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Hypoallergenic mutants of Ole e 1, the major olive pollen allergen, as candidates for allergy vaccines

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Clinical and
Experimental
Allergy

Summary

Background The C-terminal region of Ole e 1, a major allergen from olive pollen, is a dominant IgE-reactive site and offers a target for site-directed mutagenesis to produce variants with reduced IgE-binding capability.

Objective To evaluate *in vitro* and *in vivo* the immunogenic properties of three engineered derivatives of Ole e 1.

Methods One point (Y141A) and two deletion (135Δ10 and 140Δ5) mutants were generated by site-directed mutagenesis of Ole e 1-specific cDNA and produced in *Pichia pastoris*. Ole e 1 mutants were analysed for IgE reactivity by ELISA using sera from olive pollen-allergic patients. Their allergenicity was also investigated in both a mouse model of allergic sensitization and in basophil activation assays. IgG1 response was assayed by immunoblotting and competitive ELISA. T cell reactivity was evaluated by proliferation assays and cytokine production in splenocyte cultures.

Results The 135Δ10 mutant showed the strongest reduction in the IgE-binding capability of sera from olive pollen-allergic patients. Rat basophil leukaemia assays identified the deletion mutant 135Δ10 as the variant with the lowest β-hexosaminidase-releasing capacity. Furthermore, the same 135Δ10 mutant induced the lowest IgE levels in a BALB/c mouse model of sensitization. All Ole e 1 mutants retained their allergen-specific T cell reactivity. Immunization of mice with the mutants induced IgG1 antibodies, which cross-reacted with Ole e 1 and Ole e 1-like allergens from ash, lilac and privet pollens. The ability of the human IgE to block the binding of anti-Ole e 1 mutant-specific mouse IgG1 antibodies to natural Ole e 1 demonstrated that Ole e 1 mutants are able to induce *in vivo* antibodies reactive to the natural allergen.

Conclusion The 135Δ10 mutant with reduced allergenicity, intact T cell reactivity and capacity to induce blocking antibodies could provide a suitable candidate vaccine for efficient and safer therapy of olive pollen allergy.

Keywords allergy, hypoallergen, mouse model, Ole e 1, olive pollen allergen

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Introduction

The prevalence of atopic diseases such as asthma, food allergy and hayfever has increased in industrialized countries over the last 20 years, affecting up to 30% of the population [1]. Current treatments for allergy include avoidance of exposure, pharmacotherapy, which relieves only the symptoms, and conventional allergen-specific immunotherapy. Although the latter treatment has been shown to be effective in many patients, it may occasion-

ally lead to anaphylactic side reactions. Thus, the development of novel therapies has become a desirable aim. Genetic engineering has proven to be a useful tool to convert many important allergens into hypoallergenic derivatives, which have the potential to be safe immunotherapeutic agents as they exhibit little or non-IgE-binding capacity, contain T cell epitopes and induce protective blocking antibodies [2, 3].

Among the outdoor allergen sources, olive tree (*Olea europaea*) pollen is one of the most important causes of

pollinosis in Mediterranean countries and some areas of Australia and North America [4]. To date, 10 allergens from olive pollen have been identified and characterized [5–7]. Ole e 1 has been identified as the most relevant allergen of olive pollen, affecting approximately 80% of allergic patients [8, 9]. It represents the main sensitizing agent within the protein family of Ole e 1-related allergens, which includes Lol p 11 from rye-grass [10], Pla l 1 from plantain [11], Che a 1 from chenopod [12] and its taxonomical relatives Fra e 1 from ash [13], Lig v 1 from privet [14] and Syr v 1 from lilac [15]. Therefore, this allergen has been our first target to design hypoallergenic derivatives for allergen-specific immunotherapy. Ole e 1 has been purified [16], cloned [17] and produced as recombinant protein in the yeast *Pichia pastoris* with biochemical and immunological properties equivalent to those of the natural allergen [18, 19]. Previous studies have been performed in order to identify the human T cell [20] and B cell [21, 22] epitopes of Ole e 1. Recently, a continuous immunodominant IgE-epitope has been identified at the C-terminus of Ole e 1 by using overlapping synthetic short peptides and recombinant larger fragments [22]. This region offers targets for site-directed mutagenesis in order to disrupt the binding of IgE to Ole e 1. This strategy has been successfully applied to a number of important allergens for the development of hypoallergenic molecules for immunotherapy [23–31].

