

Hypoallergenic Variants of the *Parietaria judaica* Major Allergen Par j 1: In vitro and in vivo Evaluation

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Introduction

Type I immediate hypersensitivity is a genetic immune disorder affecting almost 20% of the population living in the industrialized countries leading to production of IgE against harmless substances. In particular, pollen grains of trees, grasses and weeds contain a number of water-soluble proteins that, in contact with the respiratory mucosa, release a number of allergens capable of inducing immediate symptoms of allergy due to allergen-induced cross-linking of mast cell-bound IgE (asthma, rhinitis and anaphylactic reactions) and late symptoms due to T cell activation and subsequent production of proinflammatory cytokines and the recruitment of eosinophils and macrophages. IgE antibodies have also been involved in the uptake and presentation of allergens on specific Th2 response (1). While immediate and late symptoms can be tackled by pharmacological therapies, allergen-specific immunotherapy (SIT) has been shown to be the only curative treatment effective in many clinical trials (2). However, SIT is actually performed with crude extracts which are a mixture of several pollen proteins whose allergenic composition is difficult to standardize. In addition, the administration of immunologically efficient concentration of allergen is often not feasible because of non-standardized extracts and, anyway, can lead to very dangerous life-threatening systemic reactions. In addition, the extract-based SIT can induce sensitization towards new allergens (3,4). From this point of view, the production of recombinant pure hypoallergenic derivatives of allergen may represent an improvement of great importance leading to a tailored specific immunotherapy.

Parietaria pollen allergens are multivalent glycoproteins derived from dicotyledonous weeds of the *Urticaceae* family including *Parietaria judaica* (*Pj*), *P. officinalis* (*Po*), *P. lusitanica*, *P. cretica* and *P. mauritanica*. Immunologically, *Pj* and *Po* represent the most relevant species and their pollens represent one of the main outdoor sources of allergens in the Mediterranean area (5). The composition of the allergenic extracts of the *Pj*

pollen has been extensively characterized. The two major allergens, Par j 1 and Par j 2, with molecular weights of 11.344 Da and 11.344 Da, respectively, are specific lipid transfer proteins. Their structure, defining the allergenic epitopes, is wound around an alpha-helix. Par j 1 and Par j 2 represent the two

Here we describe the production of hypoallergenic derivatives of Par j 1 and improved SIT.

Materials and Methods

Site-directed mutagenesis of Par j 1 variants

The Par j 1 mutagenesis was performed by site-specific mutagenesis using the Par j 1 forward and loop 2 forward sequence as a template. BamHI and PstI restriction sites were introduced by the same enzymatic reaction. HindIII restriction sites were introduced by the same restriction enzyme. The open reading frame was

Table 1. Primers used for the site-directed mutagenesis of the Par j 1.0101 nucleotide sequence to create the restriction sites.

Par j 1 loop 1 Forward

Par j 1 loop 1 Reverse

Par j 1 loop 2 Forward

Par j 1 loop 2 Reverse

The coding sequence of the pQE30 vector was amplified by PCR using the amino-terminal and carboxyl-terminal primers (Pharmacia). SDS-PAGE analysis of the proteins.

pollen has been extensively studied (6) and by using DNA recombinant technology, the two major allergens (Par j 1 and Par j 2) (7,8) have been cloned and immunologically characterized. The Par j 1 and Par j 2 allergens are two small glycoproteins of 14.400 and 11.344 Da, respectively, belonging to the same family of plant proteins named non-specific lipid transfer protein (ns-LTP) characterized by a remarkable homogeneity of structure, defining a class of folds composed by four α -helices and a C-terminal region wound around an internal cavity (9,10). Despite of their three-dimensional homologies, Par j 1 and Par j 2 allergens show an independent pattern of IgE recognition (8) and represent the two major allergens of the *Pj* pollen (7,11).

Here we describe a Polymerase Chain Reaction (PCR)-based approach to design hypoallergenic derivatives of the Par j 1 allergen to develop new strategies for improved SIT.

Materials and Methods

Site-directed mutagenesis, expression and purification of the wild type Par j 1 and its variants

The Par j 1 mutants were generated by alanine substitution using a PCR strategy of site-specific mutagenesis. Loop 1 and loop 2 mutants were generated using the loop 1 and loop 2 forward and reverse primers (see Table 1 for details) and using the Par j 1 sequence as a template (12). The loop 1 fragment was purified, digested with the BamHI and PstI restriction enzymes and ligated into the Par j 1 clone linearized with the same enzymes. The loop 2 fragment was purified, digested with the PstI and HindIII restriction enzymes and ligated into the Par j 1 clone linearized with the same restriction enzymes. Recombinant clones were sequenced and the mutations and the open reading frame were confirmed.

