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Recombinant hypoallergens for immunotherapy of *Parietaria judaica* pollen allergy

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TABLE OF CONTENTS

1. ABSTRACT

Recombinant allergens are a promising alternative to crude allergen extracts for diagnosis and therapy of allergic diseases. Genetically modified allergen derivatives with reduced allergenic activity but retaining their immunogenicity have also been produced to increase safety and specificity of allergen-specific immunotherapy. When a limited number of allergens are responsible for most of the allergenic activity, fusion proteins comprising these major allergens can be used to simplify vaccine development. Three different allergen fusions of Par j 1 and Par j 2, the major allergens from *Parietaria judaica*, were characterized. Two of them (Q1 and Q2) showing reduced allergenicity but conserved immunogenicity represent suitable candidates for allergen-specific immunotherapy against *P. judaica* pollen allergy.

2. INTRODUCTION

Type I allergy, a genetically determined IgE-mediated hypersensitivity, affects almost 25% of the population in developed countries. It is characterized by the formation of IgE antibodies to otherwise innocuous antigens (allergens) from pollen, mites, moulds, food, insect venoms, and animal dander, which can activate a variety of immune cells via the high- and low-affinity receptors for IgE. Allergen-induced cross-linking of IgE bound to effector cells (i.e., mast cell and basophil) via the high-affinity receptor (Fc-epsilon-RI) leads to release of inflammatory mediators (histamine, leukotrienes) and thus to the symptoms of type I allergy, such as allergic rhinitis, conjunctivitis, asthma, food allergy, dermatitis, and anaphylactic shock. IgE-mediated presentation of allergens to T-cells causes T-cell activation and release of pro-

inflammatory cytokines and thus, chronic T-cell- and eosinophil-mediated tissue inflammation. In order to induce strong effector cell activation and thus inflammatory responses, an allergen must be able to cross-link effector cell-bound IgE antibodies efficiently. This process requires the presence of at least two IgE epitopes on the allergen surface. IgE antibodies of allergic patients may recognize either "continuous epitopes" consisting of a row of consecutive amino acids or "discontinuous or conformational-dependent epitopes" which are composed of amino acids from different portions of the allergen brought into proximity by the molecule fold.

Allergic tissue inflammation can be mitigated using anti-inflammatory drugs and immunosuppressive agents. However, specific immunotherapy (SIT) is the only allergen-specific approach for the treatment of type I allergy and for preventing its progression to severe disease manifestations (1) and involves the administration of increasing doses of allergen extracts to patients in order to induce allergen-specific unresponsiveness. While several studies have shown clinical effectiveness of allergen-specific immunotherapy, the underlying mechanisms are not fully understood.

3. CURRENT ALLERGEN EXTRACTS FOR SPECIFIC IMMUNOTHERAPY

SIT has been used for the treatment of allergic diseases since 1911 as a result of the pioneering clinical work of Noon and Freeman (2, 3). Since then, the practice of immunotherapy has changed substantially over the last two decades as standardized extracts have been developed, optimal doses have been established, new routes and schedules for extract administration have been determined. But despite these advances, whole aqueous extracts of natural allergen source materials such as pollens, mites, moulds, and animal danders are still the basis for therapeutic preparations that are currently used in clinical practice.

Although several controlled clinical studies have demonstrated that the use of these preparations for SIT is clinically effective (4), there are still some disadvantages, mainly due to the extract complexity and variability (5), which cannot be overcome with existing technical means:

- Extract complexity and variability
    - Extracts contain numerous undefined components, some of which may promote allergic immune responses: These extracts are hydrosoluble complex mixtures of biomolecules, mainly proteins, glycoproteins and carbohydrates that may contain many irrelevant proteins and other macromolecules from the source material. The composition is determined to a large extent by the quality of the raw material and the method of extraction and purification. Although raw materials are provided by certified suppliers and produced under controlled conditions, sometimes there are differences. For example, the allergen content of mould cultures is highly dependent on the time, growth medium, and conditions of culture; climatic factors, soil composition, and pollution can influence the allergenicity of pollen extracts. In the same way, different raw material could be used for mite allergenic extracts: whole cultures of mites, including the nutrient medium and faecal particles, or purified mite bodies.
    - Extracts are standardized in terms of total allergenic activity to ensure that the overall potency of the preparations is constant. Complementarily, the content of one or two individual major allergens is often estimated, but extracts can also contain other important allergens with widely varying potencies and ratios.
    - Some extracts lack or contain low amounts of important allergens. 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 while some other allergens may be present in relatively small amounts that render them ineffective for diagnosis or therapy.
    - Extracts can be contaminated with unwanted materials or allergens from other sources: dog dander extract contaminated with house dust mite allergens or pollen extracts contaminated with mould allergens.
    - Relevant allergens may be instable and prone to degradation in the complex extract, particularly if proteases are present.
    - Proteases from mites, weed pollen, and fungal extracts have been involved in the degradation of bioactive peptides therefore the use of extracts containing these enzymes can exacerbate the overall bronchoconstrictive effect detected in asthmatic lungs (6).
- Cannot be tailored to the patient's sensitization profile and therefore may induce new sensitizations.
- Do not suit the international quality standards for vaccines since they cannot be compared between different products and batches or they do not allow a precise monitoring of the underlying treatment.