In this study, three Ole e 1 mutants containing changes in the C-terminal end of the molecule were produced in *P. pastoris*, and their immunological properties were evaluated both *in vitro* and *in vivo*. The Ole e 1 mutants were shown to be hypoallergenic, but retained T cell reactivity.

Materials and methods

Sera, antibodies

Sera from 13 olive pollen-allergic patients with a positive reaction to Ole e 1 were included in the study. These patients were diagnosed on the basis of a clinical history of allergy to olive pollen and a positive skin-prick test (SPT) and CAP-FEIA System (Pharmacia AB, Uppsala, Sweden) classes 3–6 to olive pollen. Written informed consent was obtained from all patients. A pool of sera from non-allergic individuals was used as a negative control.

Seven mAbs (F1–F7) against Ole e 1 were obtained in mice and characterized as described [21, 32, 33]. Polyclonal serum against Ole e 1 was prepared as reported [18].

Isolation of natural and recombinant Ole e 1, and preparation of Oleaceae-pollen extracts

Natural Ole e 1 (nOle e 1) was purified from olive pollen as described [16]. Wild-type (WT) rOle e 1 was produced in

P. pastoris as reported [18]. Protein concentrations of purified proteins were determined after hydrolysis with 5.7 N HCl at 105 °C for 24 h, in sealed tubes under vacuum, by amino acid quantifying on a Beckman 6300 (GMI Inc., Ramsey, MN, USA) amino acid analyser.

Pollen from olive (*Olea europaea*), ash (*Fraxinus excelsior*), privet (*Ligustrum vulgare*) and lilac (*Syringa vulgaris*) were purchased from ALK-Abelló (Madrid, Spain). Pollen protein extracts were prepared as described [34]. Protein extracts were analysed by SDS-PAGE [35] and Coomassie blue staining, and stored at –20 °C until use. Protein concentrations were determined according to [36], with BSA (Sigma Chemical Co., St. Louis, MO, USA) as a standard.

Site-directed mutagenesis of Ole e 1

Mutants of Ole e 1 were generated by PCR using the construct pPIC9/Ole e 1 (GeneBank/EMBL databank accession number X76395) as a template and the following specific primers: 5′O1, 5′-CGTCTCGAGAAAAGAGAG-GATGTTCCGCA-3′, the *Xho*I site is underlined. 3′Y141A, 5′-CGTCCGCGGTACATGTTGGGCGGngcATTCC-3′, the *Sac*II site is underlined and bases exchanged are indicated in lowercase; 3′-135Δ10, 5′-CGTCCGCGGTCAATAGAC-TTGTGCACATTTGG-3′, the *Sac*II site is underlined; and 3′-140Δ5, 5′-CGTCCGCGGTACATTCCCACTTATTATAGAC-3′, the *Sac*II site is underlined. PCR products were cloned after restriction digestion into the plasmid pCR2.1 included in the TOPO TA-cloning kit (Invitrogen, Groningen, the Netherlands) and the recombinant construct used to transform TOP10 *Escherichia coli* cells. The expected sequences of the different clones were confirmed after selection.

