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## Strategies for Recombinant Allergen Vaccines and Fruitful Results from First Clinical Studies

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The prevalence of allergic airway disease attributable to inhalant allergens such as pollen has been increasing steadily. A European-based survey of allergic rhinitis published in 2004 reported a prevalence of 23%, with values as high as 29% in some countries [1]. The only potentially curative and specific treatment for IgE-mediated type I allergy is allergen-specific immunotherapy, a causal therapy based on administration of the disease-eliciting allergens or derivatives thereof with the objective of reducing allergic symptoms. Once a prescribed dose or a maximum tolerated dose has been achieved, this dose can be administered repeatedly at intervals until it is deemed to have been therapeutically effective. The treatment has been shown to be particularly beneficial in allergic rhinitis, mild and moderate asthma, and insect venom hypersensitivity.

Whole aqueous extracts of natural allergen source materials such as pollens, mites, molds, and animal epithelia are the basis for therapeutic preparations that are currently used in clinical practice. The extracts are standardized in terms of total allergenic activity, or potency, and possibly the concentration of one individual major allergen, whereas product consistency is assessed in terms of protein and allergen profiles determined by various techniques including electrophoresis and immunoblotting. An extract may contain numerous proteins only some of

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which are allergens. The composition is determined to a large extent by the quality of the raw material and the method of extraction and purification. Raw materials are provided by certified suppliers and produced under controlled conditions. Nevertheless, there are differences. For example, the allergen content of mold cultures is highly dependent on the time and conditions of culture; climatic factors or pollution can influence the allergenicity of pollen; and, while some manufacturers use whole cultures of house dust mites, including the nutrient medium and fecal particles, others use purified mite bodies. In addition, many extracts derived from natural materials contain endotoxin [2].

There is clearly considerable scope for improving allergen vaccines to obtain more precisely defined preparations that are the basis for achieving improved quality and consistency, which, in turn, can lead to enhanced clinical efficacy with respect to diagnosis and treatment. The use of recombinant DNA technology seems to provide a realistic means of achieving such improvements. Furthermore, the technology provides the possibility to create allergen derivatives with reduced IgE reactivity, that is, hypoallergenic molecules that have a reduced risk for inducing undesirable allergic reactions during the course of immunotherapy but retain their therapeutic activity.

## Recombinant allergens

Many hundreds of allergens have been identified, and, often, several allergens originate from one source. For example, more than 20 allergens have been characterized from the house dust mites *Dermatophagoides pteronyssinus* and *D. farinae*, more than 20 have been identified as coming from *Aspergillus fumigatus*, and *Ambrosia artemisiaefolia* (short ragweed) is a source of nine different allergens. Although numerous allergens have been cloned for research purposes, only a few have been developed to the stage at which they can be used in clinical studies.

Once an allergen source has been identified, mRNA is isolated and used as a template to synthesize cDNA. IgE immunoscreening of cDNA libraries can then be used to select cDNA encoding allergens that most closely resemble their natural counterparts. Allergen-specific monoclonal antibodies can also be used. Alternatively, DNA-based screening techniques may be used, such as polymerase chain reaction (PCR) amplification, which relies on the use of oligonucleotide primers designed based on known amino acid sequence data for the allergen. Phage surface display technology has also been used to great advantage to identify a number of allergens. A physical linkage between the gene product and its cDNA facilitates isolation of the specific cDNA from large libraries [3,4].

The next step is to insert the cDNA into a suitable vector (viral, plasmid DNA), which enables it to be expressed and produced in a suitable host organism. Bacterial host organisms, such as *Escherichia coli*, produce the recombinant proteins without posttranslational modification, and, in many cases, it is possible

to obtain the allergens with reactivity comparable with the native form. When correct folding and posttranslational modifications, including glycosylation, become an issue, a eukaryotic expression system such as the yeast *Pichia pastoris*, Baculovirus in host insect cells, and various plants including the tobacco plant *Nicotiana benthamiana* [5] and barley [6] may be used. Nevertheless, these systems do not necessarily introduce glycosylation that is comparable with that of