For these reasons, highly heterogeneous immune responses have been observed in patients undergoing SIT based on allergen extracts (7, 8). Early evidence that immunotherapy is effective was drawn from anecdotal reports from patients who received such therapy and the physicians who observed their responses. Since the 1950s, a number of randomized, double-blind, placebo-controlled trials of immunotherapy have been conducted to define the parameters that determine under what circumstances the procedure is effective. Most of those clinical trials showed a positive effect when therapy was given in a specific way.

Since there are many factors that can affect the outcomes of such trials, at this point the answer to whether immunotherapy is effective depends on how it is used. Nevertheless, with the application of recombinant DNA technology in the field of allergen characterization, pure recombinant allergens have become available for the formulation of defined and safer allergy vaccines (9, 10).

4. POTENTIAL BENEFITS AND DISADVANTAGES OF RECOMBINANT ALLERGENS

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 routine management of allergic disease (11-13). Some of the advantages and benefits that may be achieved with these preparations can be summarized as follows:

- Represent pure molecules with defined physicochemical and immunologic properties that can be modified to obtain advantageous characteristics. Special attention should be paid to the purity of the recombinant proteins in order to avoid product- and process-related impurities and adventitious contaminants. In comparison with natural allergen extracts, there is no risk that unrelated allergens, infectious agents, or irritants can contaminate a preparation.

- Standardization, product consistency, and declaration of the concentrations of the active pharmaceutical ingredients are easily achieved on the basis of mass units. They can fit the international quality standards for vaccines and be precisely compared to give consistent and reproducible products.

- Vaccines can be exactly tailored according to the patient's sensitization profile allowing a precise monitoring of SIT efficacy. Potencies and ratios can be exactly adjusted for each molecule and therefore, different allergens stemming from the same source can be mixed together in appropriate amounts 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.

One potential disadvantage of recombinant allergens can be that the IgE binding affinity towards them may be less than with respect to their native counterparts. This could be due to the existence of several isoallergens with different IgE binding properties or to expression problems caused for improper folding or lack of post-translational modifications (14). Although this fact is a pitfall for their use as diagnostic tools, it could also be an advantage in the search for a safer immunotherapy. The fact that many allergenic extracts contain multiple allergens can be another disadvantage because of the substantial resources that should be required to make commercially available all the clinically relevant recombinant allergens from a specific allergen source.

5. STRATEGIES FOR THE MODIFICATION OF RECOMBINANT ALLERGENS TO IMPROVE SIT

Modification of allergens to improve conventional SIT should try to achieve the production of molecules with strongly reduced allergenic activity (hypoallergens), while preserving structural motifs necessary for T-cell recognition (T-cell epitopes) and for induction of protective antibody responses that can antagonize IgE-mediated effects. Antibody-binding structures on allergens are mainly dependent on intact tertiary structure; in contrast, T-cells recognize linear peptides. B cell epitope binding to IgE are prerequisites for sensitization against the native allergen and elicitation of adverse reactions. Modified allergens, that lack IgE binding or effector cell degranulation capacities, might avoid the uptake of allergens by antigen-presenting cells (i.e. specific B cells) mediated by the interaction of conformational epitopes of the allergen with specific IgE which leads to higher Th2 cytokine and IgE production. These modified allergens preferentially use phagocytic or pinocytic antigen uptake mechanisms in dendritic cells, B cells, and macrophages independently of the allergen structural features inducing in this way a balanced Th0 or Th1-like cytokine production by T-cells, low IgE and high IgG production by B cells (15).

The most commonly used methods of chemical modification involve treatment with formaldehyde and glutaraldehyde (16). The resulting intra- and intermolecular cross-linking changes the three-dimensional structures of the proteins and consequently reduces the number of exposed IgE-binding epitopes. Several successful products (allergoids) derived by chemical modification of the extracts are commercialized on the European market. These products with reduced allergenicity can facilitate the administration of fewer but higher doses of allergen and, by reducing the side effects and length of treatment schedules, can increase patient compliance. The experience with these chemically modified preparations encouraged the idea of designing hypoallergenic vaccines based on recombinant proteins. Recombinant DNA technology provides the opportunity to use genetic engineering techniques to create such hypoallergenic variants. The advantages of this approach are that the new molecules can be precisely defined and the design features validated with respect to the specific immunotherapeutic application.

Several approaches are currently under development to overcome the problem of therapy-induced IgE-mediated anaphylactic side effects. They include the adsorption of allergen extracts to novel adjuvants to delay systemic release of allergens, the coupling of allergens to immunomodulatory DNA sequences, and the design of allergen-derived peptides or recombinant allergen derivatives with reduced allergenic activity.

5.1. Chemically modified allergens

The methods used to produce allergoids can be applied to recombinant allergens, but a disadvantage is that the end product cannot be defined in molecular terms.

Oligomerization produced by chemical modification of allergens seems either to destroy or to mask structural B-cell epitopes resulting in significantly reduced IgE binding activity while maintaining immunogenicity. Conjugation with synthetic immunoestimulatory CpG sequences induces Treg and Th1 immune response, thus may function as Th1-driving adjuvants, and may influence IgE reactivity interfering with cross-linking of Fc-epsilon-IgE receptors on mast cells and basophils (17).

