

Arbeiten aus dem Paul-Ehrlich-Institut
11th International Paul-Ehrlich-Seminar, Bad Homburg 2005
Verlag Chmielorz

Clinical Evaluation of Recombinant Allergens

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Introduction

Recombinant DNA technology has been used extensively to clone and sequence several hundred different allergens, and the detailed characterization of these molecules is leading to a better understanding of the basis of allergic responses. The availability of such proteins also provides the opportunity to develop new preparations for both allergy diagnosis and allergen specific immunotherapy [1,2]. The recombinant proteins offer many potential advantages over the allergen extracts that are traditionally used for therapy. They can be produced in high purity to exacting pharmaceutical specifications and standardization, product consistency, and the declaration of absolute concentrations of the active pharmaceutical ingredients are relatively easy to achieve. The composition of a therapeutic preparation is confined to the most relevant allergens, by contrast with allergen extracts which contain substantial amounts of irrelevant non-allergenic material. They contain defined amounts of pure proteins in fixed relative amounts, possibly in equimolar concentrations.

The so-called 'wild type' recombinant allergens, which exhibit IgE-reactivity comparable with that of the natural allergen, may supersede natural allergen extracts used in specific immunotherapy. However, the success of the treatment is dependent primarily on the T-cell reactivity and immunogenicity of the proteins rather than IgE-reactivity. This view is supported by results obtained with hypoallergenic derivatives produced by chemical modification with agents such as formaldehyde and glutaraldehyde. The reduced IgE-reactivity of the preparations confers a smaller risk for inducing IgE-mediated side-reactions and allows a maximum therapeutic dose to be achieved in a relatively short course of injections by comparison with the native allergen [3,4]. The experience gives grounds to believe that it should be possible to use recombinant DNA technology to engineer allergen derivatives which combine the benefits of hypoallergenic characteristics with the advantages of recombinant proteins [5].

The question as to which allergens have to be included in a therapeutic preparation will have to be considered for each allergen source in turn. In many cases it may not be appropriate to represent all allergens and therefore it will be essential to identify those which make the most important contributions to sensitization and symptoms. Eleven different grass pollen allergens have been characterized for example, but only some of these are of 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 directed against the whole pollen extract [6]. In other cases there is a real prospect that one allergen or allergen derivative will suffice to achieve a substantial improvement in clinical symptoms, e.g. cat (Fel d 1) and birch (Bet v 1), the rationale being that one major allergen dominates and accounts for most or all of the specific IgE directed against the one sensitising agent.

Here we present and discuss results from two clinical studies of allergen specific immunotherapy with recombinant allergen preparations, one using a mixture of five grass pollen allergens in a randomised double-blind placebo controlled design, and the other a hypoallergenic derivative of the major birch pollen allergen Bet v 1 in a randomised, open comparative trial with a native allergen extract. In both cases the objective was to assess efficacy and safety in subjects suffering from seasonal allergic rhinitis/rhinoconjunctivitis, either with or without bronchial asthma GINA I or II. The results may be seen as being relevant to specific immunotherapy for inhalant pollen allergens in general, since the role of IgE antibodies and the pathology of the allergic disease is independent of the allergen under investigation.

Methods

Five grass pollen allergens, Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, and Phl p 6, and the major birch pollen allergen Bet v 1 were cloned from cDNA expression libraries derived from pollen of *Phleum pratense* (Timothy grass) and *Betula verrucosa* (birch) respectively. The cDNAs were sub-cloned into appropriate expression vectors and expressed in *Escherichia coli* [7]. The recombinant proteins were purified using various chromatographic techniques including hydrophobic-interaction, ion-exchange and size-exclusion, and analysed to confirm identity and purity. Bet v 1 was prepared as a stable folding variant with hypoallergenic characteristics and designated Bet v 1-FV.