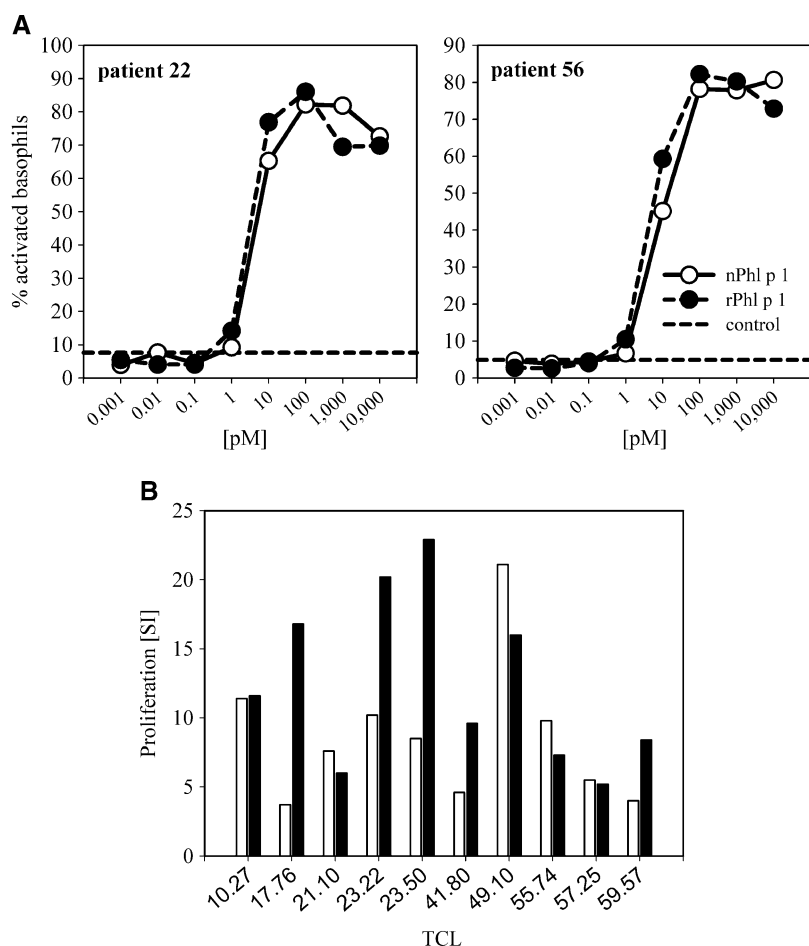


Fig. 1. Comparison of natural glycosylated and recombinant nonglycosylated Phl p 1. (A) Peripheral blood mononuclear cells from grass pollen-allergic subjects were incubated with various concentrations of the allergens or the diluent solution. Basophil activation was measured by fluorescence activated flow cytometry in terms of expression of the surface marker CD203c. (B) Lymphocyte proliferation, expressed as a stimulation index (SI), was determined in terms of  $^3\text{H}$ -thymidine incorporation by T-cell lines (TCL) derived from grass pollen-allergic subjects. White bars denote natural glycosylated Phl p 1; black bars denote nonglycosylated recombinant Phl p 1.

the natural glycoprotein. One example of this is the expression of the house dust mite allergen Der p 1 in *Pichia pastoris*, which results in inappropriate hyperglycosylation [7].

Following a fermentation process and the induction of gene expression, the heterologous recombinant protein is recovered from the cell culture and purified using appropriate chromatographic methods. Optimal production conditions must be determined for each protein to ensure that correct and reproducible secondary and tertiary structures are achieved. Once these are established, the use of contemporary technology enables pure protein to be produced with consistent pharmaceutical quality [8,9].

The so-called “wild-type” recombinant allergens exhibiting IgE reactivity comparable with that of the natural allergen are particularly suitable for diagnostic applications and may also substitute for natural allergens used in specific immunotherapy. The success of the latter is dependent primarily on the T-cell reactivity and immunogenicity of the protein. The allergen Phl p 1 from timothy grass is naturally glycosylated, but the presence of the carbohydrate moiety does not seem to be important for IgE reactivity. When compared for their ability to activate peripheral blood basophils and T-cell lines from grass pollen-allergic subjects, the purified natural molecule and a recombinant nonglycosylated form obtained by expression in *E coli* gave essentially identical results (Fig. 1).

### Characterization and quality of recombinant allergens

The production of recombinant proteins depends initially on the creation of a master cell bank incorporating a DNA construct including the expression vector together with the cDNA encoding the protein. This cell bank is the starting point for all routine production batches. Each production run starts with an aliquot of cells derived from the one cell bank, ensuring that the identical protein is always produced.

Quality control of the recombinant proteins is undertaken using an array of analytical methods. Identity is confirmed by reactivity with specific antibodies on immunoblot, peptide fingerprinting, and N-terminal amino acid analysis. Purity is determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and native PAGE [10], both in conjunction with protein staining; by size exclusion high-performance liquid chromatography (HPLC); and by reverse phase HPLC, taking account of peak symmetry. Allergen concentration is expressed in terms of absolute protein concentration determined from the UV absorption at 280 nm and the specific extinction coefficient of the particular protein, and confirmed as appropriate by specific antibody immunoassays and integrated HPLC peak areas. It is important to be able to determine the potency, and, with natural allergen equivalents, this can best be achieved by measuring IgE-binding activity in an inhibition immunoassay. The use of such a potency

measure in preparations for immunotherapy assumes a relationship between IgE reactivity and antigenicity. This measurement is clearly not appropriate for recombinant allergen derivatives that have been specifically designed to achieve a hypoallergenic character while conserving or even enhancing antigenic activity. In such cases, IgE reactivity provides little more than an indication of batch consistency. It will be necessary to establish new definitions of potency, possibly based on determination of antigenicity, but it is likely that no one method will be generally applicable.

Levels of possible contaminants, including endotoxin and host cell proteins, are also measured. Endotoxin (lipopolysaccharide) is derived from the outer membrane of gram-negative bacteria and can be detected in many allergen extracts [2]. This contaminant is a potential problem for recombinant allergens expressed in gram-negative bacteria such as *E coli*, but the chromatographic purification steps for the protein can be designed to ensure that it is rendered effectively free of contamination.

Only some of the previously described techniques can be applied in the routine quality control of allergen extracts. Furthermore, interpretation is made difficult because of the complexity of the preparations, the one exception being the specific allergen immunoassays. Consequently, the levels of characterization and standardization that can be achieved for recombinant preparations are far superior to that for allergen extracts.

Further methods can be used to confirm aspects of protein structure, identity, and potency, but, in many cases, these methods are suitable for use in development of the proteins and not in routine quality control. Techniques include circular dichroism spectroscopy to determine secondary structure; mass spectroscopy for measurement of molecular mass; basophil activation, measured in terms of expression of the activation marker CD203c [11], and basophil histamine release to assess potency; and T-cell reactivity determined using allergen-specific T-cell lines or clones to confirm immunoreactivity.

In contrast to pure recombinant proteins, allergen extracts may contain many irrelevant proteins and other macromolecules from the source material. Furthermore, relevant allergens may be unstable and prone to degradation in the complex extract, particularly if proteases are present. A possibility exists that extracts may be contaminated with unrelated allergens. One particular example that has been reported is the contamination of a dog dander extract with house dust mite allergens, which gave rise to false-positive skin test results [12]. In animal-derived extracts, such as cat and dog dander or poultry feathers and meat, there is a theoretical risk of viral contamination, although this risk can be excluded by the use of accredited material from certified suppliers.

