

ORIGINAL ARTICLE Allergens

Characterization of a Par j 1/Par j 2 mutant hybrid with reduced allergenicity for immunotherapy of *Parietaria* allergy

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Summary

Background *Parietaria* pollen is one of the major cause of pollinosis in the southern Europe. Specific immunotherapy is the only treatment able to modify the natural outcome of the disease restoring a normal immunity against allergens.

Methods We designed a recombinant molecule (PjEDloop1) comprised of genetic-engineered variants of the major allergens of the *Parietaria* pollen (Par j 2/Par j 1). Purity and chemical–physical properties of the derivative were analysed by RP-HPLC chromatography and Photon Correlation Spectroscopy. Immunological activity was evaluated by means of Western blotting, ELISA inhibition and PBMC proliferation assay in 10 *Parietaria* allergic patients. Basophil activation was studied in six subjects. The immunogenicity of the hybrid was studied looking at the immune responses induced in a mouse model of sensitization.

Results The PjEDloop1 hybrid was produced as a purified recombinant protein with high stability in solution. Western blot, ELISA inhibition and basophil activation test showed that the PjEDloop1 displays a remarkable reduced IgE binding and anaphylactic activity. CD3 reactivity was conserved in all patients. Mice immunization with the rPjEDloop1 induced antibodies and T cell responses comparable to that obtained by the wild type allergens. Such antibodies shared the specificities to rPar j 1 and rPar j 2 with human IgE antibodies.

Conclusion Our results demonstrated that a mutant hybrid expressing genetically engineered forms of the major *P. judaica* allergens displayed reduced allergenicity and retained T cell reactivity for the induction of protective antibodies in vaccination approaches for the treatment of *Parietaria* pollinosis.

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Introduction

Pollen grains contain a number of water-soluble proteins that, in contact with the respiratory mucosa of allergic patients, release a number of molecules capable of inducing immediate symptoms of allergy due to allergen induced cross-linking of mast cell-bound IgE (allergic reaction). While immediate and late symptoms can be tackled by pharmacological therapies, allergen specific immunotherapy has been shown to be the only curative treatment effective in many clinical trials. However, currently used allergen extracts have the drawback of inconsistent composition, and in some cases, due to chemical–physical properties, do not contain the rele-

vant allergens for the patient's individual sensitization [1–3]. Furthermore, it has been demonstrated that pollen extracts contain pro-inflammatory substances acting as adjuvants [4–6] and proteolytic activities [7, 8] which can alter the epithelial integrity contributing to the sensitization and pathogenesis of allergy.

On the other hand, clinical trials with recombinant allergens using a mixture of grass pollen allergens [9] or the major birch pollen allergen (Bet v 1) [10] were successful and proved the efficacy of this novel approach. In addition, the development of strategies concerning the design of genetically modified allergens with hypoallergenic activity has been proved to be clinically effective as well [11, 12].

Parietaria spp represents a common cause of pollinosis in the Mediterranean area [13, 14]. Almost 30% of all the allergic subjects in southern Europe present a skin prick test (SPT) reactivity towards this pollen and more than 50% of these subjects have experienced bronchial asthma with high levels of bronchial hyperresponsiveness [15, 16].

Parietaria judaica (Pj) pollen is a well suited allergy model as it is composed of two major components (Par j 1 and Par j 2) belonging to the lipid transfer protein (LTP) family which are responsible for IgE induction in almost all the Pj allergic patients [17–19] and inhibiting up to 95% of the total IgE binding activity of the *P. judaica* extract (PjE) [20]. 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 [21] so they can be used as marker molecules for diagnosis and therapy of the Urticaceae family. 3D modelling by homology was used to resolve the three-dimensional structures of the Pj major allergens showing that they attain an α - α - α - β common structure [22]. Using the model as a template, an immunodominant IgE epitope was mapped in the NH₂-terminal region of these allergens (loop1 region lying between aminoacids 1 and 30) [22].

In this article we described a rational approach for the design of a hybrid molecule generated by fusion of engineered forms of the Par j 1 and Par j 2 allergens. The loop1 region of both allergens were selectively targeted by site-directed mutagenesis. The derivative was expressed in *E. coli* and the immunological and chemical–physical properties of the hybrids studied by *in vitro* and *in vivo* assays.

Materials and methods

Mutagenesis, expression and purification of recombinant Par j 1/Par j 2 hybrids

Site-specific mutagenesis of the Lys23, Glu24 and Lys27 amino acids [numbers are referred to wild type (w.t.) Par j 1 (#X77414) and Par j 2 (#X95865) sequences] was performed using a PCR-based strategy of mutagenesis. In particular, 1 ng of the Par j 1 [17] and Par j 2 [18] clones were amplified using the following primers: (Pj1for 5'-ATTGGATCCCAAGAAACCTGCGGGACTATG-3' and Pj1revloop1 5'-AAACTGCAGCACCCGcTGACGGCgCTgcCTCTTCC-3') and (Pj2for 5'-GTGGGATCCGAGGAGGCTTGCGGGAAAGTGGTGCAG-3' and Pj2revloop1 5'-AAACTGCAGCACTCCGcCGACGGCgCTCCTCCC-3'), respectively. Bold letters indicate the restriction enzyme sites introduced for cloning (*Bam*HI for the forward primers and *Pst*I for the reverse primers), lower case letters display the nucleotide changes introduced for mutagenesis. Alanine

substitutions were introduced by PCR amplifying two 118 nucleotide fragments containing the coding regions for the first 30 amino acids of each allergen bearing the above reported substitutions. Then, the PCR fragments were digested with the *Bam*HI and *Pst*I restriction enzymes and inserted into the Par j 1 [23] and Par j 2 [24] clones previously linearized with the *Bam*HI and *Pst*I restriction enzymes. The derivatives were named Pj1loop1 and Pj2loop1, respectively (see Fig. 1 panel A for details).