5.2. Folding variants

Recently, a hypoallergenic folding variant of rBet v 1 has been described after a minor modification of the purification process of rBet v 1 (18). This folding variant, rBet v 1-FV, adopted a stable random coil structure and exhibited hypoallergenic properties as judged by immunoassay inhibition tests, basophil activation, and histamine release. T-cell reactivity was completely conserved and rBet v 1-FV showed strong immunogenicity in mice. First data of clinical trial are promising but final data on clinical efficacy will be important (19).

5.3. Genetically modified allergens

Genetic engineering allows targeted modifications of a protein in order to alter specific properties, such as allergenicity, in a predictable manner. Detailed information concerning IgE-binding epitopes, T-cell epitopes, and three-dimensional structure can help in the design of such molecules. Approaches for the alteration of a gene include changing specific base-pairs (site-directed mutants), introduction of a new fragment of DNA into the existing gene (chimeric or hybrid molecules, head-to-tail fusions), and deletions (9, 20).

5.3.1. Site-directed mutants

Construction of variants through mutations introduced by site-directed mutagenesis either directly in IgE-binding epitopes to compromise their activity, or 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; alteration of the surface charge in antigenic regions may modify folding and the ability to react with antibody. Several examples of these kind of hypoallergenic variants are been reviewed elsewhere (20): Lol p 5, Par j 1, latex Hev b 6.02 and Hev b 5, apple Mal d 1, egg Gal d 1, and peanut Ara h 1, Ara h 2, and Ara h 3, and mite Der p 2, Der f 2, and Lep d 2.

5.3.2. Deletion mutants

The deletion of short sequences DNA segments encoding for IgE-binding epitopes is another way for modifying allergen genes providing that T-cell epitopes are not disturbed. This approach was successfully used for the timothy grass pollen allergen Phl p 5a (21).

5.3.3. Allergen fragments

Disruption of the three-dimensional structure by fragmentation could be used to reduce the anaphylactic potential of allergens due to destruction of conformational IgE-binding epitopes. IgE recognition of continuous epitopes might also depend on their conformation, which might only occur in the context of the folder allergen molecule. Bet v 1 fragments showed reduced anaphylactic potential and induced protective IgG antibodies that inhibited allergen-induced release of inflammatory mediators (12).

5.3.4. Allergen oligomers

Recombinant oligomers are produced by linking several copies of a gene in sequence forming a homomer. Three linked copies of full-length Bet v 1 expressed in *E. coli* showed reduced anaphylactic potential but similar T-cell proliferation capacity, rabbit IgG blocking antibodies, and cytokine production upon stimulation of Bet v 1-specific T-cell clones than those of the monomer (12). However, recent results have concluded that oligomerization is not a universal strategy to reduce allergenicity as it has been found in the case of the Bet v 1 homologue, Dac g 1 (22).

5.3.5. Allergen fusions

An alternative approach consists of linking copies of different genes in sequence forming a heteromer. The fusion of Par j 1 and Par j 2 allergens from *Parietaria judaica* (fusion Q1, see below) showed hypoallergenic properties (23). On the other hand, the tetramer composed of Phl p 6–Phl p 2-Phl p 5–Phl p 1 appears to retain the IgE reactivity of the individual allergens while immunogenicity is enhanced (24). These results clearly indicate that oligomerization does not ensure hypoallergenic properties, thus the success of this technique is allergen dependent.

5.3.6. Chimeras: allergen hybrids and shuffled molecules

Hypoallergenic variants could also be created by gene rearrangement to produce modified protein sequences (25). Two rearrangement strategies may be used:

- Gene shuffling which consists in the fragmentation of two or more related genes and their random reassembly. The large number of new sequences necessitates intensive screening to identify candidate molecules (26).

- Cutting a gene at preselected points and reassembling before expression to create a ‘‘mosaic’’ protein. This approach can be extended by mixing gene fragments from different allergens (27).

Reduction of IgE reactivity by both approaches could be achieved by minimizing the number of epitopes or reducing the affinity of IgE antibodies towards these epitopes in order to avoid cross-linking of IgE receptors on effector cells. Nevertheless, the shuffled products should be able to influence the T-cell population of an allergic individual by either shifting the typical Th2 towards a Th1 response or by inducing T-cell tolerance.