Table 1. Primers used for mutagenesis: numbers indicate the relative position of the oligonucleotides on the Par j 1.0101 nucleotide sequence. Upper-case letters indicate coding sequences, lower-case letters indicate the restriction enzyme used for cloning.

	1	29
Par j 1 loop 1 For	5'ggatccCAAGAAACCTGCGGGACTATGGTGAGAGC	3'
	91	58
Par j 1 loop 1 Rev	5'ctgcagCACCCCGCTGACGGCGCTGCCTCTTTCC	3'
	91	130
Par j 1 loop 2 For	5'ctgcagCGGCGCCAAAAGAGCGGCCGGGGCGACGGCGACG	3'
	423	405
Par j 1 loop 2 Rev	5'aagcttGGCTTTTTCGGGTGCGGG	3'

The coding sequence of the wild type Par j 1 and its derivatives were expressed in the pQE30 vector (Qiagen). This vector expresses a recombinant protein with an amino-terminal hexa-histidine tag allowing purification using a HisTrap Kit (Pharmacia). SDS-PAGE gels and Coomassie Brilliant Blue staining checked purity of the proteins.

Western blot analysis

IgE-binding activity was assayed by Western blotting as previously described (10) using single sera (diluted 1:5) from *Pj* allergic patients (n=13).

ELISA

ELISA detection was performed by adding 200 μ l of a solution containing 5 mg/ml of antigen in coating buffer (sodium carbonate buffer, pH 9.5) to each well of polystyrene plates overnight at room temperature. Anti-Par j 1 rabbit preimmune and immune sera were diluted at a concentration of 35 ng/ml. Colorimetric analysis was performed as previously reported (12).

Skin Prick Test

Four patients with Skin Prick Test (SPT) sensitivity to *Pj* commercial extract were tested with serial dilutions of the rPar j 1 and its loop 1 derivative. Proteins were used at 10, 1 and 0.1 μ g/ml diluted in IXPBS. About 20 μ l of these solutions were placed on the patient's forearms and skin reactivity tested after 20 min. Histamine and IXPBS were used as positive and negative controls, respectively.

PBMC proliferation assay

Peripheral blood mononuclear cells from *Pj*-allergic patients were cultured in triplicate at 2×10^5 cells/well in 96-well flat bottom culture plates. The antigens were added to the culture medium at a concentration of 2 μ g/ml. Thymidine incorporation and the stimulation index were determined as described (12).

Results and Discussion

Par j 1 and Par j 2 allergens represent the two major allergens of the *Parietaria judaica* pollen binding most of the *Pj*-specific IgE and eliciting an antibody response in the majority of the *Pj*-allergic patients (6). Sequence data analysis had shown that these allergens belong to the same family of proteins named ns-LTP, and by 3D-modeling analysis we were able to show that they are composed of four α -helices generating two regions (loop 1 and loop 2), which are exposed to the solvent and contain amino acid residues with properties (bulky and/or charged side chains) potentially able to form an epitope. In addition, previous work has shown the presence of an immunodominant IgE epitope in the first 30 AA of the Par j 1 allergen (10). For these reasons, we decided to perform a PCR-based strategy of mutagenesis by alanine substitutions of some loop 1 and loop 2 amino acids residues to study their contributions on the overall allergenicity of the molecule. Mutated Par j 1 proteins were cloned and expressed in the pQE30 vector (Qiagen) and recombinant proteins were purified using the HisTrap Kit (Pharmacia) following the manufacturer's instructions. Fractions were analyzed on a 16% SDS-PAGE gel and purity and concentration were determined by Coomassie Brilliant Blue staining (Figure 1, panel B). Purified proteins were transferred to PVDF membranes (Millipore) and analyzed for their IgE-binding activity by Western blotting using single sera from *Pj*-allergic patients (n=13). This analysis showed a very homogeneous result with a reduction in the IgE-binding activity for the loop 1 mutant in all the patients and with no significant differences for the loop 2 mutant (see Figure 1, panel A for a representative patient). Starting from this observation, we decided to test *in vivo*

the anaphylactic reaction. For these reactions, by skin prick test, the patients exhibited a reduced response to the wild type allergen and a higher response against histamine. The lowered IgE reactivity was not a limitation. Proliferation of the rPar j 1 and its loop 1 derivative retains T cell reactivity.