Allergens were adsorbed separately to aluminium hydroxide. The *Phleum pratense* allergens were combined in approximately equimolar amounts and formulated with a total protein concentration of 50µg/ml. This was administered by subcutaneous injection in increasing doses at 7 day intervals up to a maximum of 40µg total protein comprised of 10µg each of Phl p 1, Phl p 5a and Phl p 5b, and 5µg of Phl p 2 and Phl p 6. The birch pollen allergen variant Bet v 1-FV was formulated at a concentration of 100µg/ml and the dose was increased from 0.5µg to a maximum of 80µg. Grass pollen allergic subjects were treated on a perennial basis over a period of approximately 20 months encompassing two pollen seasons, with injection intervals being extended to 14, 28, and finally 42 days. The maintenance dose was reduced by 50%

during the pollen seasons. The birch pollen allergic subjects received one course of pre-seasonal injections during the five months prior to the pollen season.

A Symptom Medication Score was the primary outcome measure to assess efficacy in both studies. A validated rhinitis quality of life questionnaire (RQLQ) was used as a secondary endpoint in the grass pollen study and was completed by subjects during the baseline visit prior to each pollen season and every 2 weeks during the seasons [8]. The questionnaire following the maximum pollen count was used for analysis.

Conjunctival (grass) and nasal (birch) provocation tests were performed before therapy (inclusion criterion) and after the second grass pollen season (2003) or the first birch pollen season using standardized lyophilised 6-grass and birch pollen allergen extracts respectively. Initial conjunctival test concentration was 5 BU/ml (1 drop), and this was increased in half-log steps until a positive reaction was obtained or a maximum concentration of 5,000 BU/ml was reached.

Specific IgE antibodies were measured using the Allervance system (Allergopharma, Reinbek, Germany). Specific IgG1 and IgG4 antibodies were detected using microtitre plates coated with either allergen extract 10 µg/ml (Allergopharma), or purified natural allergens (1 µg/ml), or monoclonal anti-IgG1 (5 µg/ml) and anti-IgG4 (0.5 µg/ml) respectively.

Results and Discussion

Recombinant grass pollen allergens

The combined symptom medication score showed a significant difference between groups after the 18 month treatment period, with median average daily scores of 4.92 versus 8.05 for active and placebo treatment respectively, a difference of 38.9% ($p = 0.044$) [9]. The symptom score alone showed a 36.5% improvement for active treatment compared with placebo (3.38 versus 5.32; $p = 0.015$). There was also a reduction in the need for medication reflected in a lower medication score. This result compares favourably with the mean 45% additional improvement in disease severity above the response to placebo seen in 43 rhinitis studies, including 1,120 actively treated patients [10].

Quality of life was assessed in respect of seven questionnaire domains and also as a whole (28 questions). Improvements in favour of active treatment were noted during the first pollen season, but these were even larger in the second pollen season (2003) when significant and clinically relevant differences in favour of active treatment were determined for the whole questionnaire and 5 of the seven domains (Figure 1). Scores for the active treatment group were at least 0.5 points lower than placebo for total score and all domains except sleep. The conjunctival provocation test showed a clear trend to a higher threshold allergen dose in the treatment group, with 21/28 of subjects tolerating higher allergen concentrations. However this improvement was not statistically different from that for the placebo group ($p = 0.081$).

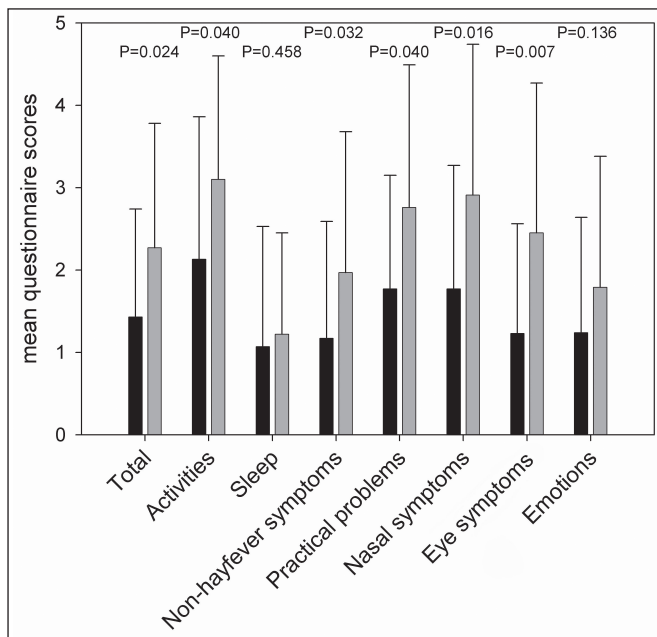


Figure 1: Rhinitis quality of life questionnaire for recombinant grass pollen study. Mean scores ($\pm$ S.D.) for active (black bars) and placebo (grey bars) subjects from the questionnaire completed following the maximum pollen count in the second year of the study (2003). Mann-Whitney U-test.

