

EDITORIAL

NOVEL STRATEGIES FOR THE DEVELOPMENT OF A VACCINE FOR *PARIETARIA* ALLERGY

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Specific immunotherapy is a well established and clinically proved strategy to cure allergic reactions. The impressive boost of knowledge derived from DNA recombinant technology applied to this field allowed the identification, cloning and expression of several clinically relevant allergens. Recombinant allergens can be easily produced in a pure and reproducible way with immunological properties comparable to natural allergens and matching the requirements of pharmaceutical companies. *Parietaria* pollinosis is a major health problem in the Mediterranean basin with prolonged symptoms. In this review we will discuss the rational approaches to design hypoallergenic derivatives of the major allergens of this pollen, their immunological properties and possible clinical future implications.

Allergic diseases are global health problems affecting more than 25% of the population living in the industrialized countries (1) with a worldwide increase in the prevalence of asthma and allergic rhinitis since 1960 (2).

With the introduction of DNA recombinant technologies in the field of allergen characterization, it became possible to identify the allergenic repertoire of several species, and the immunological characterization of pure recombinant allergens made possible the commercialization of tests with recombinant allergens for routine diagnosis of allergy and for monitoring the effects of SIT. In addition, clinical trials with recombinant allergens (3-4) were successful and proved the clinical efficacy of these molecules. Historically, the first immunotherapy trials with recombinant allergens was performed with genetically modified allergens with hypoallergenic activity proving that this approach has also been clinically effective (5). To date, several strategies

have been proposed to generate hypoallergenic derivative as summarized in Table I.

Parietaria is a genus of dicotyledonous weeds belonging to the Urticaceae family which is composed of several cross-reactive allergenic species which represents a common cause of pollinosis in the Mediterranean area (6-7). Almost 30% of all the allergic subjects in southern Europe present a Skin Prick Test (SPT) reactivity for *Parietaria* pollen and more than 50% of these subjects have experienced asthma with severe bronchial hyper-responsiveness (8). The extraordinarily long persistence in the atmosphere of the *Parietaria* pollen is responsible for prolonged symptoms in the Mediterranean area. In some areas, like southern Italy, some patients have year-long symptoms, therefore the development of new drugs for an improved and safer Specific Immunotherapy (SIT) is needed.

Parietaria pollen is a well-suited allergy model since it is composed of two major components (Par

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j 1 and Par j 2) which are responsible for the major clinical symptoms inducing an IgE production in almost all the Pj allergic patients (6-9-10) and inhibiting up to 95% of the total IgE binding activity of the *Parietaria* extract (11). To date, the Par j 2 allergen has been a well established marker allergen for the diagnosis of genuine sensitized *Parietaria* allergy (12). Moreover, using purified recombinant Par j 1 and Par j 2 allergens, it has been shown that these molecules display an IgE repertoire common to the *Parietaria* species (13) so they can be used as molecules for diagnostic and therapeutic uses of all the components of the *Urticaceae* family.

Minor cross-reactive allergens (Par j 3 and Par j 4) have also been isolated, showing a very low incidence of sensitization in geographical areas with a high load of *Parietaria* pollen (14-15).

Extract-based SIT used in *Parietaria* allergy vaccination presents several major disadvantages. In general terms, extracts are usually poorly characterized mixtures containing non-allergenic and allergenic components whose concentration is not easily assessable in terms of absolute concentration of allergens and relative quantification of major and minor allergens. In addition, it has been demonstrated that pollen extracts contain pro-inflammatory substances acting as adjuvants (16-17) and proteolytic activities which can alter the epithelial integrity contributing to the sensitization and pathogenesis of allergy. In particular, *P. judaica* pollen extract contains several elements involved in the degradation of bioactive peptides (18-19) and other non-peptide antigens with immunomodulatory activity (20), suggesting that these components, contained in the extract, can skew the immune response towards a Th2 phenotype.

Starting from these observations, there have been a few attempts to design hypoallergenic derivatives of the Par j 1 and Par j 2 major allergens by means of genetic engineering. In this review we will discuss their structures, immunological properties and possible clinical future implications.