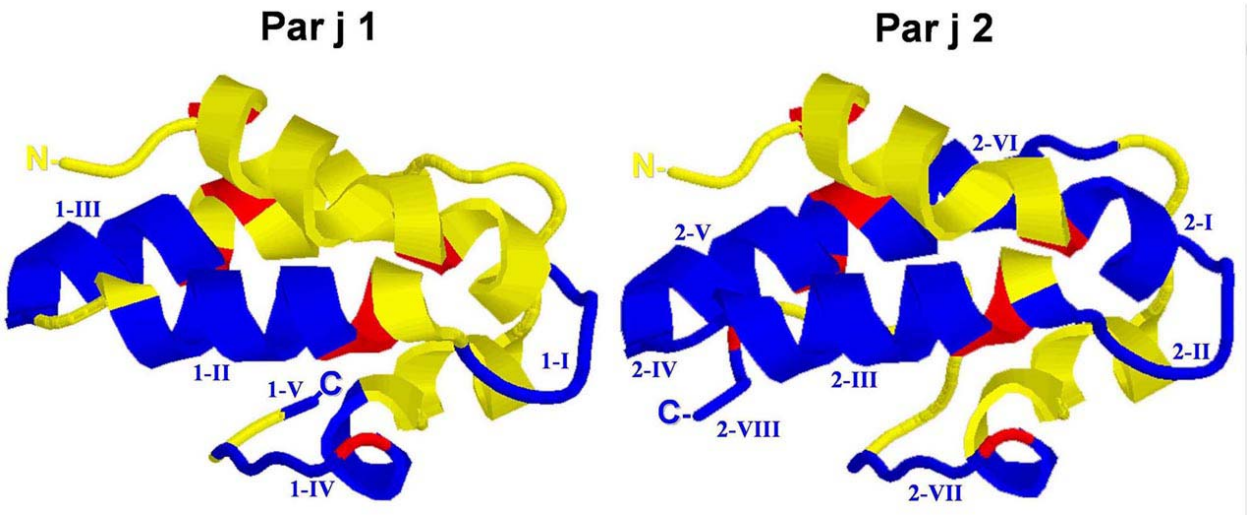

Figure 1. 3D model of Par j 1 y Par j 2. IgE antibody binding sites are marked in blue and cysteine residues involved in the four disulfide bridges are marked in red.

6. RECOMBINANT HYPOALLERGENS FROM *PARIETARIA JUDAICA*

Parietaria pollen is one of the main outdoor sources of allergens in the Mediterranean area but the presence of these weeds has also been described in Southern United Kingdom, temperate regions of Central and East Europe, Australia and California. (28). The prevalence of sensitization to *Parietaria judaica* pollen is 50% to 80%. The long time flowering season provokes almost perennial symptoms in allergic patients.

The composition of the *P. judaica* pollen allergenic extracts has been studied in detail and two major allergens (Par j 1 and Par j 2) have been described (29-30). They belong to a family of plant protein named 'non-specific lipid transfer protein' (ns-LTP) which folded in a characteristic alfa-alfa-alfa-alfa-beta structure that is stabilized by four disulfide bonds (31). Continuous and discontinuous IgE-epitopes from these allergens together are responsible for almost all of the IgE-binding activity in *P. judaica* pollen (32-34) (Figure 1).

Mixtures of a few recombinant allergens can reproduce the allergenic complexity of the natural extracts from which they come if they contain the most important IgE epitopes present in the extracts. Component resolved diagnostic can help to determine the convenience of the specific immunotherapy with whole allergen extracts. Thus, in the case of *P. judaica* pollen allergy, the major allergen rPar j 2 could de used as marker of real sensitization and two minor allergens (rPar j 3 and to rPar j 4) as markers of sensitization to not related allergen sources containing these cross-reacting allergens. Only patients who are sensitized to the major allergen Par j 2 should receive immunotherapy with wall pellitory pollen extracts, whereas patients without sensitization to Par j 2 will presumably not benefit from therapy with this pollen extract. The incorporation of the cross-reactive pollen allergens, profilin and calcium-binding protein, should allow identifying patients with broad cross-reactivities, who may be less suitable for SIT (35-38) (Figure 2).

6.1. Rationale

The first important step is the selection of the predominant allergen sources. Selection criteria may include the frequency of sensitization, the clinical relevance, the magnitude of IgE responses, and the extent to which IgE epitopes are represented in a given allergen source. One allergen could be suffice to achieve a substantial improvement in clinical symptoms when the major allergen dominates and accounts for most or all of the specific IgE response directed against the sensitizing agent. Based on that, we chose *P. judaica* pollen allergy model since it is basically a two component allergenic system and the mixture of both Par j 1 and Par j 2 allergens is able to inhibit up to 95% of the total IgE-binding activity of the *P. judaica* pollen extract (31). This fact suggests that the two minor and cross-reactive allergens, profilin (Par j 3) and calcium-binding protein (Par j 4) which have been recently described in the *Parietaria* pollen are remarkably less important from a clinical point of view (35, 36). Although Par j 1 and Par j 2 share similar IgE epitopes, the inclusion of Par j 1 and Par j 2 in a single hybrid molecule would extend the T-cell repertoire and induce strong protective antibody responses, as described for grass pollen hybrids (37). The use of hybrid technology can overcome the problems of low representation and poor immunogenicity of certain allergens as well as simplify production and registration procedures (24).

