

The recombinant major allergen of *Parietaria judaica* and its hypoallergenic variant: *in vivo* evaluation in a murine model of allergic sensitization

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Summary

Background Par j 1 represents the major allergenic component of *Parietaria judaica* pollen. Its three-dimensional structure is stabilized by four disulphide bridges. A family of three-dimensional mutants of the recombinant Par j 1 (rPar j 1) allergen, showing reduced allergenicity and retained T cell recognition has been recently developed by site-directed mutagenesis.

Objective To develop and characterize a murine model of IgE sensitization to rPar j 1. To evaluate similarities between the murine model and the human IgE response. To investigate in this model the recognition of a hypoallergenic mutant of Par j 1, and to study the immune responses elicited in mice by the mutant itself.

Methods BALB/c mice were sensitized by two intraperitoneal immunizations with rPar j 1 in alum on days 0 and 21. Allergen-specific serum IgE and IgG responses were studied by direct ELISA and immunoblotting, ELISA inhibition and competitive ELISA. Cell proliferation was evaluated in splenocyte cultures.

Results Sensitization with rPar j 1 induced high levels of IgE and IgG1 vs. low levels of IgG2a. Mouse antibodies specific to rPar j 1 were able to compete with human IgE for recognition of rPar j 1. IgE from mice immunized with rPar j 1 showed a significantly reduced binding activity towards the hypoallergenic variant rPjC, which lacks three disulphide bridges. On the contrary, rPjC was recognized by IgG1 and IgG2a antibodies as well as rPar j 1. The proliferative response to rPjC by splenocytes from mice immunized with rPar j 1 was comparable to that stimulated by rPar j 1. Immunization with rPjC induced low levels of IgE antibodies to the rPjC itself, while IgG and proliferative responses were similar to those induced by rPar j 1.

Conclusion Conformational variants of allergens, displaying reduced allergenicity accompanied by retained IgG and T cell recognition, offer a safe, specific and flexible approach to immunotherapy of type I allergy. Our mouse model of IgE sensitization to a recombinant allergen, mimicking the human response to its native counterpart, could provide valuable information for pre-clinical testing of such hypoallergenic molecules.

Keywords disulphide bridges, hypoallergens, mouse model, *Parietaria judaica*, recombinant allergens, site-directed mutagenesis, specific immunotherapy, T and B epitopes.

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Introduction

Type I allergy represents a relevant health problem in industrialized countries and affects almost 15–30% of the European population [1]. Allergic symptoms such as rhinoconjunctivitis, asthma, atopic dermatitis or anaphylactic shock are elicited by environmental allergens. Among inhalant allergens, *Parietaria judaica* pollen is one of the main causes of allergy in the Mediterranean countries [2]. The composition of the allergenic extract of *P. judaica* pollen has been extensively studied [3–4]. The major allergen Par j 1, recognized by 95% of the sera from *Parietaria*-allergic

subjects, has been characterized, purified and cloned [5–7]. The recombinant Par j 1 (rPar j 1) protein produced in *Escherichia coli* shows molecular and immunological properties comparable to its native counterpart [6]. A three-dimensional model of Par j 1 has been proposed on the basis of the structural homology with a family of plant proteins named ‘non-specific lipid transfer proteins’ (nsLTPs), showing an α – α – α – α – β structure with four disulphide bridges necessary for the correct folding of the protein [8]. The same study reported the importance of the cysteine pairing in the formation of an immunodominant IgE epitope in the 1–30 region of the molecule, involving amino acid residues K21, K23, E24 and K27 comprised in the loop between cysteines 14 and 29. According to these observations, disulphide bonds have been shown in other allergens to contribute to the overall allergenicity of the molecule [9–15].

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Although allergic symptoms can be controlled by pharmacological treatment, specific immunotherapy (SIT) represents the only curative approach toward type I allergy. The clinical efficacy of SIT performed with classical allergenic extracts has been documented in several clinical studies [16], although the underlying mechanism leading to desensitization is less clearly understood. One major disadvantage of SIT is that the administration of crude allergenic preparations may cause severe anaphylactic side reactions [16]. In addition, the use of complex allergenic mixtures may lead to sensitization towards new allergens [17, 18]. The use of recombinant allergens as substitutes for native counterparts has been proposed to overcome these difficulties [19]. Indeed, they can be used for novel forms of immunotherapies tailored according to the sensitization profile of the patient [20]. However, the risk of unwanted anaphylactic reactions still remains, since pure allergenic recombinant preparations used *in vivo* may be very potent in evoking IgE responses. To overcome these problems, it is now possible to modify allergens in a targeted way so that their IgE reactivity is reduced but the T cell response remains not affected. These modified allergens could lead to an improved and safer immunotherapy, which combines the advantage of reducing the risk of IgE-mediated anaphylactic side-effects with the possibility of down-regulating the broad repertoire of T cell specificities [21–22]. On the basis of a detailed knowledge of the IgE-binding regions, hypoallergenic recombinant derivatives can be generated by recombinant DNA technology applied to allergen engineering [23]. In particular, site-directed mutagenesis targeted to critical positions in the amino acidic sequence of allergens has been successfully employed to generate hypoallergenic molecules that are equally recognized by allergen-specific T lymphocytes, but display a significantly reduced IgE-binding capacity [21–22, 24–26]. Recently, this approach has been applied to the development and characterization of a family of three-dimensional mutants of the rPar j 1 allergen [27].