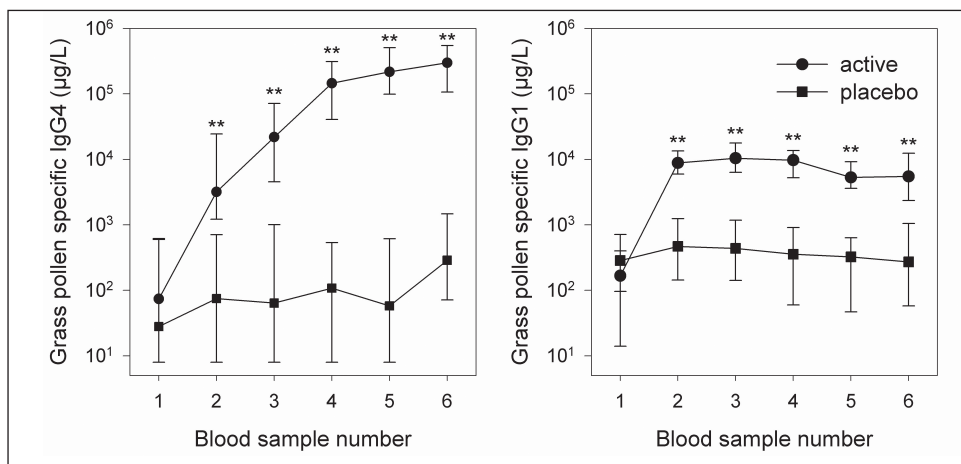


Figure 2: Grass pollen specific IgG4 and IgG1 responses. Median values with 25th/75th percentiles for subjects on active and placebo treatment. Blood samples were collected 1. before IT; 2. after initial dosage increase and before pollen season 2002; 3. after pollen season 2002; 4. 12 months after commencing IT; 5. before pollen season 2003; and 6. at end of study. ** $P < 0.001$, differences between the groups.

All subjects in the active treatment group showed specific IgG isotype responses to a *Phleum pratense* extract (Figure 2). Allergen specific IgG1 concentrations peaked during the first 12 months with an approximately 60-fold increase. IgG4 antibodies showed a continuing upward trend, achieving an approximately 4000-fold

increase in concentration by the end of the study. Differences between the groups were statistically significant ($p < 0.001$) after the initial dosage increase and then at all subsequent time points. Investigations with individual allergens showed that the responses to Phl p 5a/b were stronger than those to Phl p 1, medians being 290 and 23 $\mu\text{g/ml}$ respectively for IgG4, and 13 and 2.1 $\mu\text{g/ml}$ for IgG1.

Four subjects in each study group had no Phl p 5a/b specific IgE prior to the study, but had IgE antibodies to Phl p 1 and other grass pollen allergens. This provided an opportunity to study the possible development of new sensitizations as a result of immunotherapy. None of these subjects developed Phl p 5a/b IgE antibodies during the 18-month course of treatment, but the 4 subjects receiving the recombinant allergen preparation showed strong IgG4 and IgG1 Phl p 5a/b specific antibody responses consistent with induction of a tolerant immune response.

The recombinant preparation showed a favourable safety profile. Most reactions were local to the injection site and involved transient erythema and swelling, consistent with allergic responses or possibly mild irritation due to the aluminium hydroxide suspension. Histamine included in the placebo to mimic allergen induced responses probably accounted for many of the reactions in this group. Systemic reactions occurred in seven subjects receiving the recombinant preparation with one isolated reaction in each case, including local or general urticaria, rhinitis, rhinoconjunctivitis and one case of asthma two days following an injection. All subjects continued therapy with either the same or higher doses without further problems indicating that the preparation was generally well tolerated.