6.2. Design

Three hybrid proteins were constructed based on the two major allergens of *P. judaica* pollen. All DNA constructs were cloned in the expression plasmid pQE-32 (Qiagen) and transformed into *E. coli* M-15 (Figure 3). The

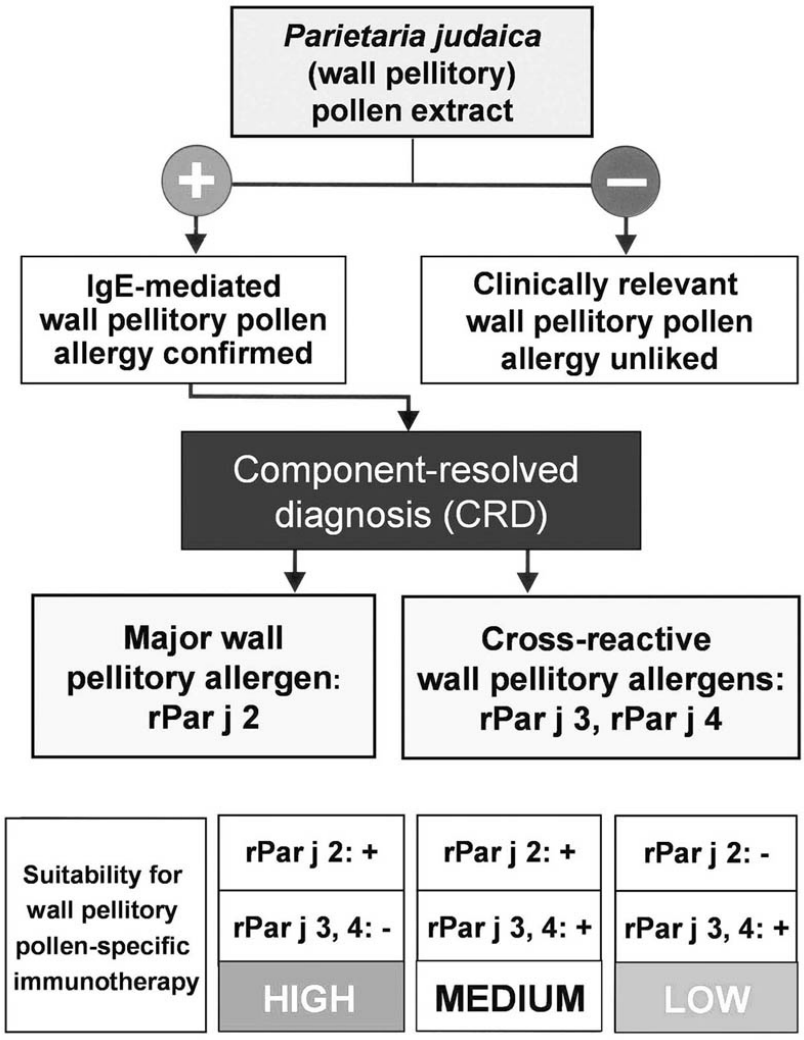

Figure 2. Decision tree for the treatment of *P. judaica* (wall pellitory) pollen allergy based on serum specific IgE.

first hybrid, called Q1, is composed of the whole sequences of both major allergens resulting in a cDNA construct with Par j 1 at the N-terminus and Par j 2 at the C-terminus. This protein was expressed as 32 kDa His-tagged fusion protein with a final yield of 2 mg of soluble protein per litre of bacterial culture. The other two hybrid proteins are deletion mutants of Q1 to remove IgE-binding epitopes: Q2 lacks the stretches from 29 to 52 residues in both components, whereas in Q3, two additional short stretches of 8 residues (72-79 in Par j 1 and 73-80 in Par j 2) were deleted. Q2 and Q3 hybrid proteins were expressed as 28 kDa His-tagged fusion proteins with a final yield of 5 and 7 mg/L culture, respectively.

Due to the fact that these fusion proteins contained a high number of cysteine residues it could be predicted that in Q1 the proportion of native-like folded protein would drastically decrease and subsequently the IgE binding activity should be reduced. Secondary structure elements of the three hybrid proteins were analyzed by CD spectroscopy showing typical spectra of proteins with random coil conformation, very different from that of the natural allergens, and therefore concluding that hybrid proteins have an almost completely unfolded state (23). In addition, in Q2 and Q3 several IgE-binding epitopes have been deleted.

6.3. Reduced IgE binding and allergenic activity of recombinant hybrid proteins

The reduced IgE reactivity of the recombinant hybrids was demonstrated by *in vitro* techniques including Western blotting, specific IgE, and EAST-inhibition, and by *in vivo* methods such as skin testing.

6.3.1. Western blotting

Western blotting with a pool of sera from wall pellitory pollen-allergic patients showed only IgE binding to the purified natural allergens nPar j 1 and nPar j 2 and to the recombinant hybrid Q1 (Figure 4). Recombinant hybrid Q2 is slightly recognized by IgE whereas Q3 has no IgE