While these molecules have been studied for their ability to interact with human antibodies, T cells and monoclonal antibodies, a limited number of studies have up to now approached the *in vivo* evaluation of such hypoallergens in a suitable animal model [28–32]. Such murine models of IgE sensitization could be particularly useful for pre-clinical testing of new candidate molecules, and could represent unique tools for understanding the immunological mechanisms involved in the induction of IgE responses.

In the present study, we first established and characterized a murine model of IgE sensitization to rPar j 1. We then evaluated the similarities between the murine model and the human IgE response. Finally, we investigated in this model the recognition of a hypoallergenic mutant of Par j 1, as well as the features of the immune response elicited in mice by the mutant itself.

Materials and methods

Recombinant and native antigens

rPar j 1 and the rPjC mutant (Cys4 → Ser, Cys29 → Ser and Cys30 → Ser) were produced according to Bonura et al. [27].

All the recombinant allergens were expressed in a prokaryotic vector containing a six histidine tag (6xHis tag) for their affinity purification. The concentration of recombinant proteins was determined by densitometric analysis of SDS-PAGE gels stained with Coomassie brilliant blue, previously validated by both the Bradford method and the measurement of OD absorbance at 280 nm.

Pollen from the wall pellitory (*P. judaica*) was purchased from Allergon AB (Angelholm, Sweden). *P. judaica* pollen extract (PjE) was prepared as previously described [33], lyophilized and stored at 4 °C until use. Protein concentration was determined by the method of Bradford [34] with bovine serum albumin (BSA) as standard (BioRad, Richmond, CA, USA).

Animals and immunizations

Eight-week-old female BALB/c mice were purchased from Charles River Laboratories (Milan, Italy). Animals were maintained in the Animal Care Unit of the Istituto Superiore di Sanità according to the local guidelines for animal care. In a preliminary set of experiments, several immunization protocols were tested, including administration of different concentrations of protein (up to 10 µg/mouse), and repeated immunizations (up to six times) every 2 weeks. We obtained the highest production of specific IgE antibodies with the lowest dose (1 µg of the recombinant allergen/mouse adsorbed to 2.5 mg of Al(OH)₃, on days 0 and 21). Therefore, in the present study, groups of five mice were immunized as above by the intraperitoneal route. Before each immunization, a suspension was prepared by mixing a 100 × antigen solution (500 µg/mL) to the commercial alum gel suspension (13 mg/mL, 200 µL/mouse, Sigma–Aldrich, Milan, Italy) in order to obtain a final antigen concentration of 5 µg/mL (1 µg/mouse plus 2.5 mg of Al(OH)₃). Control groups received Al(OH)₃ only, at the same time points. Blood samples were collected from the retro-orbital plexus 7 days after the last immunization. Individual sera were stored at –20 °C until analysed.

ELISA, ELISA inhibition and competitive ELISA

Microtitre plates (Greiner Bio-One, Frickenhausen, Germany) were coated with the recombinant allergens (5 µg/mL) or PjE (20 µg/mL) in carbonate/bicarbonate buffer, pH 9.6, for 3 h at 37 °C and overnight (o.n.) at 4 °C, respectively. Unless otherwise indicated, serum samples and antibodies were diluted in phosphate-buffered saline (PBS), 0.5% w/v BSA, 0.05% v/v Tween-20 and plates were washed between each incubation step with PBS, 0.05% v/v Tween-20. Non-specific-binding sites were blocked with PBS, 1% w/v BSA for 2 h at room temperature (r.t.). Serum samples were added in duplicates (diluted 1 : 20 for IgE measurement, 1 : 100 for IgG, IgG1 or IgG2a) and incubated o.n. at 4 °C in the IgE ELISA or 2 h at r.t. in the IgG ELISA. Pre-immune sera from each individual mouse were used in parallel as controls. After washing, bound antibodies were detected by adding a biotin-conjugated rat monoclonal anti-mouse IgE (BD Biosciences Pharmingen, San Diego, CA, USA) for 5 h at r.t. followed by avidin-peroxidase (Sigma–Aldrich) at 2.5 µg/mL for a further 30 min, or peroxidase-labelled rat monoclonal anti-mouse

IgG, IgG1, IgG2a for 1 h at r.t. All the secondary antibodies were diluted 1:500. The peroxidase substrate (ortho-phenylenediamine chloride, OPD (Sigma-Aldrich)) for IgG determination, and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid), ABTS (Sigma-Aldrich) for IgE assay was then added, and absorbance was determined with an ELISA reader (BioRad) at 492 or 405 nm, respectively.