Rationale

Parietaria major allergens belong to the Lipid Transfer Protein (LTP) family, a wide distributed group of Pathogens-Related proteins (PR-14), characterized by a highly conserved three-

dimensional structure with four α -helices and eight cysteine residues forming 4 disulphide bonds according the invariant order Cys4-Cys52, Cys14-Cys29, Cys30-Cys75 and Cys50-91. 3D modelling by homology (21) and enzymatic digestion (22) have shown that the Par j 1 and Par j 2 allergens attain a three-dimensional structure consistent with that of the LTPs. This family of proteins is characterized by a high resistance to pepsin digestion and heat denaturation (7). Such a stability and biochemical properties have suggested to be an important factor in determining possible systemic reactions during the course of SIT (23), therefore the design of engineered hypoallergenic derivative is one of the possible options for safer therapeutic products.

The knowledge of the three-dimensional structure of these allergens represents the scientific background for a rational approach to the design of hypoallergenic derivatives. *In silico* studies demonstrated that the amino acid lining between the helices 1 and 2 contains a cluster of amino acid residues within the region presenting side chains exposed to the solvent surface and containing the properties to form epitopes (21-24) (Fig. 1). *In vitro* assays performed by means of site-specific mutagenesis together with the use of reducing conditions and gene fragmentation demonstrated the cogency of this prediction allowing the mapping of an immuno-dominant IgE epitope in the region between amino-acids 1 and 30 whose conformational structure is stabilized by the Cys14-Cys29 disulphide bond (21).

Disulphide bond variants

On the basis of the detailed knowledge of the IgE-binding regions of the Par j 1 and Par j 2 allergens (21-24), Bonura and co-workers studied the correlation between the disulphide bond pairing and the binding to the IgE antibodies from Pj allergic patients. To address this question, serine to cysteine substitutions in positions 4, 29, 30, 50 and 52 of the Par j 1 allergen were performed. Using this strategy, the Authors were able to generate a family of four independent mutants: rPjA mutant lacking the Cys14-Cys29 and Cys30-Cys75 bridges; rPjB mutant lacking the Cys50-Cys91 and Cys4-Cys52 bridges; rPjC mutant lacking the Cys14-Cys29, Cys30-Cys75 and Cys4-Cys52 bridges; rPjD mutant

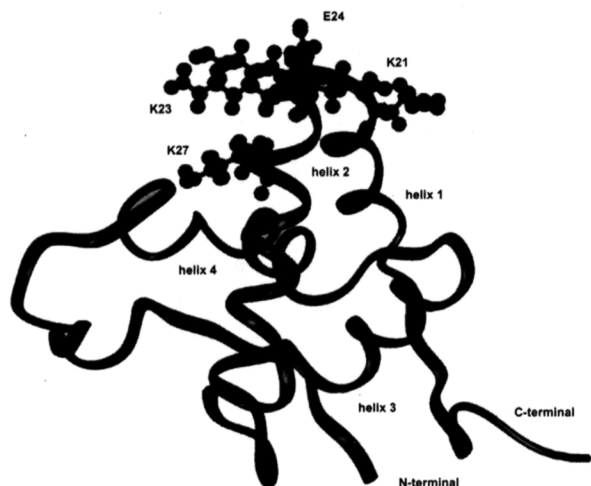


Fig. 1. Solid ribbon structures represent the 3D model of Par j 1 allergen. Side chains composing the immunodominant IgE epitope in the NH₂-terminal region of the Par j 1 are shown in ball and stick and labelled. The amino-acids involved in the IgE binding are indicated in one letter code and numbers indicate the position in the primary sequence.

lacking all the disulphide bridges (Fig. 2) (25). These derivatives were expressed in *E. coli* as recombinant proteins and their immunological properties were studied using independent techniques. *In vivo* and *in vitro* analysis showed that IgE binding is dependent on the cysteine pairing. In particular, site-directed mutagenesis showed that the disruption of the Cys14-Cys29 and Cys30-Cys75 bridges dramatically affected the binding to human IgE (rPjA). On the other hand, the loss of the Cys50-Cys91 and Cys4-Cys52 bridges seems to have a minimal effect since a strong IgE binding activity and a positive SPT are still present. This strategy of mutagenesis did not affect the T-cell recognition since proliferation assays performed with PBMC from Pj allergic patients showed that all the derivatives retained T cell reactivity (25).