A hybrid molecule containing a head-to-tail dimer of the Pj2loop1 and Pj1loop1 derivatives was generated by PCR amplifying the Pj2loop1 clone with the Pj2for and Pj2dim (5'-CGCGGATCCATAGTAACCTCTGAAAATAGT-3') oligonucleotides (bold letters indicate the restriction enzyme recognition site inserted for cloning). The DNA fragment was digested with the *Bam*HI restriction enzyme and inserted into the Pj1loop1 clone previously linearized with the *Bam*HI restriction enzyme (PjED-loop1). Furthermore, a head-to-tail hybrid expressing the w.t. Par j 2 and Par j 1 sequences was generated (named PjED). Briefly, a DNA fragment containing the full-length coding region of the w.t. Par j 2 sequence (#X95865) was generated by PCR using the Pj2for and Pj2dim oligonucleotides. The PCR fragment was digested with the *Bam*HI restriction enzyme and inserted within the *Bam*HI restriction site of the w.t. rPar j 1 clone [23] (see Fig. 1 panel A for details). Engineered hybrids were cloned into the pQE30 vector (Qiagen, Milan, Italy) and expressed into *E. coli* NM15 strain (Qiagen). The recombinant derivatives express a fusion protein of 255 AA containing a 12 amino-terminal fusion peptide, a hexa-histidine tag and two additional aminoacids (G and S) in position 115 and 116 as a consequence of the cloning procedures (sequence of the *Bam*HI cloning site) (see Fig. 1 panel A for details). Wild type recombinant Par j 1 and Par j 2 allergens were expressed as his-tagged proteins of 151 AA (putative M.W. 16384,94 Daltons) and 115 AA (putative M.W. 12743,68 Daltons) respectively. Engineered hybrids and w.t. allergens were purified as previously described [20] and analysed on a 16% SDS-PAGE gel. Purity and concentration were determined by Coomassie brilliant blue staining and densitometric analysis. Recombinant proteins for proliferation assays and basophil activation test were further on purified using a Detoxi-gel endotoxin removing gel (Pierce, Rockford, IL, USA) and tested for the endotoxin content using the Multi-test Limulus Amebocyte Lysate pyrogen plus test (Bio-Whittaker, Milan, Italy) (endogenous endotoxin content ≤ 0.003 ng LPS/ μ g of recombinant protein).

Physical–chemical analysis of the rPjEDloop1 hybrid

Recombinant PjEDloop1 hybrid was analysed by RP-HPLC chromatography, by dimensional analysis

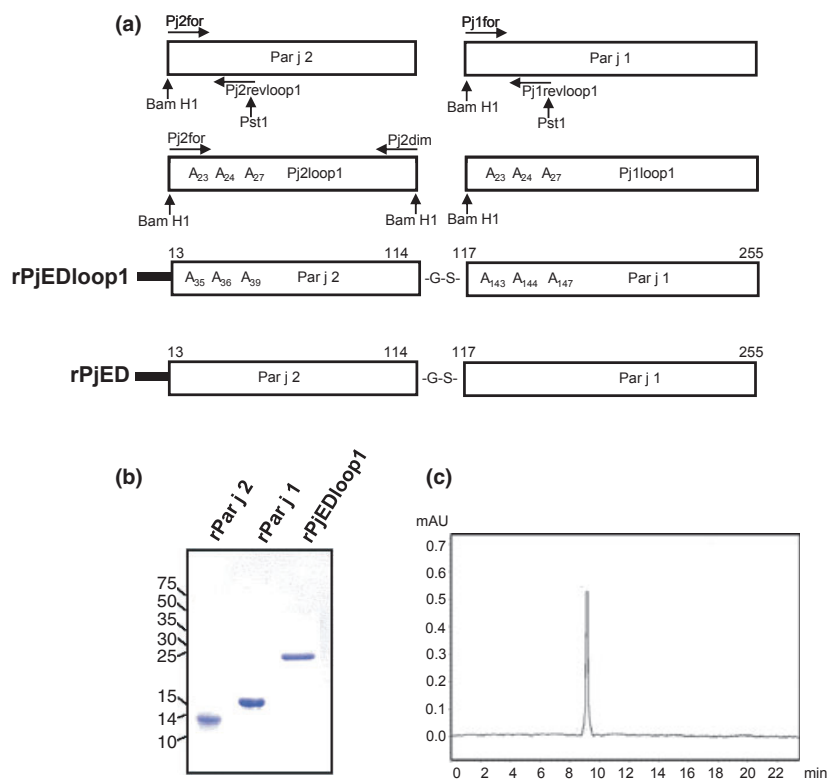


Fig. 1. Structure and chemical-physical analysis of the rPjEDloop1 hybrid. Panel A: schematic representation of the engineered hybrids. Solid bars indicate the amino-terminal tag; open boxes the coding regions of the Par j 2 and Par j 1 allergens. Arrows indicate the positions of the oligos and restriction enzymes used for the mutagenesis. A_s indicate the position of the mutated amino acids. Numbers show the size of the protein. Panel B: Coomassie brilliant blue stained SDS-PAGE of the purified recombinant proteins used for the assays. Molecular Weights are indicated on the left side. Panel C: Reversed phase HPLC elution profile of the rPjEDloop1 hybrid.