In the IgE-ELISA inhibition experiments, plates were coated with rPar j 1 as previously described, serum samples were pre-incubated for 3 h at r.t. with decreasing concentrations of the allergen used as inhibitor (PjE: from 100 to 0.16 µg/mL; rPar j 1: from 5 to 0.04 µg/mL; rPjC: from 5 to 0.04 µg/mL) in dilution buffer. Binding of IgE was then evaluated as described above.

In the competitive ELISA, rPar j 1-coated plates were pre-incubated o.n. at 4 °C with serial dilutions (1:20, 1:40, 1:80) of mouse immune sera anti-rPar j 1 or with the pre-immune sera as controls. After washing, plates were incubated o.n. at 4 °C with a pool of sera from Parietaria pollen-allergic patients, diluted 1:20 in dilution buffer. Bound human IgE antibodies were then detected with alkaline phosphatase-labelled goat anti-human IgE (KPL, Gaithersburg, MD, USA) diluted 1:1000 in dilution buffer, for 1 h at 37 °C. The alkaline phosphatase substrate *p*-nitrophenyl-phosphate (pNPP, Sigma-Aldrich) was then added, and absorbance was determined with an ELISA reader (BioRad) at 405 nm. The percentage of reduction of human IgE binding after incubation with mouse sera was determined according to the formula: % inhibition of IgE binding = $100 - [\text{ODI} / \text{ODP} \times 100]$, where ODI and ODP represent the absorbance after pre-incubation with immune serum and pre-immune serum, respectively.

Immunoblotting analysis after SDS-PAGE

rPar j 1 (1 µg/lane) and PjE (10 µg/lane) were separated by 15% SDS-PAGE under reducing conditions [35], and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) according to Towbin et al. [36]. Nitrocellulose strips were blocked in blocking buffer (PBS, 0.05% v/v Tween-20, 3% w/v gelatin) for 1 h at r.t. and then incubated o.n. at r.t. with 1:50 (IgE) or 1:1000 (IgG) mouse immune sera or with pre-immune sera as controls, diluted in blocking buffer. After washing, strips were exposed to 1:1000 biotin-conjugated rat monoclonal anti-mouse IgE (BD PharMingen) for 3 h at r.t. followed by avidin-peroxidase (Sigma-Aldrich) at 2.5 µg/mL, or 1:5000 peroxidase-labelled goat anti-mouse IgG (Bio-Rad) for 1 h at r.t., all diluted in blocking buffer. The peroxidase substrate (3,3'-diaminobenzidine, Sigma-Aldrich) was then added to develop the colorimetric reaction.

Spleen cell proliferation assay

Two weeks after the last immunization, mice were killed by cervical dislocation. Individual spleens were aseptically removed and minced, and cell suspensions were prepared. After erythrocyte lysis, spleen cells were resuspended in complete medium (RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 25 mM Hepes, 100 IU/mL penicillin, 100 µg/mL streptomycin) and counted. Proliferation

assays were performed by plating cell suspensions at a concentration of 2×10^5 /well in 96-well round-bottomed plates (Costar, Corning Life Sciences, Acton, MA, USA) in triplicate, and stimulating the cells with 2 µg/mL of rPar j 1 or rPjC, or medium alone as controls. Tritiated thymidine (1 µCi/well) (ICN Pharmaceuticals, Biomedical Division, Milan, Italy) was added on day 6, and cells were harvested 18 h later on Microbeta filter mats using a 96-well harvester. The stimulation index (SI) was calculated with the following formula: mean c.p.m. in allergen-stimulated cultures/mean c.p.m. in controls.

Statistics

Data represents mean values $\pm$ SEM, with the number of mice/group indicated in each figure. Differences between mean values were analysed by the Mann-Whitney rank sum test and the Wilcoxon signed-ranks test. *P*-values <0.05 were considered statistically significant.