The subject of effective therapeutic and diagnostic allergen concentrations has attracted a great deal of attention. The acceptable safe dose for an allergen extract may be dictated by the contribution of one or two allergens to the extract's biologic potency, defined in terms of IgE reactivity. Some other allergens may be present in relatively small amounts that render them ineffective for diagnosis or therapy. Working with pure recombinant molecules provides the opportunity to

formulate preparations in which all of the components are consistently present in effective concentrations.

### **Hypoallergenic variants of recombinant allergens**

The idea of hypoallergenic allergen derivatives is not new, and, indeed, several successful products derived by chemical modification of the extracts are on the European market. The most commonly used methods of chemical modification involve treatment with formaldehyde and glutaraldehyde using an approach first adopted in the development of vaccines against tetanus and diphtheria. The resulting intra- and intermolecular cross-linking changes the three-dimensional structures of the proteins and consequently the IgE-binding epitopes, resulting in so-called “allergoids.” The reduced IgE reactivity can be demonstrated using *in vitro* techniques, such as RAST inhibition and basophil activation, and *in vivo* methods including skin testing and nasal provocation [13]. The immunogenicity of the preparations can be demonstrated by the ability to activate allergen-specific T cells and induce allergen-specific IgG-antibody responses [14–16]. The advantage of such preparations is that they have a reduced risk for inducing IgE-mediated side reactions and allow a maximum therapeutic dose to be achieved in a relatively short course of injections when compared with the native allergen [16,17]. The experience with these hypoallergenic preparations suggests that it should be possible to combine the benefits of the hypoallergenic nature with the advantages offered by recombinant proteins.

Recombinant DNA technology provides the opportunity to use genetic engineering techniques to create such hypoallergenic variants. Detailed information concerning IgE-binding epitopes, T-cell epitopes, and three-dimensional structure can help in the design of such molecules. The advantages of this approach are that the new molecules can be precisely defined and the design features validated in respect to the specific immunotherapeutic application [18,19].

Hypoallergenic allergen variants can be created by various production methods. One approach is chemical modification. The methods used to produce allergoids, that is, formaldehyde and glutaraldehyde treatment, can be applied to recombinant allergens, but a disadvantage is that the end product cannot be defined in molecular terms. Conjugation with particular unrelated chemical entities may influence IgE reactivity. The coupling of an average of four 22-base CpG-oligonucleotide sequences per molecule of the major ragweed allergen Amb a 1, for example, resulted in a threefold increase in the concentration of allergen required to achieve 50% inhibition in an IgE ELISA and a similar 30-fold increase for the basophil histamine release assay [20]. In a second approach, peptides represent continuous, discontinuous, or portions of T- or B-cell epitopes expressed or synthesized singly or in combination to create a new molecular form. Peptide mixtures derived by proteolytic digestion of allergen extracts were considered for immunotherapy more than 20 years ago

but were dismissed because of the problems of consistent production and standardization. The availability of detailed information on protein structure now allows a designer approach. In the major cat allergen, Fel d 1, a mixture of overlapping 16 to 17 amino acid peptides spanning most of the sequence of both of the two chains was selected on the basis of T-cell reactivity and the ability to bind to MHC molecules [21]. The peptide mixture was shown to be effective in modifying surrogate markers of allergy, including cutaneous responses to allergen challenge and ex vivo parameters of T-cell activation [22,23]. An alternative strategy is to combine several T-cell determinants to form a large hybrid peptide, as has been achieved for the two major allergens of Japanese cedar [24]. In this case, the hybrid failed to bind allergen-specific IgE and was substantially more effective in inducing T-cell proliferation than a mixture of the individual peptides.

A third approach involves fragmentation of the allergen so that the correct molecular folding of the intact molecule is lost, as are conformational IgE-binding epitopes. This method has been used successfully in the birch pollen allergen Bet v 1, which can be expressed as two hypoallergenic fragments [25]. In a fourth approach, recombinant oligomers are produced by linking several copies of a gene in sequence (homomer) or several different genes in sequence (heteromer). A trimeric form of Bet v 1 has been shown to be hypoallergenic [26]. A construct of the group 5 and 6 allergens of *Phleum pratense* has been shown to have partial hypoallergenic properties [27]. On the other hand, Phl p 2-Phl p 6, Phl p 6-Phl p 2, and Phl p 5-Phl p 1 hybrids appear to retain the IgE reactivity of the individual allergens while immunogenicity is enhanced [28]. These results clearly indicate that oligomerization, per se, does not ensure hypoallergenic properties, and it must be concluded that the success of the technique is allergen dependent.

A fifth strategy involves producing variants through mutations introduced by site-directed mutagenesis either directly in IgE-binding epitopes to compromise their activity, or at sites outside the epitopes in positions such that they influence the conformation of the molecule and thereby IgE-binding activity. Substituting single amino acids can have dramatic effects on the overall structure. Disulfide bonds can be destroyed by replacing one or more cysteine residues, resulting in degeneration of tertiary structure and conformational changes, an approach used successfully to produce variants of Der p/f 2 with reduced IgE reactivity [29,30]. Alteration of the surface charge in antigenic regions, such as by substituting aspartic acid (negative charge) for alanine (nonpolar) or vice versa, may modify folding and the ability to react with antibody. The deletion of short sequences can also be used to good effect providing that T-cell epitopes are left undisturbed [31]. PCR amplification in conjunction with oligonucleotide primers may give rise to allergen variants with little or no IgE-binding activity as a result of chance mutations. Various variants of Bet v 1 have been characterized [32]. The isoform Bet v 1a was identified based on high IgE reactivity, whereas the Bet v 1d isoform, which differed by seven amino acid residues, was found to have little IgE reactivity. Cutaneous reactivity was 100-fold lower, and a conjunctival provo-

cation was negative in 48 patients tested, whereas 38 of these patients reacted to the Bet v 1a molecule [33]. Both isoforms show good T-cell reactivity, although failure in both cases to stimulate a number of T-cell clones indicates that some T-cell epitopes are compromised.