Expression and purification of Ole e 1 mutants

The DNA fragments were subcloned into the expression vector pPICZαA and electroporated in *P. pastoris* KM71 cells [37]. The Ole e 1 mutants were detected by SDS-PAGE in the supernatant of the 72-h cultures after methanol induction. Large-scale production of mutants was carried out under similar conditions using the colony, which gave the best yield in the small-scale experiments. Ole e 1 mutants were purified from the extracellular media by three chromatographic steps using DEAE-cellulose ion-exchange, Sephadex G-75 size-exclusion and RP-HPLC columns, as described for WT rOle e 1 [18]. Purity was checked by SDS-PAGE and N-terminal amino acid sequence determination.

Sensitization of animals

Female, 6–8-week-old BALB/c mice ($n = 5/\text{group}$) were obtained from Harlan Interfauna Ibérica SA (Barcelona,

Spain). Animals were maintained at the Animal Care Services of the Faculty of Biology (Complutense University, Madrid, Spain), according to the local guidelines for animal care and use. The studies were approved by the Animal Experimentation Ethics Committee of the Complutense University.

Sensitization was performed by i.p. injections with 1 µg nOle e 1, WT rOle e 1 or the mutants adsorbed to 2 mg aluminium hydroxide in 150 µL of PBS at days 0, 7, 14 and 21, as described [38]. Controls received PBS or adjuvant without antigen at the same time-points. Blood samples were collected from the retro-orbital plexus 7 days after the last immunization. Individual sera were prepared and stored at -20°C until use.

ELISA and competition ELISA

Human IgE (hIgE) quantification was performed by indirect ELISA [34]. Recombinant protein-coated plates were incubated with individual sera (diluted 1:10) from Ole e 1-allergic patients for 2 h. The binding of hIgE was detected by mouse anti-hIgE antibody (diluted 1:5000, ALK-Abelló), followed by peroxidase-labelled goat anti-mouse IgG (diluted 1:5000, Pierce Chemical Co, Rockford, IL, USA). Peroxidase reaction was developed with *o*-phenylenediamine chloride (Merck & Co. Inc., Whitehouse Station, NJ, USA) as a substrate. OD was measured at 492 nm.

In the competition ELISA procedure [38], nOle e 1-coated plates were simultaneously incubated with pooled sera of mice sensitized to each Ole e 1 mutants (diluted 1:5000) and non-diluted sera from allergic patients, using non-allergic sera for control purposes. Binding of mouse IgG1 was detected by peroxidase-conjugated rabbit anti-mouse IgG1 (diluted 1:5000, Rockland, Gilbertsville, PA, USA) as described [38]. The inhibition percentage of mouse IgG1 binding to nOle e 1 after the incubation with human allergic sera vs. control sera was determined according to the formula: % inhibition = $(1 - \text{OD}_A/\text{OD}_N) \times 100$, where OD_A and OD_N represent OD after incubation with human allergic sera and non-allergic sera, respectively.

Total IgE (diluted 1:40) antibodies were measured by a sandwich ELISA using the OptEIA mouse IgE set (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Previous studies performed by our group showed that total IgE consists primarily of specific IgE in sensitized mice [38], indicating that specific IgE can be estimated by measuring total IgE levels in mouse sera.

SDS-PAGE and immunoblotting

SDS-PAGE was performed in 15% (w/v) polyacrylamide gels. Recombinant proteins were stained with Coomassie Blue or transferred onto nitrocellulose membranes

(Hybond ECL, Amersham, Buckinghamshire, UK) [39]. Membranes containing blotted proteins were incubated with mAbs and polyclonal serum, all of them specific for nOle e 1 as described [21].

Immunodetection of blotted WT rOle e 1 and pollen extracts with IgG1 (diluted 1:5000) from Ole e 1 mutant-sensitized mice was performed as described [38].

Proliferation and cytokine assays

Seven days after last immunization, spleens from nOle e 1-sensitized mice were removed aseptically, minced, filtered through sterile filters and a cell suspension was prepared as described [38].