Results

Mice immunized with rPar j 1 in alum produce high levels of specific IgE and IgG1 antibodies that are able to recognize native Par j 1

Allergen-specific IgE and IgG responses were analysed by rPar j 1-specific ELISA after two immunizations with rPar j 1 in alum on days 0 and 21. Production of IgE to rPar j 1 could be detected in the mouse sera 1 week after the last immunization and could be still revealed up to serum dilutions of 1:320 (Fig. 1a). No rPar j 1-specific IgE response was observed in control mice immunized with alum alone. Specific IgE induced by rPar j 1 were able to recognize in immunoblotting (Fig. 1b, lanes 1) the Par j 1 component of the whole PjE and the rPar j 1 itself. The molecular size of rPar j 1 appeared to be larger than the band corresponding to the native allergen in PjE probably because of the presence of the His tag, which influences the migration of proteins in SDS-PAGE. Some degradation products with lower molecular size were also recognized by mouse antibodies. No reactivity was observed when blotted allergens were incubated with pooled mouse pre-immune sera (Fig. 1b, lanes 2). Likewise, in ELISA inhibition experiments PjE could inhibit in a similar fashion the binding of mouse IgE (elicited in mice by rPar j 1 immunization), to rPar j 1 adsorbed onto the plate (Fig. 1c).

We then measured rPar j 1-specific IgG responses induced after sensitization with rPar j 1 in alum. High levels of specific IgG, in particular those of the IgG1 subclass, were produced whereas a low production of rPar j 1-specific IgG2a was observed (Fig. 2a). As already shown for IgE, mouse anti-rPar j 1 IgG antibodies reacted with nitrocellulose-blotted whole PjE (Fig. 2b). Taken together, these results indicate that rPar j 1 is a good immunogen and that it retains the IgE and IgG epitopes as its native counterpart represented in PjE.

Mouse antibodies specific to rPar j 1 compete with human IgE for recognition of the same molecule

Next, we evaluated, by a competitive ELISA, whether rPar j 1-specific mouse antibodies induced in our model were able to

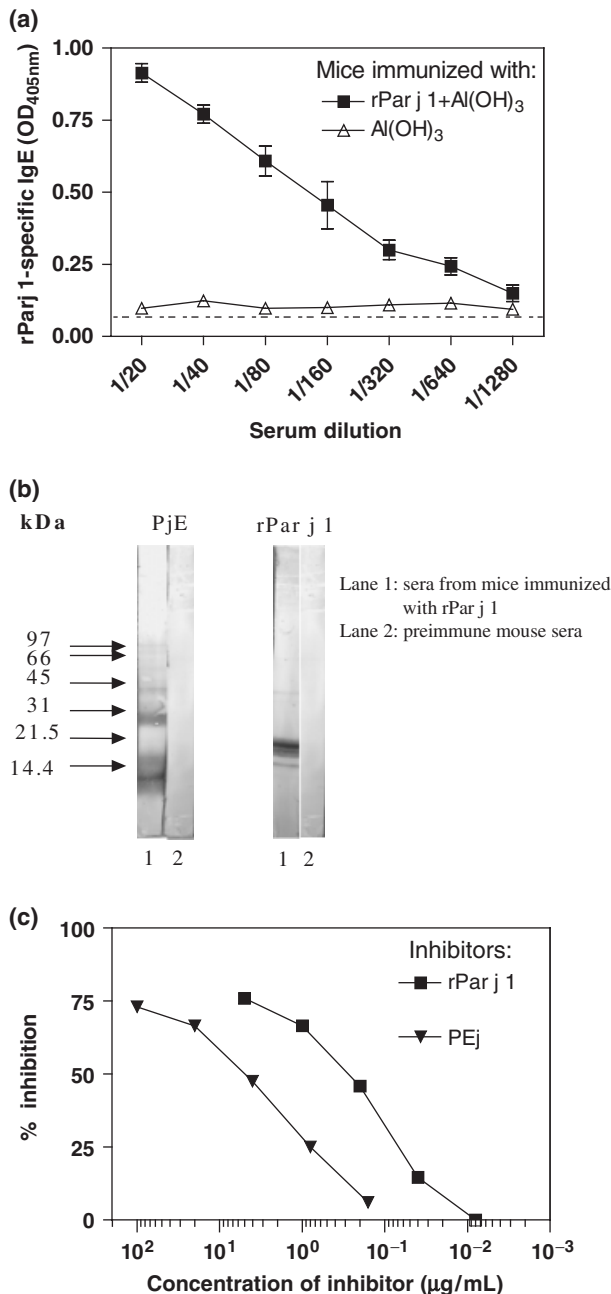


Fig. 1. Specific IgE response to recombinant Par j 1 (rPar j 1) in mice intraperitoneally sensitized with rPar j 1 in Al(OH)₃, and bled 7 days after the last immunization. (a) Titration of serum IgE specific to rPar j 1; dotted line represents the background value (negative control without antigen and immune serum). (b) Immunoblotting analysis after SDS-PAGE showing IgE-binding activity to Pj extract (PjE), and rPar j 1, of pooled sera from mice immunized with rPar j 1 in Al(OH)₃. Lane 1: mouse immune sera diluted 1 : 50; lane 2: mouse pre-immune sera diluted 1 : 50. (c) Inhibition of mouse IgE binding to rPar j 1, by PjE and rPar j 1. Data are representative of five or six experiments. Titration results are expressed as the mean of individual sera from mice ($n = 5$) $\pm$ SEM.