A sixth strategy for creating hypoallergenic variants involves gene rearrangement to produce modified protein sequences. This rearrangement may be achieved by a random approach using “gene shuffling,” also referred to as molecular breeding, which gives rise to a large number of new sequences and necessitates screening to identify candidate molecules [19,34]. Alternatively, a gene may be cut at preselected points and reassembled before expression to create a “mosaic” protein [35]. The latter approach can be extended by mixing gene fragments from various allergens.

### **Selection of allergens for a therapeutic vaccine**

In addition to the advantages in quality, the expectation is that recombinant allergens or their hypoallergenic derivatives will be better than allergen extracts in terms of the degree of therapeutic benefit they can confer and the specificity of the diagnosis.

In many cases, it will not be realistic to represent all allergens from a particular source; therefore, it will be important to identify the most important allergens that contribute to sensitization and symptoms. Grass pollen, for example, has 11 different allergens that have been identified and characterized in detail, some of which are known to occur as several different isoforms. Only some of these allergens have major general importance, being associated with a high prevalence of sensitization in the grass pollen-allergic population and together accounting for a large part of the specific IgE response directed against the whole pollen extract. The group 1 and 5 allergens are strong candidates for inclusion in a therapeutic vaccine. More than 90% of grass pollen-allergic subjects react to group 1 allergens and as many as 85% to group 5 [36,37]. There are two isoallergens of the timothy grass pollen allergen Phl p 5, the principal difference being that two 14/15 amino acid sequences are missing from the N-terminal half of Phl p 5b when compared with Phl p 5a. The molecules show extensive homology. Although there is a high degree of IgE antibody cross-reactivity, the immunologic differences in terms of T-cell reactivity may justify the inclusion of both isoallergens in a vaccine. The allergens Phl p 2 and Phl p 6 show prevalences of sensitization in the range of 40% to 60% and 60% to 70%, respectively, and are classified as major allergens. Although as many as 80% of grass pollen-sensitized subjects may have IgE antibodies directed to the group 4 allergen, the concentrations are relatively low compared with those specific for Phl p 1 and 5, as is specific skin test reactivity. Further allergens including Phl p 7, 10, 11, and 12 are classified as minor allergens and are not strong contenders for inclusion in a vaccine. The prevalence of sensitization to the calcium-binding

protein designated Phl p 7 is about 10% [38], and subjects reacting to Phl p 10 (cytochrome c) are seldom found [36]. Both Phl p 11 (profilin) and Phl p 13 are glycosylated, and a substantial part of the IgE reactivity appears to involve cross-reactive carbohydrate determinants.

It seems reasonable to conclude that any preparation for grass pollen-specific immunotherapy must include the group 1 and group 5 allergens. These allergens may be supplemented by other relatively important allergens, such as group 2 and 6 and possibly group 4. A study conducted using two purified natural allergens of *Phleum pratense*, subsequently identified as Phl p 5 and Phl p 6, found that they were significantly less effective clinically than a partially purified whole allergen extract, and one can speculate that this result might have been attributable to the lack of Phl p 1 [39,40].

Similar considerations will have to be applied in preparations for house dust mites. Although more than 20 allergen groups have been identified in association with *D pteronyssinus* and *D farinae*, the group 1 and 2 allergens, which were the first to be identified, are apparently the most important and account for a large proportion of the specific IgE response directed against the mite extracts.

In other instances, there is a real prospect that one allergen or allergen derivative will suffice to achieve a substantial improvement in clinical symptoms (eg, cat [Fel d 1] and birch [Bet v 1] allergens), the rationale being that one major allergen dominates and accounts for most or all of the specific IgE response directed against the one sensitizing agent.

One concept that has been propagated is patient-tailored immunotherapy [41]. The availability of pure recombinant allergens will make it possible to perform component-resolved diagnosis and to produce IgE reactivity profiles for individual patients. Furthermore, the information will help to elucidate the role played by cross-reacting allergens in clinically relevant sensitizations. With this information, it would be possible to define a vaccine to match an individual's sensitization profile. One day this may become a reality; the more immediate prospect is the introduction of standard combinations of allergens from one source, or in some cases single allergens, to provide immunotherapy for the sensitizations most frequently implicated in allergic disease.

### **Recombinant hypoallergenic variants of birch pollen allergen Bet v 1**

The IgE-binding reactivity of allergens such as Bet v 1 from birch pollen is dependent on their three-dimensional structure. Cleaving the cDNA and expressing the two parts separately results in two allergen fragments (amino acid residues 1–73 and 74–159) showing only minimal allergenicity and a random coil conformation [25]. The cleavage point was chosen to prevent compromising any of the recognized T-cell epitopes. Three-dimensional mapping of the Bet v 1 molecule revealed that the IgE-binding structure involved residues 9 to 22 and 104 to 123, regions that are segregated between the two fragments,

resulting in the loss of IgE-antibody binding activity. It has been proposed that a similar approach could be used for the grass pollen allergen Phl p 6 [42], but experience has shown that the approach is not generally applicable.