using Photon Correlation Spectroscopy (PCS) and by surface charge using principles of laser Doppler velocimetry and phase analysis light scattering (M3-PALS technique). RP-HPLC chromatography was performed using a Shimadzu system (Shimadzu Instrument, Milan, Italy) and a C₅ column Jupiter (Phenomenex, Houndsfield, UK) packed with 5.0 µm particles, with dimensions 250 × 4.60 mm and 300 Å of silica porosity. The UV detection was performed at 280 nm. The elution was conducted with a gradient flow of 1 mL/min for 60 min of a eluent mixture composed by CH₃CN/CF₃COOH 0,1% (solvent A) v/v and H₂O/CF₃COOH 0,1% (solvent B) v/v; this mixture was incremented from 0% to 60% of organic phase. Size characterization and Zeta potential measurements were carried out with a Nano Zetasizer ZS (Malvern Instruments Ltd, Worcestershire, UK) on protein solution at 25° and 37°C by using, as dispersing medium, phosphate buffer (1 × PBS).

Human IgE binding activity

Sera from 10 consecutive SPT positive *P. judaica* allergic patients were used for the IgE binding analysis. The study has been approved by the Institutional ethical

committee and donors signed an informed consent before undergoing sampling. Immunoblot and ELISA inhibition experiments were performed as previously described [23]. Briefly, inhibition assay was performed by adding serum samples (diluted 1 : 5) in phosphate-buffered saline (PBS), 0.5% w/v BSA, 0.05% v/v Tween-20 and pre-incubated over night at room temperature with increasing concentrations of the allergens used as inhibitors (serial dilutions from 10 to 0.1 µg/mL). The percentage of inhibition of human IgE binding was calculated as follows: $100 - [\text{ODI}/\text{ODP} \times 100]$, where ODI and ODP represent the absorbance after pre-incubation with treated serum and untreated serum, respectively. Quantification of the IgE binding on immunoblot was performed by densitometric analysis using the a Chemi Doc apparatus and the Quantity ONE software (Bio Rad, Milan, Italy).

PBMC proliferation assay

PBMC from 10 Pj allergic patients were resuspended in 1 × PBS pH 7.2 (1 × 10⁷ cells/mL) and labelled with a solution containing 5 µM 5(6) Carboxy-Fluorescein Diacetate Succinimidyl Ester (CFDA-SE) (Molecular Probe)

as previously described [20]. Cells were incubated with the antigens (rPar j 1 plus rPar j 2 [mix] and rPjED-loop1) at a concentration of 1 and 10 µg/mL for 7 days. Then CFSE-labelled cells were stained with an anti-CD3 PE antibody (Caltag) for 20 min at 4°C in the dark. Background proliferation was measured in the unstimulated sample (medium alone). A minimum of 50 000 events were collected and analysed on a FACScalibur flowcytometer (BD, Milan, Italy). Analysis gates were set on live lymphocyte by forward and side scatter profile. Results were analysed using the WinMDI 2.8 software.

Flow cytometry-based basophil activation test

Basophil activation assay was performed looking at the activation of the CD63 antigen by flow cytometry upon antigen stimulation. In particular, K-EDTA peripheral blood was obtained from five consecutive Pj allergic patients and one non allergic subject (negative control). Blood aliquots (50 µl) were incubated with serial dilutions (1–1000 ng/mL) of the recombinant allergens [rPar j 1 plus rPar j 2 (mix) and rPjEDloop1] following the manufacturer's instructions (Flow2 CAST; Buhlmann, Schönenbuch, Switzerland). An anti-FcεRI mAb was used as positive controls. The data analysis was performed with the WinMDI 2.8 software based on a first gate including the entire basophil CCR3⁺ population with low Side Scatter. Within this population, the percentage of CD63⁺ cells was compared to the total amount of basophiles previously gated. Activation was expressed as Stimulation Indices (S.I.). S.I. were calculated according to the following formula: %CD63⁺ Allergen stimulated cells divided by %CD63⁺ unstimulated cells. S.I. ≥ 2 were considered positive.