compete with *Parietaria*-specific human IgE for recognition of the same allergen. As shown in Table 1, pre-incubation of the rPar j 1-coated ELISA plate with serial dilutions of pooled mouse immune sera inhibited the binding of IgE from allergic patients. This finding suggests that antibodies produced in mice after the sensitization protocol shared the specificity to rPar j 1 with human IgE-specific antibodies.

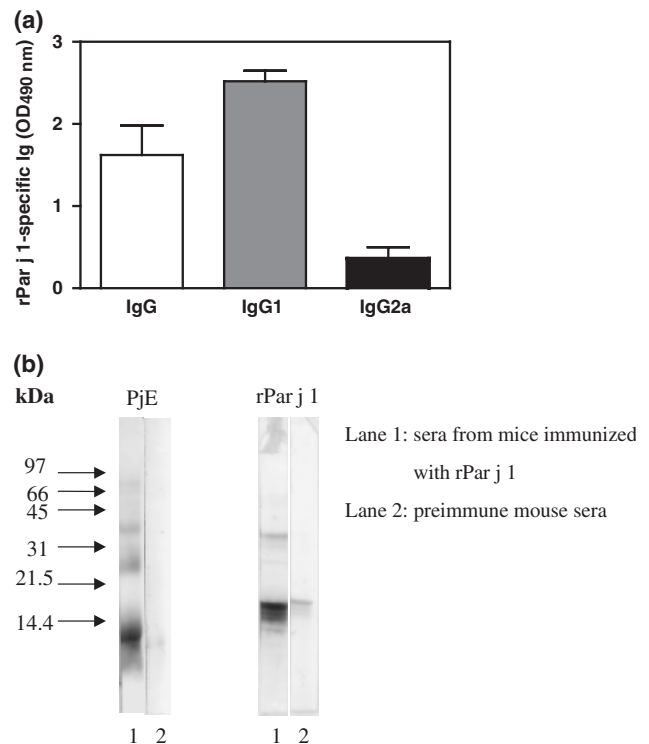


Fig. 2. Specific IgG response to recombinant Par j 1 (rPar j 1) in mice intraperitoneally sensitized with rPar j 1 in Al(OH)₃. (a) Total IgG, IgG1 and IgG2a subclasses. Sera were used at 1 : 100 dilution. Data are representative of five experiments. Results are expressed as mean of individual sera from mice ($n = 5$) $\pm$ SEM. (b) Immunoblotting analysis after SDS-PAGE showing IgG-binding activity to Pj extract (PjE), and rPar j 1, of pooled sera from mice immunized with rPar j 1. Lane 1: mouse immune sera, diluted 1 : 1000; lane 2: mouse pre-immune sera, diluted 1 : 1000.

Table 1. Mouse anti-rPar j 1 antibodies inhibit binding of rPar j 1-specific human IgE*

Mouse sera (dilution)	Human IgE binding after incubation with pre-immune mouse sera (ODP)	Human IgE binding after incubation with mouse anti-rPar j 1 immune sera (ODI)	Inhibition of human IgE binding (%)
1/20	0.844	0.124	85.3
1/40	0.682	0.112	83.6
1/80	0.639	0.151	76.4

*ELISA plates coated with rPar j 1 were pre-incubated with mouse anti-rPar j 1 immune sera or with the corresponding pre-immune sera. Then, a pool of sera from *Parietaria*-allergic patients was added, and bound IgE antibodies were detected. The percentage of reduction of human IgE binding after pre-incubation with mouse sera was determined according to the formula: % inhibition of IgE binding = $100 - (\text{ODI}/\text{ODP} \times 100)$, where ODI and ODP represent the absorbance after pre-incubation of the ELISA plate with immune serum and pre-immune serum, respectively. Human IgE binding in the absence of murine antibodies was 0.986 OD. Background mean value (negative control without antigen) was 0.095 OD.