The immunological properties of one of these mutants were further investigated in a murine model of IgE sensitization. In this paper, the Authors initially studied the ability of a conformational mutant of the Par j 1 (rPjC) (Fig. 2) to be recognized by murine rPar j 1-specific IgE (26). The IgE binding

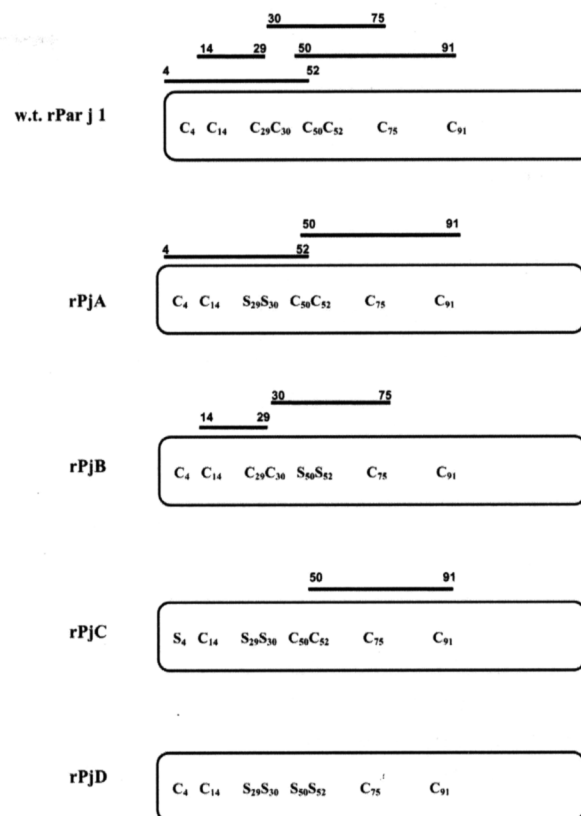


Fig. 2. Schematic representation of the wild-type rPar j 1 allergen and its four disulfide bond variants: PjA (lacking Cys14-Cys29 and Cys30-Cys75 bonds), PjB (lacking Cys50-Cys91 and Cys4-Cys52 bonds), PjC (lacking Cys4-Cys52, Cys14-Cys29 and Cys30-Cys75 bonds) and PjD (lacking Cys4-Cys52, Cys14-Cys29, Cys50-Cys91 and Cys30-Cys75 bonds). C indicates cysteine residues and numbers indicate their positions on the wild-type sequences; S indicates Ser→Cys substitutions. Bars indicate the presence of disulphide bridges according to the order: Cys4-Cys52, Cys14-Cys29, Cys30-Cys75, Cys50-Cys91.

of this hypoallergenic derivative was significantly reduced, mimicking the results described by Bonura and co-workers with human IgE antibodies (25). On the other hand, the ability of the rPjC to bind murine serum Par j 1-induced IgG was not affected by the changes in the three-dimensional structure of the allergen. Therefore, the ability of the rPjC derivative to induce an immune response was evaluated, showing that the i.p. injection in mice induced a poor IgE response when compared to that

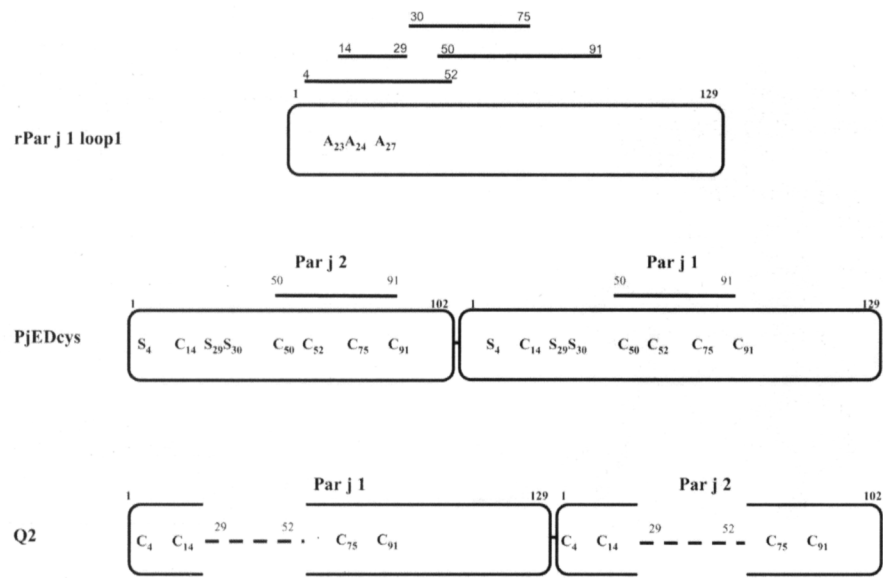


Fig. 3. Schematic structure of the engineered Par j 1 and Par j 2 allergens. Bars indicate the presence of disulphide bridges according to the order: Cys4-Cys52, Cys14-Cys29, Cys30-Cys75, Cys50-Cys91. Numbers indicate the amino-acid positions on the wild-type sequence; boxes show the coding regions of the Parj1 and Parj2 allergens; A and S indicate the positions of the mutated residues; dashed lines show deleted stretches.