Sensitization of mice and evaluation of specific antibody levels

Eight-week-old female Balb/c mice were purchased from Harlan Nossan (Udine, Italy). Animals were housed in the Animal Care Unit of the Istituto Superiore di Sanità and treated according to the local guidelines for animal care (D.L. 116/92). Groups of eight mice were intraperitoneally immunized with 3.65 µg of the rPjEDloop1 (0.150 nM) or with an equimolar mixture containing 2 µg of rPar j 1 and 1.65 µg of rPar j 2 (w.t. mix) (0.150 nM of each recombinant allergen), according to a protocol previously published [25]. Specific IgE, IgG1 and IgG2a responses by individual mice immunized with either the mixture of monomers or the rEDloop1 were monitored by ELISA as previously described [25]. The w.t. mix and the whole PjE were used as coating antigens. Sera were diluted 1 : 20 for IgE analysis, 1 : 10000 for IgG1 analysis and 1 : 50 for IgG2a analysis. Results were expressed as Optical Densities at 490 nm or as Arbitrary

Units, which were calculated from a standard curve obtained with a pool of hyperimmune mouse sera. rPar j 1 (2 µg/lane), rPar j 2 (2 µg/lane), and PjE (10 µg/lane) were separated by 15% SDS-PAGE under reducing conditions in Tris-Glicine gel, and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Nitrocellulose strips were treated in blocking buffer (PBS, 3% w/v gelatin) for 1 h at room temperature and then incubated for 3 h at room temperature with a pool of anti-rPjEDloop1 hybrid mouse immune sera, or with pre-immune sera as controls. After washing, strips were exposed to 1 : 1000 biotin-conjugated rat monoclonal anti-mouse IgG1 (BD PharMingen, San Diego, CA, USA) for 1 h at room temperature followed by avidin-peroxidase (Sigma, Milan, Italy) at 2.5 µg/mL, both diluted in PBS/Tween 0.05%. The peroxidase substrate with the colorimetric reagent 3,3'-diaminobenzidine (Sigma) was then added to develop the colorimetric reaction.

ELISA competition experiments with mouse antibodies

Competitive ELISA assays were performed as previously reported [20]. ELISA plates were coated with the w.t. mix and pre-incubated with serial dilutions of a mouse immune sera pool anti-rPjEDloop1 (1 : 25, 1 : 100, 1 : 400, 1 : 1600, 1 : 6400) or with the pre-immune sera pool as a control. Individual sera from 10 *Parietaria* pollen-allergic patients with determined Pj-specific IgE concentration (ImmunoCAP; Phadia, Milan, Italy) were diluted to the concentration of 1–2 kU/L IgE and then were added to the wells. The percentage of inhibition of human IgE binding after incubation with mouse sera was calculated as follows: $100 - [\text{ODI}/\text{ODP} \times 100]$, where ODI and ODP represent the absorbance after pre-incubation with immune serum and pre-immune serum, respectively. Mean percentage inhibition values were plotted against the serial dilutions of mouse sera pool. Data shown are representative of four experiments.

Spleen cell proliferation assay

Two weeks after the last immunization, mice were sacrificed and individual spleens were removed to obtain single cell suspensions. Proliferation assays were performed as previously described [25] with the w.t. mix and PjE (10 µg/mL). Results were expressed as S.I., calculated with the following formula: mean cpm in allergen-stimulated cultures/mean cpm in controls. Data shown are representative of four experiments.

Statistical analysis

The Mann–Whitney *U*-test (rank sum) or the Wilcoxon matched-pairs signed-rank test when appropriate were used for the non-parametric analysis of differences

between different experimental groups or different *in vitro* treatments. *P*-values of less than 0.05 were considered statistically significant.

Results

Chemical–physical analysis of the Par j 1/Par j 2 hybrids

The Par j 1/Par j 2 hybrids were produced at high yield in *E.coli* (2.5 mg of protein/litre of culture) (Fig. 1 panel B). The chromatography of the purified rPjED-loop1 on RP-HPLC resulted into a well-defined single peak. No other component has been detected (Fig. 1 panel C). Next, the analysis by PCS showed that this hybrid showed a size of about 27 nm with a polydispersity index value (PDI) of 0.485 in $1 \times$ PBS. The PDI value indicates that the protein has a homogeneous size distribution. Moreover, the measurements of the zeta potential in phosphate buffer solution ($1 \times$ PBS), showed a value of -18.1 ± 1.6 mV at 25°C. This value remains constant at 37°C demonstrating that the protein is self-stabilising in this buffer inasmuch its surface charge inhibits coalescence and enhances stability. After several freeze-and thaw cycles, the measurements showed similar values demonstrating that the hybrid presents high stability in phosphate buffer solution.

Human IgE binding activity

IgE binding activity of the PjEDloop1 derivative was studied by means of ELISA inhibition and Western blot. To address this question, the w.t. recombinant major allergens were coated in ELISA plates and incubated with single sera from Pj allergic patients that were pre-treated with the w.t. allergens, the PjED and the PjED-loop1 derivatives, respectively. The w.t. allergens and the PjED hybrid were capable of an almost complete

inhibition demonstrating that dimerization *per se* is not interfering with the IgE binding activity. On the other hand, the PjEDloop1 hybrid was capable of inhibiting the Par j 1 and the Par j 2 specific IgE with mean values of 15.7% and 34.3%, respectively. The differences in the inhibition values between the w.t. allergens, the PjED and the PjEDloop1 derivatives were statistically significant ($P \leq 0.005$) (Fig. 2 panel A).

Furthermore, the Western blot analysis was consistent with the ELISA assay. When the IgE binding activity of the PjEDloop1 hybrid was compared to the reactivity of the PjED by immunoblot, a statistically significant reduction of binding was observed ($P \leq 0.005$) confirming the relevance of the loop1 epitope for the allergenic activity of the Pj major allergens (Fig. 2 panel B).