IgE antibodies from mice immunized with rPar j 1 recognize to a lower extent the disulphide bond variant of rPar j 1, rPjC, whereas this mutant is equally recognized by IgG, IgG1 and IgG2a

Among the mutant molecules generated by Bonura et al. [27], the rPjC mutant, lacking the Cys14–Cys29, Cys30–Cys75 and Cys4–Cys52 bridges, showed a markedly reduced human

IgE-binding activity and was therefore chosen to be tested in our mouse model. When sera from mice immunized with rPar j 1 were tested in direct IgE ELISA using the rPjC mutant as antigen to coat the plate, we observed a significantly reduced binding activity of mouse IgE to this mutant molecule (Fig. 3a). This result was confirmed by IgE inhibition assays (Fig. 3b), where increasing amounts of the rPjC mutant, pre-incubated with a pool of sera from mice immunized with rPar j 1, inhibited the binding of IgE to rPar j 1 (adsorbed onto the ELISA plate) from 15.3% to 26.3%. On the other hand, when the rPar j 1-specific IgG response elicited in the same mice was analysed by ELISA, we found that the rPjC mutant

was recognized to a comparable extent as the rPar j 1 by IgG as well as by the IgG1 and IgG2a subclasses (Fig. 3c).

Mice immunized with the mutant allergen rPjC produce lower levels of IgE, and similar levels of IgG than mice immunized with rPar j 1

Having characterized the immune response induced in mice by rPar j 1 and its similarities with the human IgE response, we then studied the immune responses in mice sensitized with the mutant allergen rPjC. To this end, groups of five mice were immunized either with rPjC or with rPar j 1 in alum. The IgE response anti-rPar j 1 induced by the mutant rPjC was significantly lower than that induced by rPar j 1 (Fig. 4a, white histograms). Moreover, mice immunized with rPjC produced very low levels of specific IgE antibodies against the rPjC mutant itself (Fig. 4a). On the other hand, rPjC was able to induce a comparable IgG response as the rPar j 1 allergen (Fig. 4b: IgG1; Fig. 4c: IgG2a).

Immunization with rPar j 1 or rPjC induces similar in vitro proliferative responses to either rPar j 1 or rPjC

Finally, we characterized the proliferative response both in mice immunized with rPar j 1 and in mice immunized with the rPjC mutant.

First, we evaluated antigen-specific proliferation of splenocytes isolated from mice immunized with rPar j 1 (Fig. 5). Spleen cells were stimulated *in vitro* with rPar j 1 as well as with rPjC mutant. In all the experiments performed ($n = 6$), rPjC was able to induce a proliferative response comparable to that stimulated by rPar j 1 [stimulation indices ranging from 4.5 to 14.9 for rPjC-stimulated cultures and from 7.0 to 13.7 for rPar j 1-stimulated cultures ($P = 0.528$)], suggesting that rPjC, which shows a reduced binding activity to IgE, yet retained the relevant Par j 1-specific T cell epitopes.

Next, to study the proliferative response of mice immunized with the rPjC mutant, we stimulated splenocytes from these mice with either rPjC itself or rPar j 1 as antigens. In all the experiments ($n = 4$), the proliferative response stimulated *in vitro* by the two antigens was not statistically different [stimulation indices ranging from 4.0 to 13.0 for rPjC-stimulated cultures and from 5.0 to 11.0 for rPar j 1-stimulated cultures ($P = 0.151$)] (Fig. 5). In addition, the extent of the proliferative response in splenocytes from mice sensitized with rPjC was similar to that induced in splenocytes from mice immunized with rPar j 1.

Discussion

Recombinant allergens have become attractive candidates for improving diagnosis and immunotherapy of allergic diseases, replacing the use of poorly characterized allergen extracts. Moreover, the use of recombinant allergens modified to reduce their IgE reactivity has been proposed.

A critical point is represented by the modification of the three-dimensional structure of allergens. As a result of the linearity in T cell epitopes as opposed to conformation dependence in B cell epitopes, this approach allows a