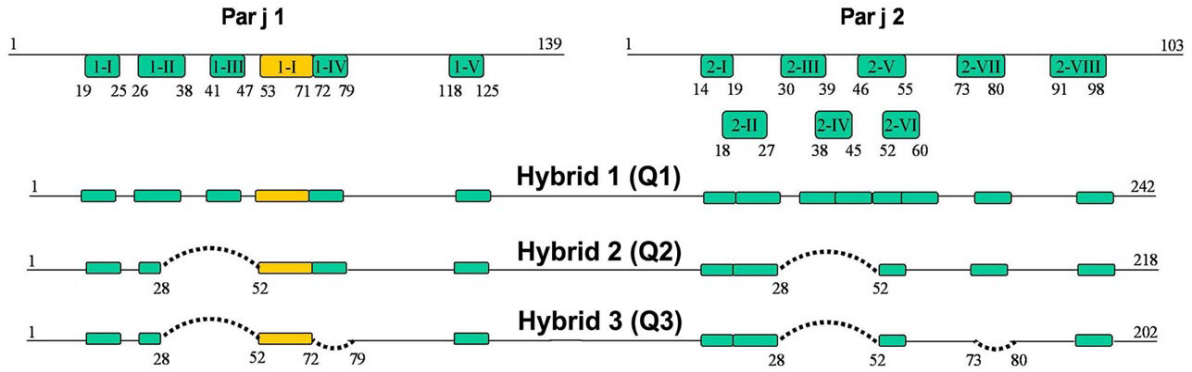

Figure 3. Schematic construction of hybrid proteins. Identified T-cell epitope regions and putative IgE-binding epitope regions were marked in orange and green, respectively. Dashed lines showed deleted stretches.

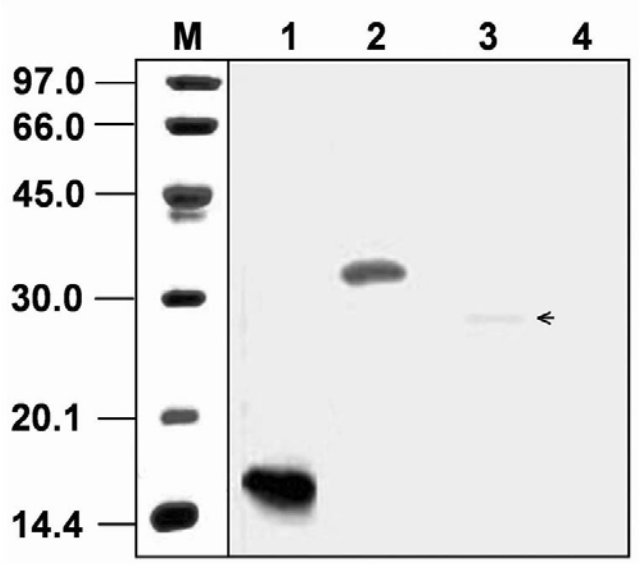

Figure 4. IgE Western blotting of natural Par j 1 and Par j 2 (lane 1), Q1 (lane 2), Q2 (lane 3) and Q3 (lane 4) incubated with the serum pool of wall pellitory pollen-allergic patients. Arrowhead indicated IgE reacting band of Q2.

binding capacity. The deletions introduced in the other recombinant hybrids (Q2 and Q3) affected drastically the IgE binding capacity of these proteins, since under the denaturated conditions of the electrophoresis, conformational IgE epitopes are less important for IgE binding.

6.3.2. IgE-binding activity

The capacity of human IgE to bind the hybrid was also tested by EAST with individual sera from allergic patients (Figure 5). Fusion Q1 showed lower IgE-binding capacity than nPar j 1 and Par j 2. In the case of deletion mutants, serum specific IgE showed almost none IgE-binding activity compared with the natural allergens. When comparing with the whole extract, 29 out of 30 patients sera showed lower IgE reactivity to hybrid Q2 and Q3 ($P<0.001$).

ELISA inhibition experiments using the pool of sera confirmed the reduced ability of the equimolecular mixture of rPar j 1 and rPar j 2 and the three hybrid proteins to inhibit IgE binding to the whole extract in patient sera (Figure 6). It was required 80 fold higher concentration hybrid protein Q1 than natural allergens to reach 50% of IgE-binding activity inhibition to the *P. judaica* pollen extract while 1500 fold higher concentration is necessary in the case of Q2 and Q3 hybrids. In no case, hybrid proteins reached the IgE inhibition obtained with the natural allergens (Par j 1 and Par j 2). These results suggested that continuous epitopes deleted in Q2 and Q3 are immunodominant epitopes in the tested patients.

6.3.3. Skin prick reactivity

The hypoallergenic nature of the recombinant hybrids was investigated *in vivo* by skin prick testing in 30 *P. judaica* pollen allergic patients (Figure 7). While the *P. judaica* extract and purified natural allergens, at 5 and 50 microg/mL, reacted positively to all the patients tested, only 16.7% and 86.7% of the patients had a positive SPT response (>7 mm^2) to fusion Q1 at the same concentrations.