The birch pollen protein has also been used as a model system to investigate the potential of oligomerization to influence IgE reactivity. Linking three copies of the Bet v 1 cDNA in sequence and expression in *E coli* resulted in a trimeric form of the protein. IgE reactivity was reduced as judged by histamine release and skin testing, but circular dichroism spectroscopy showed that the secondary structure was essentially the same as monomeric Bet v 1 [26]. Basophil activation measured in terms of CD203c expression indicated that the trimer was less hypoallergenic than the fragments [43]. The reasons for the hypoallergenic nature have not been elucidated, but steric hindrance of the IgE-binding sites seems a likely explanation. The general utility of this approach has not been confirmed.

The hypoallergenic nature of these preparations was investigated in two studies involving skin prick and intradermal testing. When skin prick testing was performed at concentrations of 100 µg/mL in birch pollen-allergic subjects who had not received specific immunotherapy, 18 of 23 and 15 of 23 failed to react to the fragment mixture and the trimer, respectively [44]. All subjects reacted to unmodified Bet v 1, but groups of nonatopic individuals and atopic individuals without birch pollen sensitivity showed negative test results to all preparations. A similar result emerged from intradermal testing. Eight of 23 and 13 of 23 of the birch pollen-allergic subjects failed to react to 1 µg/mL concentrations of the fragment mixture and the trimer, respectively. Clear dose-response effects were seen with each of the allergen derivatives. The second study produced similar results [45]; however, there was a large intersubject variation in the endpoint of the intradermal tests. Analysis of the ratios of the endpoint concentrations of Bet v 1 to each of the derivatives showed that although the derivatives were hypoallergenic in all birch pollen-allergic subjects, the degree of hypoallergenicity varied. The two Bet v 1 fragments were tested separately, and both were shown to be hypoallergenic in nature. Subjects proved to be more sensitive to all preparations when tested during the *Fagales* pollen season, suggesting that natural exposure to this tree pollen primed the subjects' response to the birch pollen allergen.

The first study of allergen-specific immunotherapy with recombinant preparations investigated the clinical effects, immunologic activity, and tolerance of a mixture of the two Bet v 1 fragments and the Bet v 1 trimer in comparison with placebo. The recombinant preparations were adsorbed to aluminium hydroxide suspensions at concentrations of 100 µg/mL, and immunotherapy was planned with a course of eight preseasonal injections of increasing concentrations from 1 to 80 µg of total protein, with further injections up until the beginning of the pollen season. Subjects were assessed at baseline, after treatment, during and after the pollen season, and after 12 months [46].

Results from 71 patients from one of the three study centers showed that both active preparations induced Bet v 1-specific IgG1, IgG2, and IgG4 antibody

responses as well as an IgA response. Responses to the trimer were stronger, and this preparation also induced IgM antibodies, indicating its good immunogenic properties. The serum antibodies were shown to be able to inhibit allergen-induced histamine release in vitro from basophils of birch pollen-allergic subjects. Sera from the trimer-treated subjects were more effective, reflecting the higher IgG antibody titers. It was possible to show a correlation between IgG1 antibody titers and an improvement in clinical symptoms, as judged by a ten-point interval scale, as well as a reduction in skin test reactivity to Bet v 1. Measurement of Bet v 1-specific IgE responses showed a threefold increase in the placebo group owing to seasonal pollen exposure, whereas the responses in the two treatment groups were blunted.

Nasal lavage fluids were collected from a randomly selected subgroup of 23 subjects at the end of the birch pollen season following the course of immunotherapy and again 12 months after the time point at which treatment had commenced [47]. Bet v 1-specific lavage fluid IgG1 levels were significantly raised at the end of the pollen season in subjects who had received the active preparations (10 trimer; 3 fragment mixture) when compared with placebo subjects. Higher levels of IgG2 and IgG4 were also detected as in serum, but these differences were not significant. There were also no apparent differences in IgA levels. There were correlations between the various IgG subgroup concentrations in serum and lavage fluid. At the end of the birch pollen season, there was a correlation between nasal IgG4 and reduced specific nasal sensitivity. Perhaps not surprisingly, the nasal antibody levels mirrored those in serum. The reduced nasal sensitivity may be accounted for by the inhibitory effect of the antibodies on basophil and mast cell mediator release as demonstrated for the serum antibodies in vitro.

Investigations of cytokine responses conducted in one of the other two study centers showed that treatment with trimer resulted in significant reductions in interleukin-5 (IL-5) and IL-13 producing cells when compared pre- and posttreatment, which was indicative of a suppression of the Th2 response [48]. There were also trends for decreased numbers of IL-4 producing cells and increased numbers of IL-12 producing cells, but the differences were not significant, most likely because of the small number of subjects (8 trimer, 10 fragment mixture, and 8 placebo). The results from measurements of immunologic parameters provide an encouraging basis for pursuing the development of hypoallergenic derivatives, but emphasis must be placed on the generation of data to provide evidence of clinical efficacy.

An alternative strategy for developing a hypoallergenic derivative came from the observation that recombinant Bet v 1 can be induced to adopt a stable random coil structure that can be clearly distinguished from the secondary structure of the native molecule by circular dichroism spectroscopy. This folding variant, designated Bet v 1-FV, exhibits hypoallergenic properties as judged by immunoassay inhibition tests and basophil activation [43,49]. The first clinical trial to test this derivative began in 2003. Subjects with birch pollen rhinitis were included in an open randomized comparative study of recombinant Bet v 1-FV and a natural

birch pollen extract, both adsorbed to aluminum hydroxide. Treatment was administered over a period of 4 months before the birch pollen season, with injections given at weekly intervals. The maximum dose of Bet v 1-FV was 80µg, fourfold higher than the Bet v 1 content of the natural allergen preparation. Preliminary results following the first year's treatment indicate that the combined