Table I. Strategies for the generation of hypoallergenic derivatives.

STRATEGY	RATIONALE	KEY PROPERTIES
Oligomers	altered presentation of IgE epitopes	maintained IgE binding activity but reduced anaphylactic activity
Mutants	site specific mutagenesis of IgE epitopes	reduced or absent anaphylactic activity
Mosaic	disruption on the 3D structure	reduced or absent anaphylactic activity
Carrier-bound allergen-derived peptides	fusion to non allergenic proteins	no IgE and no allergen-specific T-cell reactivity
Folding variants	disruption of the natural folding	Absence of anaphylactic activity
T cell peptides	use of synthetic peptides	no IgE binding activity
Fragments	disruption of the 3D structure	reduced or absent anaphylactic activity

evoked by the rPar j 1. Furthermore, when spleen cells from mice immunized with the w.t. allergen and with the rPjC mutant were studied, the Authors did not report any difference in the stimulation indexes providing evidence that the alteration of the three-dimensional structure of the Par j 1 allergen did not affect the epitopes required for T-cell activation and IgG induction (26). This kind of approach seems to be particularly powerful since it is not related to the sequences of the IgE epitopes but is based on the modification of specific structural domains and, for this reason, is feasible for all the proteins with allergenic activity belonging to the LTP family. This observation was clearly demonstrated by Bonura and co-workers using the Par j 2 allergen as a model (11).

Epitope deletion

Previous studies have shown the presence of an immunodominant IgE epitope in the first 30 aa (loop 1 region) of the Par j 1 allergen. Site specific mutagenesis allowed the identification of the amino acids involved in the IgE recognition (21). Following this observation, Colombo performed a PCR-based strategy of mutagenesis by substitution of the residues K23, E24 and K27 with alanine residues (rPar j 1 loop1) to study the contribution of these aminoacids on the overall allergenicity of the molecule (Fig. 3). This strategy allowed the characterization of a derivative with a reduced IgE binding activity in all the tested patients. Furthermore, the anaphylactic activity of this derivative was tested *in vitro* and *in vivo*. By means of basophil histamine release and skin prick test, the Author found that the Par j 1 loop1 showed reduced allergenic activity. When the anaphylactic activity the rPar j 1 loop1 mutant was compared to that one of the rPar j 1, a more than ten-fold reduction of release was reported in four out of six patients. These *in vitro* observations were consistent with the *in vivo* data where the Author demonstrated a reduced capability of the Par j 1 loop1 of inducing type I hypersensitive response in three out of four patients. Finally, a proliferation assay performed with PBMC from Pj-allergic patients showed that the derivative displayed retained T-cell reactivity (27).

This approach based on the analysis of the amino acid composition of the loop1 region of the

Parietaria LTPs may also represent a more general strategy for other components of the same protein family. In fact, the immunological relevance of the region between helices 1-2 has been reported to be relevant in other allergenic LTPs, suggesting that this region can be considered as a hot spot for the generation of hypoallergenic LTPs (28).

Hybrid

Hybrid allergenic molecules are proteins consisting of multiple allergens (either omo- or etero-multimers) fused in an engineered single molecule. The advantage of this strategy has been shown by a few reports where oligomerization induced immunological changes such as reduced allergenicity (29-30) and increased immunogenicity (31). Despite of their common evolutionary origin, Par j 1 and Par j 2 allergens display some cross-reactive and independent IgE epitopes (10), therefore the generation of a single protein comprising the key offending allergens and containing most of the B-cell and T-cell epitopes against *Parietaria* pollen in one single pharmaceutical product may reduce production costs while providing a broad usage.

A head to tail dimer expressing two disulphide bond variants of the major *Parietaria* allergens (Par j 1 and Par j 2 allergens both mutated in cysteine residues at positions 4, 29 and 30, respectively) was generated using a PCR based strategy and expressed in *E. coli* (PjEDcys) (Fig. 3).