Recombinant hypoallergenic birch pollen allergen

There was no significant difference between the daily median symptom medication scores for the Bet v 1-FV treated group and the comparator group for the three weeks encompassing the pollen season, the scores being 5.90 and 12.48 respectively [11]. The daily median score for a group of control patients receiving symptomatic treatment in a separate clinical study in the same geographic region was 14.67. Similar numbers of subjects receiving the comparator and the recombinant preparation showed increased allergen tolerance in a conjunctival provocation test.

Significant increases in both IgG1 and IgG4 antibodies specific for natural Bet v 1 were observed for both the Bet v 1-FV and comparator treated subjects, with higher median values for the group that received the recombinant preparation. This probably reflects the high dosage of Bet v 1-FV with a maintenance concentration of 80 μg per injection.

The number and nature of the adverse events recorded during the study were comparable between the two groups. Local reactions were observed in association with 16% of injections in both study groups. Four systemic adverse events were recorded on the day of injection in each study group during the dosage increase phase of treatment outside the hazel and alder pollen seasons. During the maintenance treatment phase in the period immediately preceding the birch pollen season, when other trees

of the Fagales order were in bloom, a further 21 systemic adverse events were recorded in the Bet v 1-FV group and 15 in the comparator group. These reactions included rhinitis, conjunctivitis, local urticaria, asthma symptoms including cough, and nausea. Many of them, in particular rhinitis and conjunctivitis, might be explained in terms of concomitant exposure to other tree pollens, but a causal association with immunotherapy cannot be ruled-out completely.

Conclusion

Recombinant DNA technology has delivered the prospect of a new generation of preparations for allergen specific immunotherapy. The results from these first clinical studies with recombinant grass and birch pollen allergen preparations are very encouraging and give reason to hope that recombinant products will become available for use in the routine management of allergic disease.

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Discussion

Sager: I have a question concerning the safety profile of both preparations of the recombinant allergens. In how many patients did you observe the local or even the systemic reactions?

Cromwell: I have not got those figures fresh in my head, but I can safely say that it was less than half of the patients that showed local reactions. These were characterized by reddening or local swelling at the injection site, that is to say reactions which you can normally expect in the course of allergen immunotherapy. None of these local reactions were in any way unexpected. As I have shown, in the grass study there

were seven systemic reactions which were seen in a total of five patients. In the birch study we had 25 systemic reactions in the recombinant group as compared with 19 in the comparator group, but I am sorry I cannot recall how many patients that involves. Perhaps Dr Annemie Narkus can recall the number of patients that were concerned.

Narkus: In the birch study we had only four systemic reactions outside the pollen season and these were in two patients. So the severe reactions were extremely limited. What we saw was that when the hazel and the alder pollen season started, the number of systemic reactions increased. So, with birch we should aim to achieve the maximum dose before the pollen seasons for these related species begin.

Lund: I have two questions, one regarding the grass and one regarding the birch study. First, we have learnt yesterday from the CREATE project that, especially among the grass proteins, Phl p1 is very difficult to produce in recombinant form. To what extent have you shown the similarity between each of the individual recombinant proteins in your grass vaccine mix to their natural counterparts? Second, with regard to the denatured Bet v 1 preparation you use for vaccines, have you shown that you do not have any residual correctly folded Bet v 1 in that preparation?

Cromwell: First question: we have looked at all the recombinant molecules that have been produced in procaryotic expression systems with *E. coli*. The groups 2, 5, and 6 grass molecules are the natural allergen equivalents. They are non-glycosylated proteins. Phl p 1 is naturally a glycosylated protein, and since it was expressed in *E. coli*, the glycosylation part of the molecule is missing. We have looked at these molecules in comparison to the natural molecules with a wide range of tests, including IgE-binding, T cell responses, histamine release, and basophil activation tests. We could show that the recombinant molecules had almost identical reactivities in all these tests as compared to the natural allergens. We have also conducted extensive physicochemical characterizations showing sequence identities with the natural molecules. Second, as for Bet v 1, our molecule is slightly different from the one that you saw yesterday. Regarding the Bet v 1 folding variant, we have again conducted very extensive studies with several hundred sera from birch pollen-allergic patients showing that the molecule is hypoallergenic in a very high percentage of these. There are one or two patients who show limited IgE reactivity towards the molecule, but this is less than 5% of these patients. Looking at the circular dichroism spectra of the folding variant, we showed a very pronounced and clear difference between the natural molecule and the folding variant. That difference appears to be stable and we can show consistent quality in terms of the CD spectra and other physicochemical methods used for evaluation.