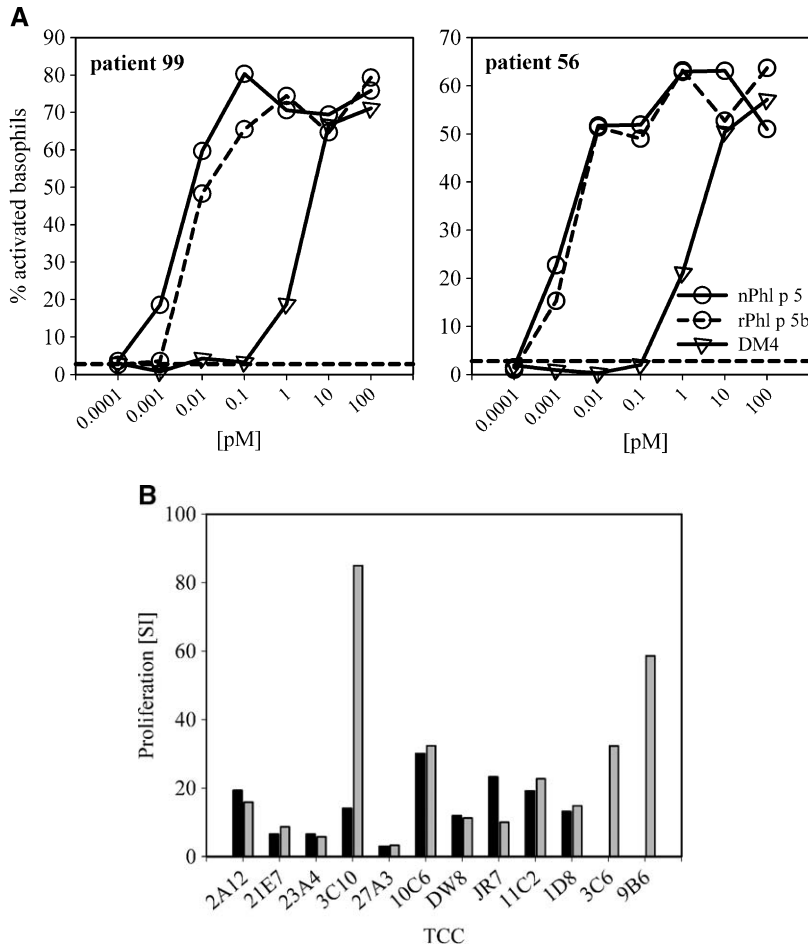


Fig. 2. Comparison of the natural timothy grass pollen allergen Phl p 5 with recombinant Phl p 5b and the genetically engineered derivative DM4. (A) Peripheral blood mononuclear cells from grass pollen-allergic subjects were incubated with various concentrations of the proteins or the diluent solution. Basophil activation was measured by fluorescence activated flow cytometry in terms of expression of the surface marker CD203c. (B) Lymphocyte proliferation, expressed as a stimulation index (SI), was determined in terms of <sup>3</sup>H-thymidine incorporation by T-cell clones (TCC) derived from nine grass pollen-allergic subjects and showed specificity for five distinct T-cell epitopes distributed throughout the protein. Cells were stimulated with rPhl p 5b (black bars) and rPhl p 5 variant DM4 (gray bars).

symptom-medication score for subjects receiving the recombinant preparation is favorably less than that for subjects receiving the natural pollen extract. The scores for both groups are superior to those of a reference group with only antisymptomatic treatment (parallel control group). Improvements in specific nasal sensitivity, as judged by a nasal provocation test, have been seen in both study groups, and the immunogenic activity of the recombinant preparation is confirmed by its ability to induce strong IgG1 and IgG4 antibody responses. Safety data indicate that the preparations are comparable with respect to the occurrence of adverse events. Again, the results are encouraging for further development of the hypoallergenic preparation, but final data on clinical efficacy will be important.

### **Recombinant hypoallergenic variants of grass pollen allergens**

Information on both T cell and IgE-binding epitopes was used as a basis for designing mutants of group 5 allergens from timothy grass and rye grass pollen [31,50]. Various candidate derivatives were produced. Although partial reduction in IgE reactivity could be achieved through introduction of point mutations, more dramatic results were obtained by creating small deletions in the molecule. Two deletions, one of 20 amino acid residues and one of 25, were introduced at sites outside five distinct T-cell epitope regions and resulted in a variant (designated DM4) with substantially reduced IgE-binding activity, skin test reactivity, and histamine-releasing activity while largely retaining T-cell reactivity (Fig. 2). Larger deletions or deletions at other sites often resulted in hypoallergenic molecules but invariably considerable loss of T-cell reactivity [31].

The DM4 mutant was subsequently tested in a group of 22 grass pollen-allergic subjects, 19 of whom reacted with at least one concentration of natural or recombinant Phl p 5b, which served as reference preparations [51]. The wheal responses to recombinant Phl p 5b were smaller than those to the natural molecule but not significantly. In contrast, the wheal response to DM4 was significantly smaller than that to the two reference preparations (Fig. 3). The measurement of IgE reactivity to each of the proteins supported these results, and, in the majority of cases, serum IgE failed to bind to the DM4.

The DM4 variant of Phl p 5b is seen as a potential candidate for a therapeutic vaccine for grass pollen-specific immunotherapy, but before planning any clinical studies, it will be necessary to develop derivatives of other grass pollen allergens to create a mixture that can account for all of the major causes of sensitization.