Basophil activation assay

The allergenic activity of the rPjED loop1 was studied comparing the capability of the hybrid to up-regulate the CD63 surface antigen in comparison to an equimolar mixture of the rPar j 1 and rPar j 2 allergens. Four out of five patients already reacted to the w.t. mix at the concentration of 100 ng/mL. All the subjects reacted to the control stimulus. In all the patients, we observed that the rPjEDloop1 hybrid did not reach the level of significance even at the highest concentration of stimulus (1000 ng/mL) (Fig. 3 panels A to E). No reactivity was observed when blood from a non allergic subject was tested (Fig. 3 panel F). These results demonstrated that the rPjEDloop1 displayed a reduced anaphylactic activity.

PBMC proliferation assay

The ability of the rPjEDloop1 to induce the proliferation of CD3⁺ cells from PBMC from 10 Pj allergic was studied by means of a flow-cytometry assay. All the

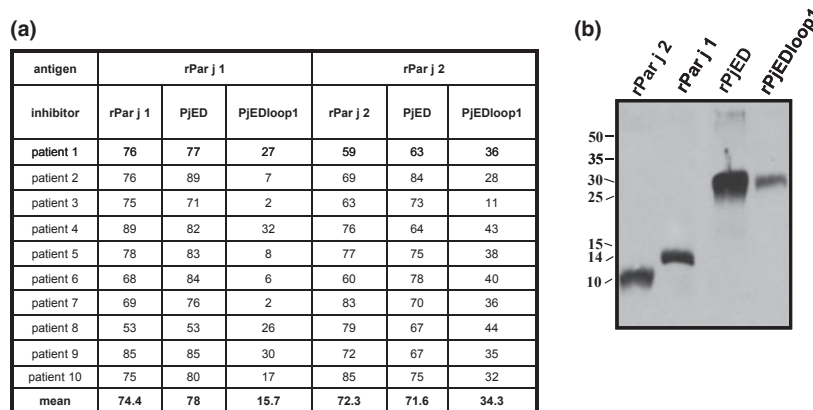


Fig. 2. IgE binding activity of the rPar j 1/rPar j 2 hybrids. Panel A: ELISA inhibition assay performed on plate-bound rPar j 1 and rPar j 2 allergens. Numbers indicate the percentage of inhibition. Mean values are indicated in the bottom lane. Panel B: Western blot analysis of the rPar j 1, rPar j 2, rPjED and rPjEDloop1 with a representative serum from one Pj allergic patient.

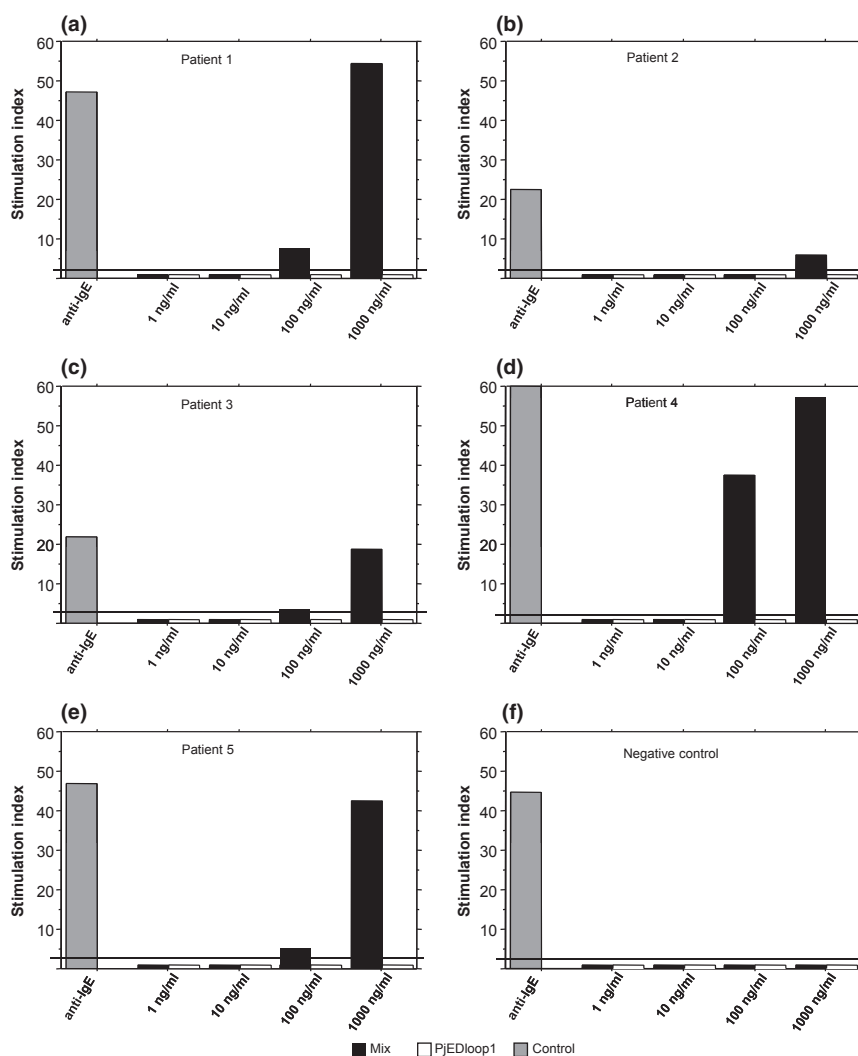


Fig. 3. Basophil activation assay. Basophils from five Pj allergic patient were stimulated with increasing concentrations of a mixture containing the rPar j 1 and rPar j 2 (mix) and with an equimolar concentration of the PjEDloop1 hybrid (panels from A to E). Panel F shows a non allergic control subject. Numbers on the y-axis express the Stimulation Index; numbers of the x-axis the stimuli used. A horizontal bar delimits the cut-off.