Unidentified: You will not be able to trace small amounts of correctly folded Bet v 1 in a CD spectrum and the antibodies you induce could – theoretically at least – stem from a contamination with correctly folded Bet v 1?

Fiebig: We have a set of monoclonal antibodies which react with the native form and the correctly folded recombinant form, and also antibodies which only react with the denatured and non-correctly folded form. Therefore we are in the position to detect

small traces of the correctly folded molecule among the non-correctly folded ones. We performed spiking experiments with small amounts of protein and we could not find any form other than this defined folding variant in the preparations using this very sensitive system.

Vieths: I would like to ask a question that I have asked before, but which is still very interesting to me. Were those patients who were not responding to immunotherapy, patients who were sensitized to allergens not contained in the cocktail or different from Bet v 1 in the case of birch?

Cromwell: This is something we have to follow-up and see whether or not, looking at serological parameters, any clear pattern emerges. We have not looked at those sera in great detail as yet. Certainly, that does remain a possibility. But, I think that I am correct in saying that all the patients who were in the study did in fact react to the group 1 allergens, whilst eight showed no IgE-reactivity towards Phl p 5. So all the patients reacted to at least some components of the cocktail.

Drachenberg: Obviously your primary endpoint was a combined symptom and medication score. How did you construct this combined score?

Cromwell: That is a detailed question and I think we should discuss that outside the session. It has recently been published in JACI and symptoms were assessed looking at nose, eyes, and lung, using the 0-1-2-3 scoring of symptoms – none, mild, moderate and severe. The medication score was compiled taking into account the pharmacodynamic and pharmacokinetic properties of the various symptomatic medications. The symptom and medication scores were simply added together.

Malling: I would like to focus a little on the systemic side effects in the birch study. Because this hypoallergenic variant had a very limited IgE reactivity and in principle it should not be able to elicit systemic side effects. Have you looked into those patients who reacted with systemic side effects? Were they the patients who had an IgE reactivity to the hypoallergenic variant?

Cromwell: No, we have not looked at that in detail. One of the other possibilities is that some of those reactions which we documented might have been due to natural exposure to pollen from related trees rather than to a reaction induced by the hypoallergenic molecule.

Malling: But natural exposures do not induce systemic side effects, they give clinical symptoms.

Cromwell: Some of those systemic effects were defined as rhinitis and conjunctivitis, and a relation to the treatment could not be categorically excluded.

Valenta: I would like to come back to the question of Prof. Malling, because I think it is very important from where we can expect systemic side effects when we treat with something that is not IgE-reactive. We have been very much concerned about

this. It seems that when you look, for example, at the peptide studies by Mark Larché in which he uses essentially T cell epitope-recognizing peptides that do not bind IgE at all. In approximately three quarters of the patients he sees severe systemic side effects. He can induce asthma by injecting T cell peptides and my guess would be that besides the IgE-mediated side effects we also have to look at T cell-mediated side effects.

Rabin: I am just curious. Can you or your colleagues comment on the difference in cost between this recombinant grass preparation and the natural ones.

Cromwell: I have no exact figures. Clearly, establishing recombinant allergen production is an expensive business, but there is no reason to believe that once a product is established and production is up and running that it is going to make any great difference for the cost of immunotherapy.

Sun: Could you tell us the dosage of your group 1 recombinant and natural allergens?

Cromwell: In the birch study, we had 20µg of the natural allergen in the extract by comparison with 80µg in the hypoallergenic recombinant preparation. So it was a four-fold higher concentration in the hypoallergenic preparation.