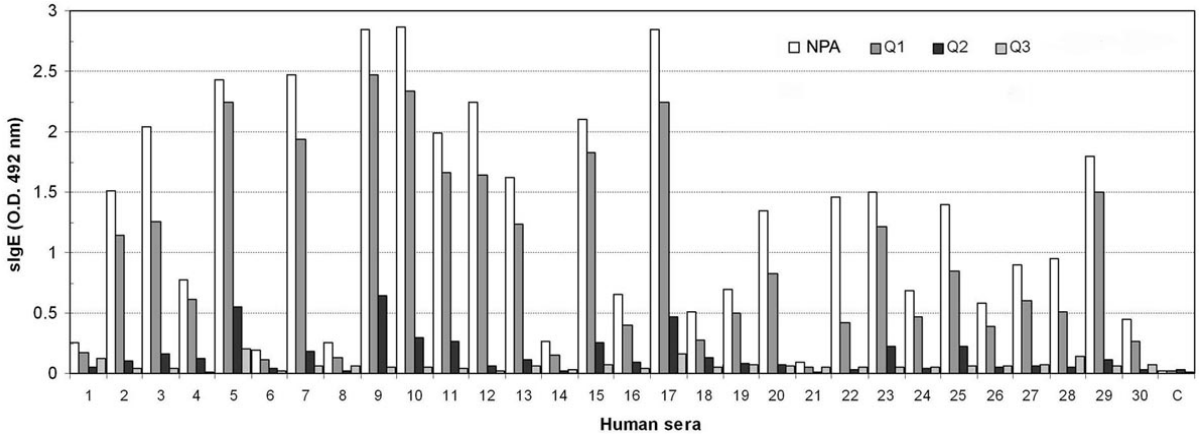

Figure 5. Specific IgE to natural purified allergens (NPA) and recombinant hybrids using individual sera from wall pellitory pollen-allergic patients (dilution 1/10) (cut-off value $O.D._{492\ nm}$ 0.055).

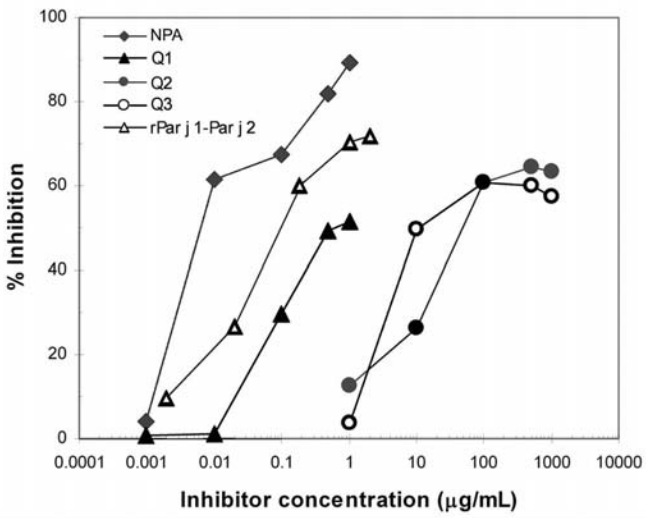

Figure 6. Binding of human IgE from the serum pool to *P. judaica* pollen extract was inhibited by natural purified allergens (NPA), equimolecular mixture of rPar j 1 and rPar j 2 (rPar j 1-Par j 2), and Q1, Q2 and Q3 hybrid proteins. Values represent the mean of triplicate measurements.

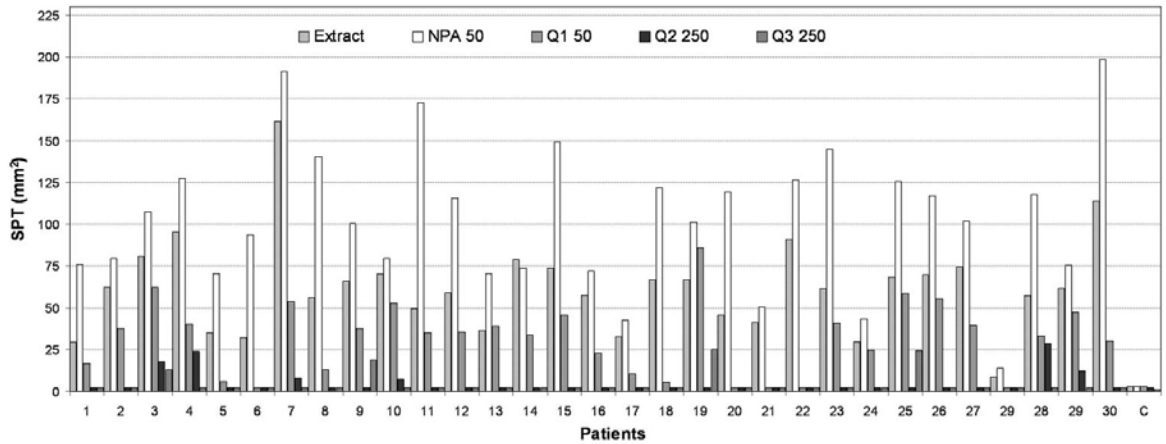

Figure 7. Cutaneous reactivity of 30 wall pellitory pollen-allergic patients to with the whole *P. judaica* pollen extract (12 microg/mL), natural purified proteins (NPA, 50 microg/mL), Q1 (50 microg//mL), Q2 (250 microg/mL) and Q3 (250 microg/mL). Values represent the mean of wheal areas on both arms.