patients reacted towards the rPar j 1 and rPar j 2 allergens. The rPjEDloop1 hybrid induced the proliferation of CD3⁺ cells in all the patients. No significant differences were observed between the cells stimulated with the w.t. recombinant allergens vs. the cells incubated with the hybrid (Fig. 4).

Humoral and cell responses in mice

Antigen-specific IgE, IgG1 and IgG2a responses were evaluated by ELISA in sera of mice immunized with the rPjEDloop1 in comparison with mice immunized with the mixture of the rPar j 1 and rPar j 2 allergens. IgG1 antibody levels induced by the hybrid and measured against the mixture of individual allergens were comparable to that induced by the mixture of monomers

(Fig. 5 panel A), whereas rPjEDloop1 immunization induced significant ($P < 0.05$) lower levels of specific IgG2a (Fig. 5 panel B). Comparable IgE levels without statistically significant differences were observed (data not shown). Titration of IgG1 antibodies in pooled sera from rPjEDloop1-immunized mice against the whole PjE demonstrated the capacity of such antibodies to recognize with high efficiency the natural allergens in PjE (Fig. 5 panel C). In Western blotting, IgG1 antibodies induced by the rPjEDloop1 immunization were also able to recognize the individual rPar j 1 and rPar j 2 allergens as well as their natural counterparts represented in PjE. Differences in the molecular weight between the recombinant and the natural allergens was due to the amino-terminal tag introduced for purification of recombinant allergens (see M & M section for details).

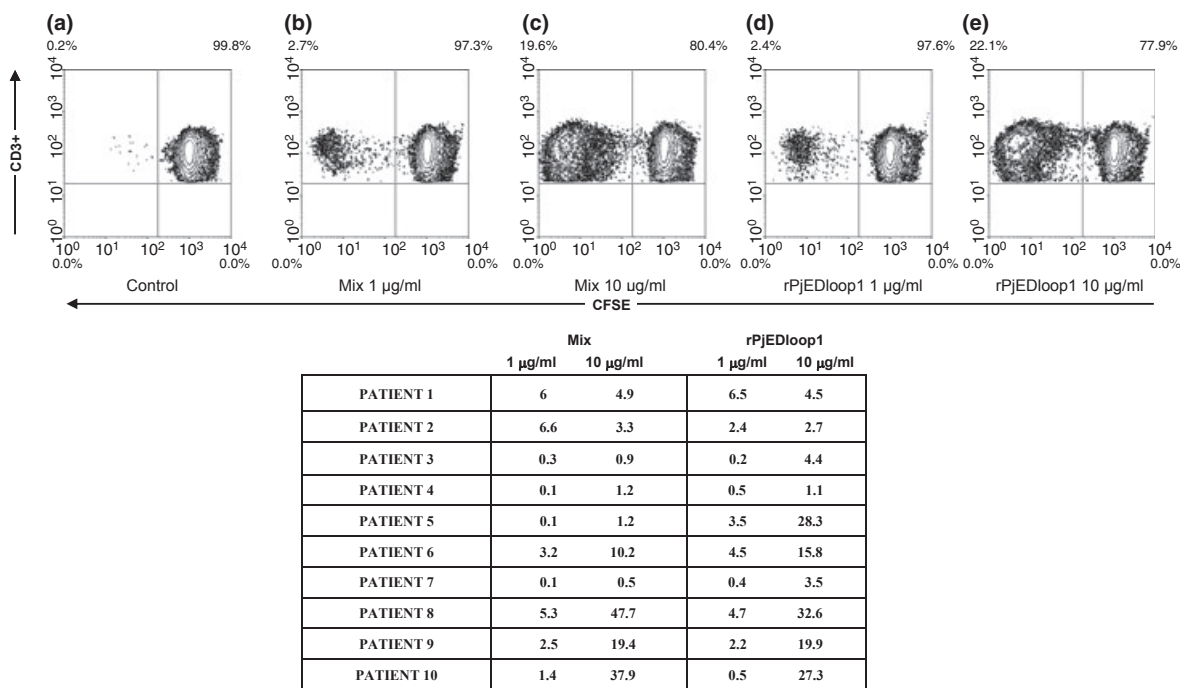


Fig. 4. PBMC proliferation assay. CFSE labelled PBMC from one representative Pj allergic patient were stimulated with the rPar j 1 and rPar j 2 mix (panel B and C) and an equimolar concentration of the PjEDloop1 hybrid (panel D and E). Panel A shows the unstimulated culture. Table summarizes the percentage of proliferating CD3⁺ cells after allergen stimulation subtracted of the proliferating cells obtained in the unstimulated cultures in 10 Pj allergic patients.