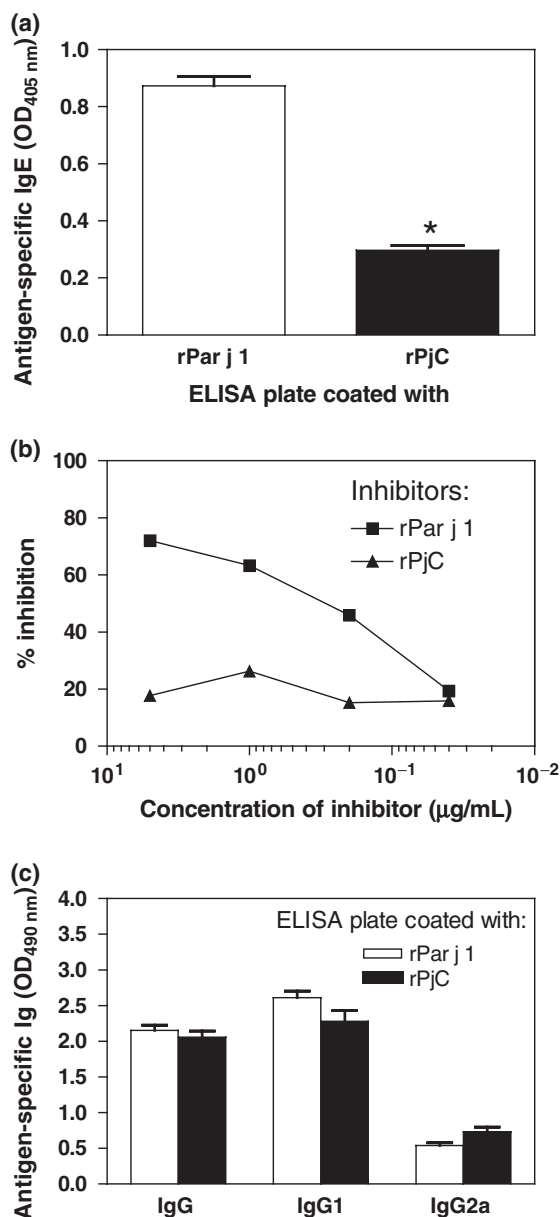


Fig. 3. (a) IgE recognition of recombinant Par j 1 (rPar j 1) and its disulphide bond variant rPjC by sera from mice immunized with rPar j 1. Sera were used at 1:20 dilution. * $P < 0.05$. (b) Inhibition of mouse IgE binding to rPar j 1, by rPar j 1 and by rPjC. (c) IgG recognition of rPar j 1 and rPjC by sera from mice immunized with rPar j 1. Sera were used at 1:100 dilution. Data are representative of four experiments. Results are expressed as mean of individual sera from mice ($n = 5$) $\pm$ SEM.

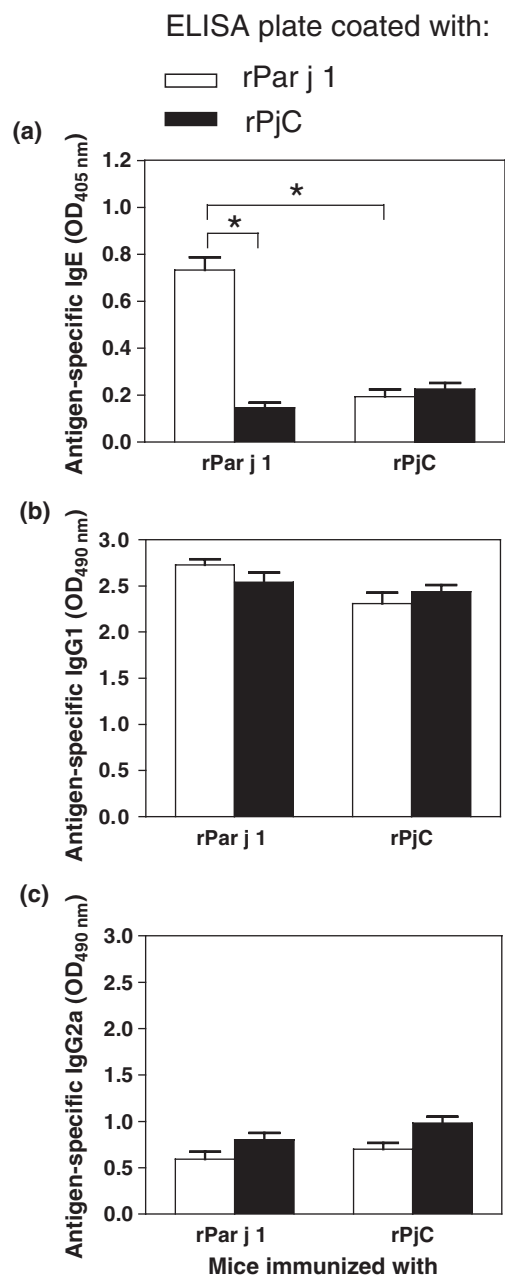


Fig. 4. Specific IgE (a), IgG1 (b) and IgG2a (c) binding to rPar j 1 (white histograms) and to rPjC (black histograms) in mice intraperitoneally sensitized with rPar j 1 or rPjC. Sera were used at 1:20 dilution for IgE detection and 1:1000 dilution for IgG detection. * $P < 0.05$. Data are representative of four experiments. Results are expressed as mean of individual sera from mice ($n = 5$) $\pm$ SEM.

differential regulation of allergen-specific T cell cytokine patterns and IgE:IgG production.