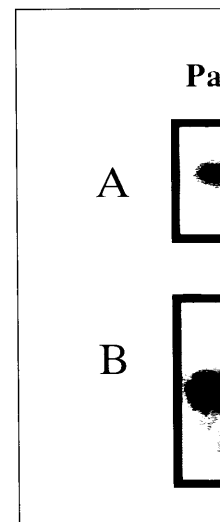


Fig. 1. SDS-PAGE and Western blot analysis of Par j 1. Panel A shows a Western blot with a single band for Par j 1. Panel B shows Coomassie Brilliant Blue staining of the same gel, showing a single band for Par j 1.

Table 2. Induction of allergic reaction by different concentrations of Par j 1. +, a positive reaction; -, a negative reaction; n.d., not determined.

Patient	10 n
1	n
2	
3	
4	

n.d. = not determined

usly described (10)

containing 5 mg/ml
each well of poly-
nit preimmune and
metric analysis was

mercial extract were
Proteins were used
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amine and IXPBS

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the anaphylactic activity of the loop 1 mutant to test the effect of the site-specific mutagenesis. For these reasons we tested the Par j 1 wild type allergen and the loop 1 mutant by skin prick test. This analysis showed that in 3 out of 4 patients, loop 1 derivative exhibited a reduced ability to induce type I hypersensitive reaction compared to the wild type allergen (see Table 2 for details). All the patients gave a positive reaction against histamine (positive control) and no reaction against IXPBS (negative control). The lowered IgE reactivity of the loop 1 mutant did not interfere with the T cell recognition. Proliferation assay was performed on PBMC from five *Pj*-allergic patients using the rPar j 1 and its loop 1 derivative as antigens and showing that the loop 1 mutant retains T cell reactivity (Figure 2).

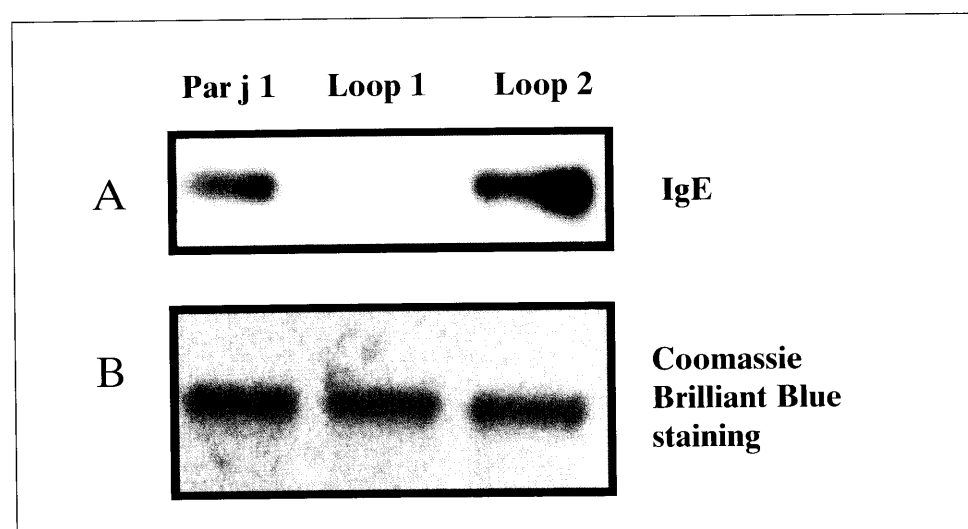


Fig. 1. SDS-PAGE analysis of the rPar j 1 allergen and its site-specific loop 1 and loop 2 mutants: panel A shows a Western blot analysis using a serum from a *Pj*-allergic patient; panel B shows a Coomassie Brilliant Blue staining of the recombinant proteins used.

Table 2. Induction of Type I immediate reaction: Four *Pj*-atopic patients were skin prick tested with different concentrations of the wild type recombinant Par j 1 and its loop 1 derivative. + indicates a positive reaction, - a negative reaction.

Patient	Par j 1			Loop 1		
	10 mg/ml	1 mg/ml	0.1 mg/ml	10 mg/ml	1 mg/ml	0.1 mg/ml
1	n.d.	+	+	n.d.	+	-
2	+	+	+	+	-	-
3	+	+	-	+	-	-
4	+	+	+	+	+	+

n.d. = not determined + = positive - = negative

In conclusion, we described the immunological characterization of a hypoallergenic derivative of the Par j 1 major allergen by means of site-directed mutagenesis. In particular we were able to show that a reduced number of point mutations can dramatically reduce the allergenicity of one of the two major *Pj* allergens preserving the overall structure of the allergen. In fact, ELISA assays showed that a rabbit polyclonal antibody made against the rPar j 1 allergen is able to bind the loop1 mutant at a level comparable to the wild type allergen (Figure 3).