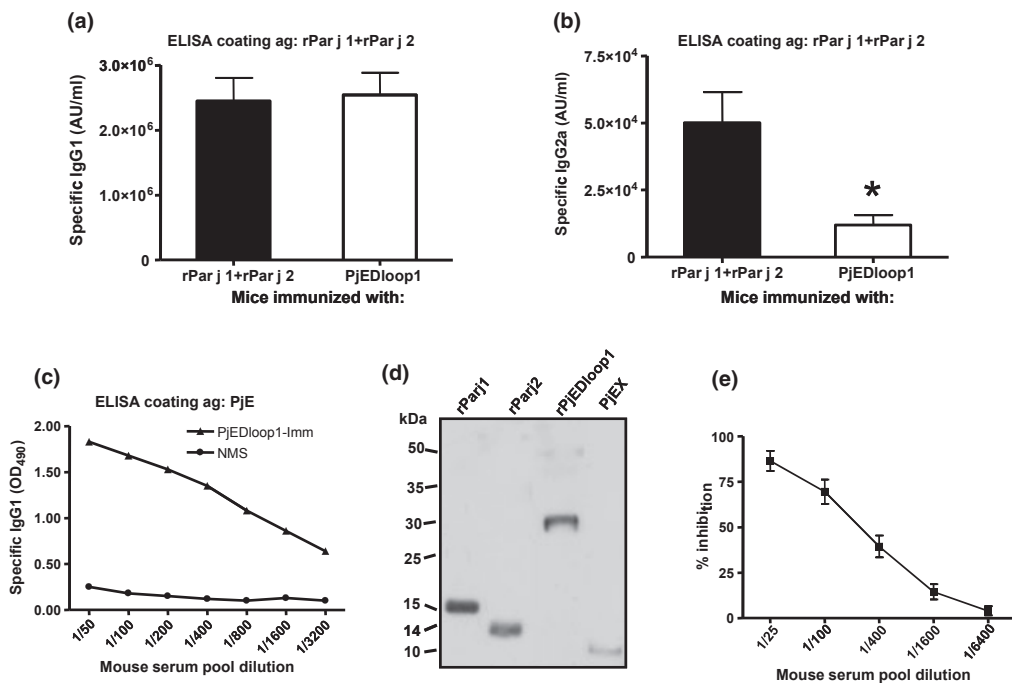


Fig. 5. Reactivity of IgG antibodies induced by immunization of Balb/c mice with the Parj1/Parj2 mix and the rPjEDloop1 proteins. Panels A-B: Specific IgG1 and IgG2a response to rPar j 1 and rPar j 2 in mice immunized with the rParj1 + rParj2 mix (black histograms) or the rPjEDloop1 (white histograms). Results are expressed as mean AU of individual sera (mice $n = 8$) $\pm$ SEM. Statistical significance: $*P < 0.05$. Panel C: ELISA titration against PjE of IgG1 in pooled sera from mice immunized with rPjEDloop1 in comparison with pre-immune sera (NMS). Results are expressed as Optical Densities (OD) at 490 nm. Panel D: IgG1 reactivity of pooled sera from mice immunized with the rPjEDloop1, evaluated against the rPar j 1, the rPar j 2, the hybrid and the Pj extract by Western blotting analysis. Panel E: Inhibition of human IgE binding to the rPar j 1 and rPar j 2 mix after pre-incubation with mouse immune sera pool. The mean $\pm$ SD percentage inhibition obtained with the 10 human sera is plotted in the dose-response curve against mouse serum dilutions.

(Fig. 5 panel D). No reactivity was observed when blotted allergens were incubated with pooled mouse pre-immune sera (data not shown). Furthermore, PjEDloop1-induced antibodies failed to react to unrelated recombinant allergens expressed in the same system and bearing amino-terminal His-tag tail (data not shown). We next examined whether mouse antibodies induced by rPjED-loop1 can compete for the binding of *P. judaica*-specific human IgE to the mixture of the single recombinant allergens in an ELISA competition experiments. As reported in Fig. 5 panel E, the binding of IgE antibodies from Pj allergic patients to the w.t. recombinant allergens was strongly inhibited by the mouse antibodies, reaching mean values higher than 90%.

Consistent with the human analysis, rPjEDloop1 immunization induced a T cell response comparable to that obtained after immunization with the w.t. recombinant allergens, as observed in spleen cells *in vitro* re-stimulated with both the mixture of rPar j 1 + rPar j 2 (Fig. 6a) and the natural PjE (Fig. 6b).

Discussion

Triggering of an allergic reaction is the consequence of the cross-linking of mast cell and basophil-bound IgE antibodies to allergens. Our group was able to show that the K21, K23, E24 amino acids located within the NH2-terminal region of the Par j 1 major allergen (loop1 region) are key elements of an immunodominant IgE epitope [22]. The aim of this article was the characterization of a hybrid with hypoallergenic features containing the most remarkable allergenic components of the Pj pollen.