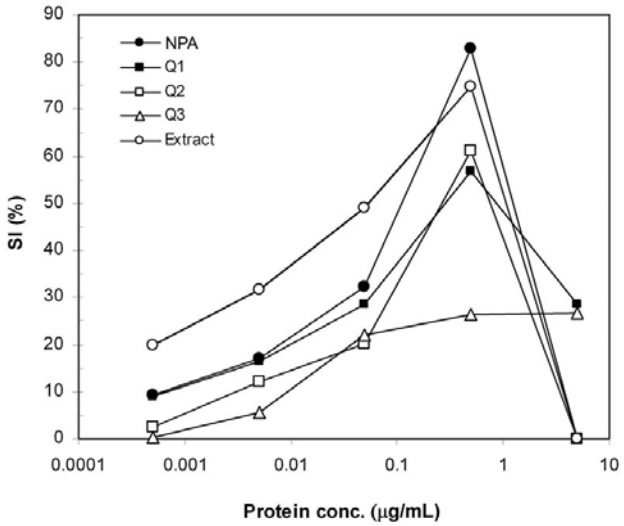

Figure 8. Doses-response curves of PBMCs proliferation. PBMCs from individual patients were stimulated with *P. judaica* pollen extract, natural allergens, and Q1, Q2 and Q3 hybrids at different concentrations. Medians of individual values are given as means of triplicate stimulation index percentages (SI%).

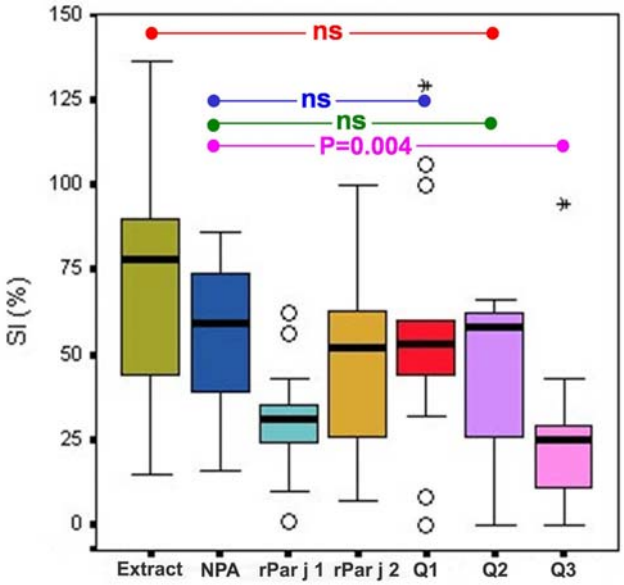

Figure 9. Induction of PBMCs proliferation. PBMCs from 13 individual patients were stimulated with pollen extract, natural allergens (NPA), rPar j 1, rPar j 2, and hybrids Q1, Q2 and Q3 at 0.5 µg/ml. Medians of individual values are given as means of triplicate stimulation index percentages (SI%).

Q2 and Q3 were able to elicit positive cutaneous responses at the higher concentration tested (250 microg/mL) in 20% and 13.3% of the patients and these positive responses were of lower intensity than those observed with the natural allergens (P<0.001). No positive reaction was observed in control subjects, either to the extract or to the purified allergens indicating the high specificity of the test, and no adverse side effects were observed in all the subjects tested.

6.4. T-cell reactivity of recombinant hybrid proteins

The maintenance of the antigenicity (T epitopes) is an essential requirement for the use of hypoallergens in SIT. In order to investigate the T-cell stimulating capacity of the hybrid proteins, specific proliferation of PBMCs from 13 *P. judaica* pollen allergic patients were analysed at different stimulating concentrations showing than 0.5 µg/ml was the most stimulating allergen concentration (Figure 8). Statistical analysis showed that the wall pellitory pollen extract, used as control, displayed a stimulating capacity similar to that obtained by the mixture of the purified major allergens, nPar j 1-Par j 2 (Figure 9). As can be expected, Q1, composed of the complete

sequences of Par j 1 and Par j 2, was able to induce proliferation to an extent similar to the whole extract and the purified major allergens. Q2 induced a similar proliferation suggesting that in this deletion hybrid the majority of Par j 1 and Par j 2 T-cell repertoire was retained. On the contrary, recombinant Q3 showed much lower allergen-specific activation capacity, suggesting that the deletion of the sequences had disrupted putative T-cell epitopes.

7. CONCLUSIONS

Considering their reduced anaphylactogenic potentials together with their conserved T-cell reactivities, genetically engineered molecules Q1 and Q2 could be used as a vaccine prototype in safe and shortened schedules of allergen-specific immunotherapy against *P. judaica* pollen allergy.

8. FUTURE PERSPECTIVES

Recently, the first clinical trial using a modified recombinant allergen preparation has been performed using Bet v 1 fragments and Bet v 1 trimer for vaccination of 124 birch pollen-allergic patients in a double-blind, placebo-controlled study (12). Active treatment induced protective IgG antibodies that inhibited allergen-induced release of inflammatory mediators. A reduction of cutaneous sensitivity and an improvement of symptoms in actively treated patients were also reported. In addition, increases of allergen-specific IgE induced by seasonal birch pollen exposure were significantly reduced in vaccinated patients.

According to these and other recent results obtained with recombinant allergen–based vaccines for birch and grass pollen allergy (11, 13), it is likely that SIT can be developed based on recombinant wild-type allergens and hypoallergenic derivatives for the most common allergen sources. Future work will have to focus on the identification of the relevant allergens in these allergen sources, the preclinical characterization and selection of the candidate vaccines, and the rational design of immunotherapy studies for measuring clinical efficacy. Furthermore, the use of defined molecules instead of whole allergen extracts will allow to investigate more precisely the mechanisms underlying immunotherapy and to develop new forms of immunotherapy.

9. ACKNOWLEDGEMENTS

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