In this context, the progress of recombinant techniques has allowed the development of different approaches to reduce the allergenic potential. Genetic engineering has enabled fractionation or oligomerization of allergens [30, 37–41], as well as the mutation or deletion of amino acids crucial for B cell epitopes, including those involved in the disulphide bond formation [10–12, 14, 15, 22, 27, 40]. Thus, hypoallergenic variants have been developed and extensively characterized for their ability to recognize human IgE and IgG antibodies,

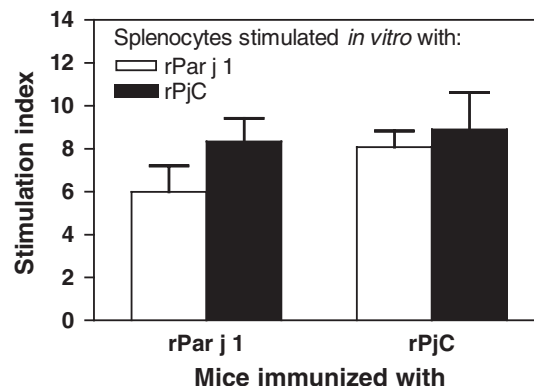


Fig. 5. Proliferation of splenocytes from mice immunized with rPar j 1 or rPjC, and stimulated *in vitro* with rPar j 1 (white histograms) or rPjC (black histograms). Results are expressed as mean stimulation index (SI) $\pm$ SEM, obtained with five individual mice. Data are representative of four to six experiments.

and to induce skin prick test and histamine release activity, as well as *in vitro* T cell activation, and binding of monoclonal antibodies. However, only a few studies have evaluated the *in vivo* activity of such hypoallergens [28–32].

Therefore, the primary aim of this study was to establish a murine model of IgE sensitization to the recombinant major allergen of *P. judaica* pollen, rPar j 1, to test an allergen mutant (e.g. rPjC) displaying, in humans, low IgE-binding activity.

We sensitized BALB/c mice with rPar j 1, and obtained high levels of specific IgE and IgG1 vs. low levels of IgG2a, indicative of a Th2-type response induced by this regimen of sensitization. IgE and IgG1 antibodies from rPar j 1-sensitized mice cross-reacted with the homologous allergen present in *Parietaria* pollen extract. Similar results were obtained in other murine models of sensitization using recombinant grass pollen allergens [42] and recombinant Ole e 1 [43]. Moreover, serum antibodies produced after immunization with rPar j 1 were able to inhibit the binding of human IgE to the same allergen. The fact that antibodies from sensitized mice bound epitopes recognized also by human IgE further supports the hypothesis that mice sensitized with rPar j 1 represent a suitable animal model for the investigation of allergen-specific IgE-binding ability.

In this setting, we studied the ability of a conformational mutant of rPar j 1 (rPjC) [27] to be recognized by rPar j 1-specific murine IgE. The binding of this modified allergen to serum IgE from mice immunized with rPar j 1 was significantly reduced, and the result was confirmed by IgE inhibition assays. On the other hand, the ability of rPjC to bind serum IgG was not affected by the changes in the three-dimensional structure of the allergen. Thus, conformational and/or discontinuous epitopes are involved in the allergenicity of rPar j 1, but not in its overall antigenicity.

T lymphocytes are crucial cellular components in the induction of IgE, as well as in the down-regulation of IgE responses during SIT [44]. The feature of the Par j 1 conformational mutant to retain the ability to activate allergen-specific T lymphocytes was proved in a proliferation assay employing splenocytes from mice sensitized to rPar j 1. Therefore, we evaluated the ability of the rPjC mutant itself to induce an immune response. Our results demonstrate that

the mutant allergen used as an immunogen induced a poor IgE response when compared to that evoked by rPar j 1, and that IgE antibodies raised against rPjC recognized significantly lesser rPar j 1. On the other hand, immunization with rPjC resulted in T cell activation and IgG responses, which were similar to those induced by rPar j 1 itself. These data provide evidence that the disruption of the three-dimensional structure of the Par j 1 allergen does not affect the T cell epitopes required for T cell activation nor those for the induction of IgG responses.

Taken together, our data demonstrate that the murine IgE sensitization model here described is a suitable system for the *in vivo* testing of conformational mutant allergens. They also demonstrate that immunization with this mutated allergen results in a lower level of IgE production, with a conserved ability to induce T cell activation. These conformational variants of allergens, displaying reduced allergenicity accompanied by retained IgG and T cell recognition, offer a safe, specific and flexible approach to immunotherapy of type I allergy. Our mouse model of IgE sensitization to a recombinant allergen, mimicking the human response to its native counterpart, could provide valuable information for pre-clinical testing of such hypoallergenic molecules.

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