### **Recombinant grass pollen allergens**

The grass *Phleum pratense* is a member of the subfamily Pooideae, which, in turn, belongs to the family Poaceae. It shows substantial cross-reactivity with

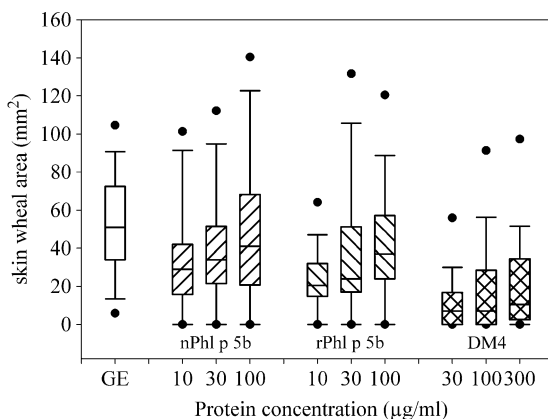


Fig. 3. Comparison of median skin test wheal responses to the natural timothy grass pollen allergen Phl p 5b with those to recombinant Phl p 5b and the genetically engineered derivative DM4. A grass pollen extract (GE) and three concentrations of each of the test proteins were skin prick tested in triplicate on the volar forearms of 22 grass pollen-allergic subjects using a random scheme. Boxes show the 25th and 75th percentiles; bars show the fifth and 95th percentiles; and dots show the outliers.

other members of the subfamily [36]; therefore, it can be considered representative of grasses found in temperate regions. Separate aluminum hydroxide adsorbates of the recombinant allergens Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, and Phl p 6 were prepared and mixed together to create a therapeutic vaccine, with approximately equimolar amounts of the individual allergens. This mixture of allergens accounts for a substantial proportion of the specific IgE sensitization developed against grass pollen; therefore, it is realistic to expect that it could be effective against a substantial part of the symptoms.

A double-blind, placebo-controlled clinical trial was undertaken with 62 grass pollen-allergic patients sustaining rhinoconjunctivitis with or without asthma [52]. The recombinant allergen adsorbate was administered in subcutaneous injections of increasing concentrations at 7-day intervals before the pollen season in 2002, starting with 0.02 µg of total protein, followed by 0.16 µg and then doubling to 40 µg of total protein (0.8 mL). The maximum dose contained 10 µg of Phlp1, 5 µg of Phlp 2, 10 µg of Phlp 5a, 10 µg of Phlp 5b, and 5 µg of Phlp 6. Maintenance injections were continued until after the subsequent pollen season with a 50% reduction during each pollen season. The median cumulative dose was 490 µg of total protein, corresponding to 122.5 µg each of Phl p 1, Phl p 5a, and Phl p 5b, and 61.25 µg of Phl p 2 and Phl p 6.

A symptom-medication score was the primary outcome measure to assess efficacy. Subjects kept diaries for 3 months over each pollen season to record the nature and severity of eye, nose, and chest symptoms and the type and dose of any medication. The intensity of symptoms was documented as follows: 0=no symptoms, 1=mild symptoms, 2=moderate symptoms, 3=severe symptoms. The same rescue medication was available to all subjects, and use was scored

taking into account pharmacokinetic and pharmacodynamic characteristics. A per protocol analysis included 24 active treatment and 25 placebo patients. A combined symptom-medication score adopted as the primary endpoint showed a 39% improvement in the active treatment group when compared with the placebo group ( $P = .044$ ). Symptoms alone improved by 37% ( $P = .015$ ), and the use of symptomatic medication decreased by 36.5% in a comparison with the placebo group.

A validated rhinitis quality of life questionnaire was a secondary endpoint [53]. Questionnaires were completed every 2 weeks during the pollen season, and the questionnaire following the maximum pollen count was used for analysis. Benefits were registered for subjects on active treatment during the first pollen season. The per protocol evaluation during the second pollen season showed even greater differences between active and placebo treatment with an overall significant benefit ( $P = .024$ ), providing further evidence of clinical efficacy. Significant effects were seen in five of seven domains tested (Fig. 4). The level of clinically relevant improvement for individual subjects was considered to be 0.5, and the mean between group differences that were judged statistically significant were all considerably in excess of this figure. Conjunctival provocation tests were performed before therapy (inclusion criterion) and at the end of the study using a standardized six-grass allergen extract. The concentration was increased in half-log steps starting with 5 biologic units (BU)/mL until a positive reaction was obtained or a maximum concentration of 5000 BU/mL (0.18  $\mu$ g of group 5 allergen/drop) was reached. A favorable trend was observed ( $P = .081$ ),

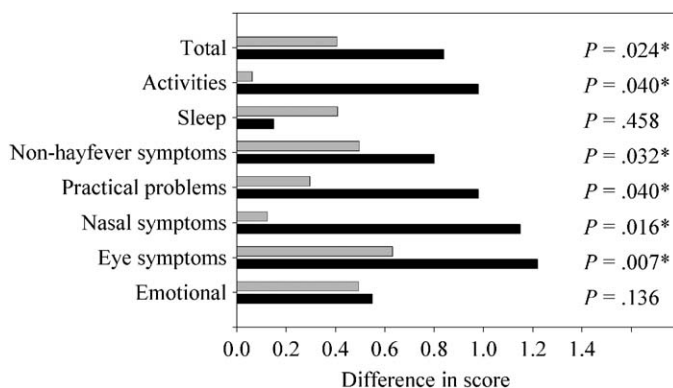
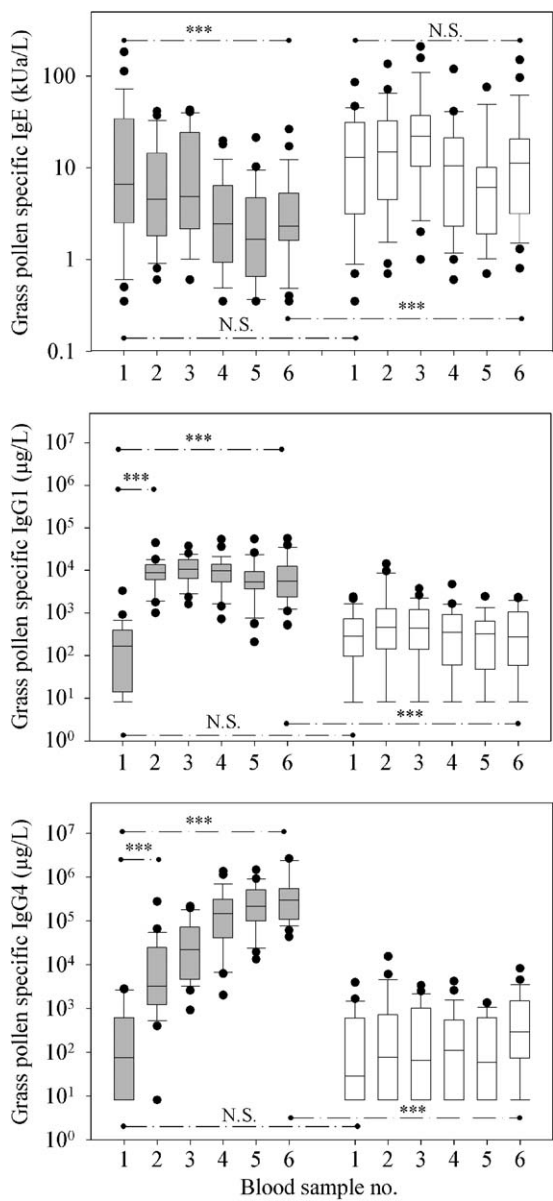