The definition of hybrid multimeric proteins with reduced allergenicity has been already introduced in the field suggesting that oligomerization can interfere with the anaphylactic activity of the allergens probably due to an altered presentation of IgE epitopes [26]. In this study we were able to compare the IgE binding

activity of two hybrids composed of the w.t. and loop1 engineered Par j 1 and Par j 2 allergens, respectively, showing that dimerization *per se* has a weak effects on both the IgE binding and anaphylactic activities. On the other hand, the mutagenesis of specific key residues in the loop1 region led to a derivative with statistically significant reduced IgE binding activity and upregulated CD63⁺/CCR3⁺ cells from Pj allergic patients. Starting from these observations, we further focused our study on the rParjloop1 properties. Chemical-physical analysis demonstrated that this molecule can be produced in large amount in *E. coli*, purified to homogeneity and displayed a stable form in solution matching some of the relevant criteria for pharmaceutical applications.

T cell response is crucial for the induction of specific T cell tolerance or anergy against allergens and for the generation of allergen-specific blocking IgG subclasses of antibodies [27, 28]. For these reasons, we compared the ability of an equimolar mixture of both the w.t. allergens and the mutated hybrid to induce human T cell proliferation showing that the derivative resembles the T cell epitopes of the w.t. allergens.

This aspect has been further analysed in immunization studies in mice by looking at the proliferation of spleen cells from mice immunized with the w.t. allergens or the rEDloop1 derivative. Upon *in vitro* re-stimulation with the rPar j 1/rPar j 2 mix and the natural PjE, comparable proliferative responses were found in the two immunization groups, demonstrating the immunological equivalence of the w.t. and the engineered hybrid.

In parallel, we compared the pattern of antibodies induced by immunization with the w.t. mix and the rEDloop1. The hypoallergenic hybrid maintained the capability of inducing a strong IgG1 antibody response, which almost completely blocked the binding of *P. judaica*-specific IgE antibodies to the individual allergens. In both experimental groups IgG1 levels were far higher than IgG2a (IgG1/IgG2a ratio in monomer-immunized

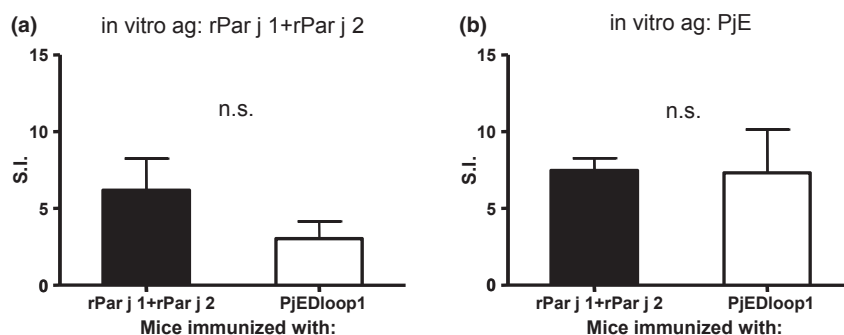


Fig. 6. Splenocytes proliferation assay. Proliferation of splenocytes from mice immunized with the rPar j 1 + rPar j 2 mix (black histograms) or the rPjEDloop1 hybrid (white histograms) stimulated *in vitro* with the rPar j 1 + rPar j 2 mix (Panel A, $P = 0.50$) or the *P. judaica* extract (PjE) (Panel B, $P = 0.70$). Results are expressed as mean stimulation index (S.I.) $\pm$ SEM, obtained from eight individual mice.

group was 50, and in mutant-immunized group was 200). The further reduction in IgG2a levels after mutant immunization could be ascribed to differences in the presentation of such dimeric molecules by antigen presenting cells, affecting the polarization of the induced T cell response. This is confirmed by the very low IFN- γ production that we observed in mice immunized with rPjEDloop1, as compared with mice immunized with the monomeric allergens, after antigen-specific in vitro re-stimulation of spleen cells (data not shown). Probably, in our system the dimeric mutant molecule is up-taken by dendritic cells and macrophages/monocytes through phagocytosis/pinocytosis whereas B cell presentation and IgE-facilitated presentation are ruled out by the alteration of B epitopes. Such presentation pathways could prevalently induce T cell tolerance, involving T regulatory cells, rather than a Th0/Th1 skewing. Further experiments on the mechanisms operative in the presentation of such modified allergens are needed to confirm these hypothesis. The significant reduction of the IgG2a isotype (IFN- γ -driven) observed in mice immunized with the rPjEDloop1 suggests the induction of a regulatory T cell response rather than a re-polarization of the Th2 response towards the Th1 phenotype, which could maintain an inflammatory effector potential, thus increasing the suitability of such hybrid derivative as an immunotherapy candidate. In addition, Western blotting experiments demonstrated that the PjEDloop1-induced IgG antibodies were able to recognize the recombinant and natural Par j 1 and Par j 2

allergens suggesting that an immunization protocol performed with the recombinant hybrid can induce antibodies capable of binding natural major *P. judaica* allergens.

Taken together, the results described in this article indicate that rPjEDloop1 is a hypoallergenic molecule resembling the human T cell epitopes. *In vivo* sensitization experiments demonstrate that it is a good immunogen in mice, inducing T cell responses and antibodies sharing the specificities to Par j 1 and Par j 2 with human IgE antibodies. These characteristics suggest that this derivative is a good candidate for clinical trials in *Parietaria* allergic patients.

Finally, epitope mapping performed by synthetic peptides [29] and phage display [30] confirmed the immunological relevance of the loop1 region in other allergenic LTPs suggesting that the strategy described in this study can be used for the generation of hypoallergenic LTPs from different sources.

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