularly in North America, where allergologists mix aqueous extracts of several different allergenic raw materials to match a patient's sensitization profile. The availability of recombinant allergens offers the prospect of taking this one step further. Detailed information on patients' individual allergen sensitization profiles can be obtained by screening with panels of recombinant or purified allergens (component-resolved diagnosis).[53] This raises the hope and indeed the expectation of personalized immunotherapy with combinations of recombinant allergens to match a patient's sensitization profile. In the case of allergen sources, such as grass pollen, ragweed pollen, and house dust mites, several different allergens have been identified, thus creating the potential for large numbers of allergen combinations. The position could eventually become even more complicated if we contemplate combining allergens from different sources.

If such patient-tailored or personalized recombinant products are to become a reality, it will first be necessary to extend the range of allergens that is available and to generate adequate data on quality and clinical efficacy before seeking regulatory approval. The regulatory framework dictated by current European guidelines only allows the registration of finished products and thus limits the possibilities to fixed allergen combinations, denying the possibility to formulate individualized or personalized products.[54] Guidelines from the European Medicines Agency in respect of the "production and quality issues"[5] and "the clinical development of products for specific immunotherapy for the treatment of allergic diseases,"[55] together with the obligatory requirement for pediatric clinical development, now necessitate very much more extensive clinical development programs than have been considered necessary hitherto (Fig 1). Conventional dose-finding studies and the demonstration of clinical efficacy with every allergen would not be feasible because of the difficulties of finding sufficient numbers of patients with appropriate sensitization profiles and the enormity of the task.

Taking these considerations into account, the best option at the present time is to develop allergen mixtures that account for the most commonly encountered sensitization profiles. One such example is the mixture of 5 *P pratense* allergens.[11] It is to be hoped that once such recombinant products have proved their worth, regulatory authorities will be open to a more pragmatic approach, allowing either the approval of single allergens with the possibility to incorporate them in personalized formulations or more flexibility in varying the formulation of allergen mixtures.

CONCLUSION

Various recombinant allergen and hypoallergenic variant preparations are now emerging as strong candidates for products for allergen-specific immunotherapy. The birch pollen allergen Bet v 1 is the focus of the pioneer work to develop the first products that are likely to be granted marketing authorization. These products have the advantage that they contain only 1 recombinant protein

in contrast to the situation with grass pollen and house dust mites, for example, for which several allergens will be necessary. Several clinical studies with recombinant preparations have now shown significant and meaningful clinical benefit for patients with rhinoconjunctivitis attributable to either birch or grass pollen. The first studies with modified cat and peanut allergens are now in progress, and it is to be expected that house dust mite preparations will enter clinical development in the near future.

What do we know?

- **Several hundred allergens have now been cloned and expressed as mature recombinant IgE-reactive proteins.**
- **A few allergens, such as Amb a 1 from short ragweed, have thus far defied attempts to express them successfully as recombinant wild-type proteins.**
- **Genetic engineering techniques have been used to create numerous hypoallergenic variants that might be considered as candidate molecules for immunotherapy.**
- **Immunotherapy with the single dominant recombinant major allergen from birch pollen is at least as effective as a whole birch pollen extract.**
- **First results of clinical studies with recombinant Fel d 1 in subjects with cat allergy suggest that this allergen alone will be sufficient to treat allergies to cats.**
- **A cocktail of recombinant major allergens from grass pollen is clinically efficacious, as judged by improvement in SMS and rhinitis quality-of-life questionnaire scores.**
- **A variant of the recombinant birch pollen major allergen Bet v 1 with substantially reduced IgE reactivity induces significant clinically relevant improvement in a combined SMS.**
- **The hypoallergenic recombinant Bet v 1 variants tested in clinical trials are immunogenic, inducing strong allergen-specific antibody responses.**
- **Diagnostic techniques now allow allergen sensitization profiles to be analyzed at the molecular level, thus raising the prospect for personalized allergen-specific immunotherapy.**

What is still unknown?

- **Will a limited number of major allergens be sufficient for effective treatment in all cases in which several allergens are associated with one source (eg, house dust mites)?**
- **Do we need to include all isoallergens in a recombinant cocktail for immunotherapy, such as Phl p 5a and Phl p 5b (approximately 65% identity) or Amb a 1 and Amb a 2 (approximately 66% identity)?**
- **Will recombinant products give rise to the same long-term benefits achieved with some whole-allergen extracts?**
- **Will personalized immunotherapy with combinations of allergens chosen to match a patient's sensitization profile become a reality?**
- **Will it be possible to develop and validate new primary end points that would allow efficacy to be demonstrated more easily and within a shorter time frame than is possible with the SMS that is the current method of choice?**
- **Are additional amplifiers or immunostimulants likely to be of additional benefit to recombinant protein immunotherapy?**
- **Will recombinant products prove to be generally superior to natural extracts?**
- **Will it be possible to extend the range of recombinant allergens in development for therapeutic application to include commercially less attractive preparations?**

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