Fig. 4. Rhinitis quality of life questionnaire for subjects undergoing immunotherapy with a mixture of five recombinant *Phleum pratense* pollen allergens. Bars show differences between active and placebo treatment groups in pollen seasons during the first and second years of the study, 2002 (gray) and 2003 (black), respectively. Questionnaires completed immediately following the maximum pollen count were used for analysis. Mann-Whitney U-test results and  $P$  values for the 2003 season are shown. (From Jutel M, Jaeger L, Suck R, et al. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol* 2005;116(3):611; with permission from the American Academy of Allergy, Asthma, and Immunology.)

with an increase in the threshold dose in favor of treatment with the active preparation. The failure to achieve a significant result was most likely attributable to the small numbers of patients.

Active treatment induced highly significant increases in IgG1 and IgG4 grass pollen-specific antibody concentrations together with a significant decrease in



IgE (Fig. 5). IgG1 increased approximately 60-fold, peaking during the first 12 months of the study. IgG4 showed a continuing upward trend, achieving an approximately 4000-fold increase by the end of treatment. Specific IgE levels were not significantly different between groups at the beginning of the study, but thereafter the active treatment group showed a downward trend with values significantly less than baseline. Four subjects in each group had no Phl p 5a/b-specific IgE before the study but reacted to Phl p 1 and other grass pollen allergens. None of these subjects developed Phl p 5a/b IgE antibodies during the study, although the four subjects receiving active treatment developed strong IgG4 and IgG1 Phl p 5a/b responses consistent with induction of a protective immune response. This observation obviously needs to be substantiated. It will also be of interest to look at the prophylactic effects of the treatment in guarding against the development of new sensitizations.

Treatment-related adverse events were seen in association with 78 injections (10.7%) in the active treatment group and 44 injections (5.9%) in the placebo group. Local reactions involving erythema and swelling near the injection site, with or without pruritus, accounted for nearly all of these events. Single systemic reactions were seen in seven active treatment and two placebo subjects, the former including general urticaria (2 cases), local urticaria (2 cases), dyspnea (1 case), rhinoconjunctivitis (1 case), and asthma (1 case) 2 days following the injection. All of these subjects continued treatment without further problems, and it was concluded that the preparation showed a favorable safety profile when compared with findings from other immunotherapy studies.

### Potential benefits of recombinant allergens for specific immunotherapy

Recombinant DNA technology has delivered the prospect of a new generation of preparations for allergen-specific immunotherapy. The first clinical studies with recombinant allergens have yielded encouraging results, suggesting that there is a good chance that such preparations will become available for use in the

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Fig. 5. Grass pollen-specific IgE, IgG1, and IgG4 antibody concentrations in blood from subjects undergoing immunotherapy with a mixture of five recombinant *Phleum pratense* pollen allergens. Median values are shown with 25th/75th and 10th/90th percentiles represented by boxes and error bars, respectively, and outliers by points. Gray bars denote the active group and white bars the placebo group. Time points: 1, before immunotherapy, 1/2002–2/2002; 2, after initial dosage increase and before pollen season, 4/2002–5/2002; 3, after the pollen season, 7/2002–9/2002; 4, after 12 months, 1/2003–3/2003; 5, before the pollen season, 4/2003–5/2003; 6, at the end of the study, 8/2003–9/2003. \*\*\* $P < .001$ ; NS, nonsignificant. (From Jutel M, Jaeger L, Suck R, et al. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol* 2005;116(3):611; with permission from the American Academy of Allergy, Asthma, and Immunology.)

routine management of allergic disease. Some of the advantages and benefits that may be achieved with these preparations can be summarized as follows:

- Although a large proportion of the proteins and other molecules in an allergen extract may have no relevance to allergy, recombinant preparations include only allergens or allergen derivatives.
- The proteins can be produced in high purity to exacting pharmaceutical standards.
- There is no risk that unrelated allergens, infectious agents, or irritants can contaminate a preparation.
- Different allergens stemming from the same source (eg, grass pollen) can be mixed together in appropriate amounts (possibly equimolar concentrations) to achieve an optimal therapeutic formulation in contrast to the composition of native allergen extracts, which is determined largely by the raw material from which they are derived.
- Standardization, product consistency, and declaration of the concentrations of the active pharmaceutical ingredients are easily achieved.
- The technology provides the means for producing precisely defined hypoallergenic derivatives that may make it possible to achieve an enhanced benefit-risk ratio.

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