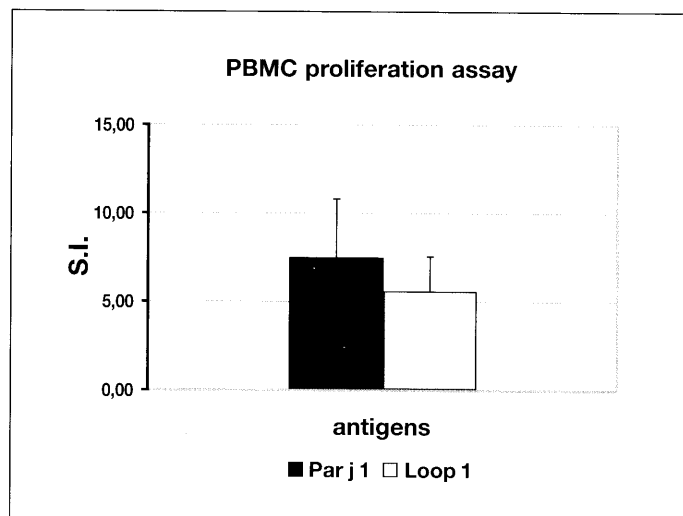


Fig. 2. PBMC proliferation assay: proliferative response of human PBMC from five *Pj*-allergic patients to wild-type rPar j 1 and to the loop 1 derivative.

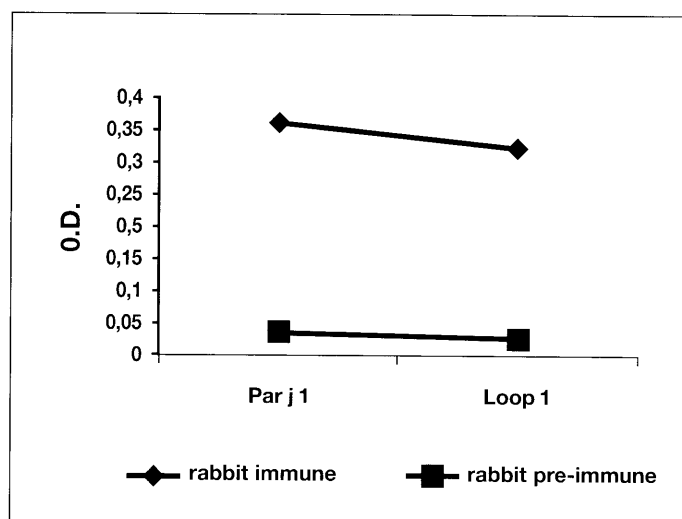


Fig. 3. ELISA detection of the IgG binding activity of a rabbit polyclonal immune and pre-immune antiserum against rPar j 1. The antigens used were the wild-type rPar j 1 allergen and its loop 1 variants.

SIT represents the only curative treatment consisting of repeated subcutaneous injections of increasing doses of the allergenic extracts. Its clinical efficacy has been demonstrated by several trials but the molecular mechanisms involved in this therapy have not been clarified yet. SIT seems to act at the T cell level with a reduction in IL-

4 and IL-5 production. It is characterized by an increase in specific antibodies. IgG antibodies have the effect of blocking antibody-dependent allergic specific IgE preventing the release of inflammatory mediators and histamine. Starting from this concept, the binding activity of the antibodies is a useful tool, at least for the assay against the Par j 1 allergen.

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4 and IL-5 production by T cells (13) and with the induction of an anergic state characterized by an increase in IL-10 production (14). However, the induction of protective specific antibodies has been documented in many reports although the production of IgG antibodies has not been recognized as a marker for a successful SIT. The presence of blocking antibodies seems to be an important event competing with the allergen-specific IgE preventing the mast-cell activation and the subsequent release of inflammatory mediators and inhibiting the IgE-mediated presentation of allergens to T cells (1). Starting from this background, the design of allergen derivatives with reduced IgE binding activity, but with a preserved three-dimensional structure, may represent an useful tool, at least in principle, to induce the production of protective antibodies against the Par j 1 wild type protein.

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