ELISA inhibition and immunoblot experiments performed with sera from allergic patients showed that the PjEDcys derivative displayed a marked reduced capability of binding human IgE and a dramatic reduction in the anaphylactic activity (100-1000-fold reduction). These data were confirmed *in vivo* by SPT demonstrating that the disruption of some key disulphide bonds on both allergens profoundly changed the allergenic properties of these molecules. When the T-cell proliferative response was investigated, the Authors reported retained T-cell reactivity.

The immunogenicity of the hypoallergenic hybrid was studied *in vivo*, looking at the pattern of antibody production in a mouse model of sensitisation. BALB/c mice were i.p. immunized with the hybrid and the induced antibodies were studied for their ability to bind the natural major allergens of the

Parietaria pollen. Despite the changes introduced by mutagenesis, the rPjEDcys derivative was able to induce a strong IgG response towards the Par j 1 and Par j 2 allergens. In addition, ELISA inhibition and Western blot experiments performed with the *Parietaria* pollen extract demonstrated that the rPjEDcys-induced IgG antibodies were able to recognize the natural allergens, suggesting that an immunization protocol performed with the recombinant hybrid can induce an antibody response capable of inhibiting IgE-mediated presentation, blocking basophil histamine release and reducing seasonal IgE production and allergic symptoms.

A similar strategy was suggested by Gonzales-Rioja and co-workers which generated hybrid proteins using the 2 major allergens of the *P. judaica* pollen. The Authors designed two hybrid proteins merging deletion mutants of the wild type Par j 1 and Par j 2 allergens. In particular, the Q2 hybrid lacks the stretches from residues 29 to 52 in both allergens (Fig. 3). In this way, the Authors deleted the region containing 4 cysteines residues (Cys 29, Cys 30, Cys 50 and Cys 52) abrogating the possibility of forming any disulfide bridge and deleting the IgE immunodominant epitope described by Colombo and co-workers (21). The hybrid was expressed in *E. coli* as a 28-kd His-tagged fusion protein and the secondary structure was analyzed by means of Circular Dichroism spectroscopy. The spectra of the hybrid protein were totally different from the natural allergens showing a complete unfolded state (32).

This hybrid molecule displayed an almost complete reduction of *in vitro* IgE reactivity and a highly reduced *in vivo* activity. On the other hand, T-cell reactivity of this hybrid molecule was analyzed by means of stimulation of PBMC to ensure that the majority of the Par j 1 and Par j 2 T-cell repertoire was retained. The Q2 derivative was able to induce T-cell proliferation to an extent similar to the whole extract and natural purified *Parietaria* major allergens.

CONCLUSION

The use of defined molecules instead of crude allergen extracts opened the way to a more precise knowledge of the sensitization profile of allergic patients (component resolved diagnosis) (33), allowing the precise definition of the molecules

responsible for the induction of the allergic symptoms and introducing the concept of new forms of recombinant allergen-based vaccines. Recently, the first results from immunotherapy trials with recombinant allergens (3-4) or biotechnology-based derivatives (5-34) have begun to become available and a first drug based on a recombinant hypoallergenic folding variant of the major birch pollen allergen is expected to come onto the market for routine SIT. The commercialization of these highly standardized forms of allergy therapeutics requires more clinical studies to confirm the advantages of this new form of immunotherapy and to increase our knowledge about the immunological mechanisms behind this treatment. From this point of view, the *Parietaria* pollen is an intriguing model for at least two reasons: first of all, because the Pj pollen contains only few proteins of clinical relevance, therefore the production of a vaccine useful for all Pj allergic patients is technically affordable; secondly, because all the strategies described in this paper refer to approaches that can be exported to other biological systems with allergenic LTPs, suggesting that *Parietaria* pollen may represent a model system to study. In fact, allergenic LTPs have been isolated in other pollen- and plant-derived foods (35). The most frequently implicated foods belong to the Rosaceae fruits but allergenic LTPs have also been detected in tree nuts, peanut, beer, maize, mustard, asparagus, grapes, cabbage, dates, orange, fig, kiwi, lupine, fennel, celery, tomato, eggplant, lettuce, chestnut and pineapple. Because of their stable structure and resistance to proteolytic digestion and heat treatment, strategies leading to the generation of hypoallergenic LTPs represent a goal of paramount importance, opening the way to standardized new forms of allergen-specific immunotherapy.

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