For T cell proliferation assays, spleen cells (2×10^5 cells/well, 0.2 mL/well) were stimulated or not, with Con A (2–0.02 µg/mL), WT rOle e 1 and the three mutants (2–0.02 µg/mL) for 96 h in 96-round-bottom microtitre plates (Costar, Cambridge, MA, USA). The cultures were pulsed with 1 µCi/well [^3H]thymidine (Hybond ECL) for 16 h and cells were harvested on microbeta filter mats using a 96-well harvester and proliferative responses were measured by β plate scintillation counting. The stimulation index was calculated as the ratio of cpm of the unstimulated control and cpm of the allergen-stimulated cells.

To measure the cytokine production, spleen cells were cultured in 48-well plates (Costar) at 10^7 cells/mL (0.5 mL/well) and stimulated with or without Con A (2 µg/mL), WT rOle e 1 and the mutants (2 µg/mL). Supernatants were taken after 48 or 72 h and stored at -20°C until analysis. Levels of IL-4, IL-5, IL-10 and IFN- γ were measured by ELISA kits (Pharmingen). Values in pg/mL were calculated according to standard curves obtained with recombinant mouse cytokines. The sensitivity of the assays was 5 pg/mL for IL-4, 60 pg/mL for IL-5, 15 pg/mL for IL-10 and 25 pg/mL for IFN- γ .

Rat basophil leukaemia cell degranulation assay

The assay was performed as described by Hoffmann et al. [40], with some modifications. Rat basophil leukaemia (RBL-2H3) cells (1.5×10^5 /well) were plated in 96-well flat-bottom plates and incubated for 24 h at 37°C under 5% CO_2 . Passive sensitization was performed with sera (diluted 1:20) from nOle e 1-sensitized mice and non-sensitized animals (control) for 2 h. Cells were stimulated with WT rOle e 1 or Ole e 1 mutants (1 µg/mL in Tyrode's buffer) for 1 h in a humidified atmosphere at 37°C . Released β -hexosaminidase was measured in supernatants using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate buffer, pH 4.5. OD was measured at 400 nm. Results are reported as percentage of total β -hexosaminidase content after lyses of cells with 1% Triton X-100. The spontaneous release was subtracted from this total value.

Results

Generation of Ole e 1 mutants

Three Ole e 1 mutants were engineered based on the mapping data of human T cell epitopes [20] and the putative continuous IgE-binding epitope [22] of Ole e 1 (Fig. 1a). Point and deletion mutations were inserted by site-directed mutagenesis into the dominant C-terminal IgE-binding epitope of the molecule that had no overlap with any of the identified T cell determinants. The point mutant Y141A involved one amino acid exchange (Tyr¹⁴¹ → Ala); the two deletion mutants, 140Δ5 and 135Δ10, lacked the 5 and 10 amino acid residues of the C-terminus of Ole e 1, respectively. Ole e 1 mutants were produced in *P. pastoris* as soluble proteins and purified after three chromatographic steps as described in "Materials and Methods". The yield of the purified proteins was around 5 mg/L of yeast culture. Purified Ole e 1 mutants showed a band around 22 kDa after Coomassie Blue-stained SDS-PAGE (Fig. 1b). Edman degradation of the

N-terminal of these recombinant proteins rendered the same sequence EDVPQ, which is according to those expected for positions 1–5 of the amino acid sequences deduced from the clones. Immunoblot analysis showed that all Ole e 1 mutants were recognized by a rabbit antiserum (pAb) raised against nOle e 1 (Fig. 1c). The recognition of Ole e 1 mutants by seven nOle e 1-specific mAbs (F1–F7) was tested by immunoblot (Fig. 1c). The three mutants lost the IgG-binding capacity when assayed against F6, whereas only 135Δ10 displayed a weaker recognition by F3 and failed to bind F4, suggesting that amino acids at positions 136–139 of Ole e 1 were responsible for this binding.

Ole e 1-derived mutants exhibit reduced IgE-binding capacity

The IgE-binding capacity of the Ole e 1 mutants was analysed by indirect ELISA using 13 sera from Ole e 1-allergic patients. The IgE-binding capacity of the three mutants

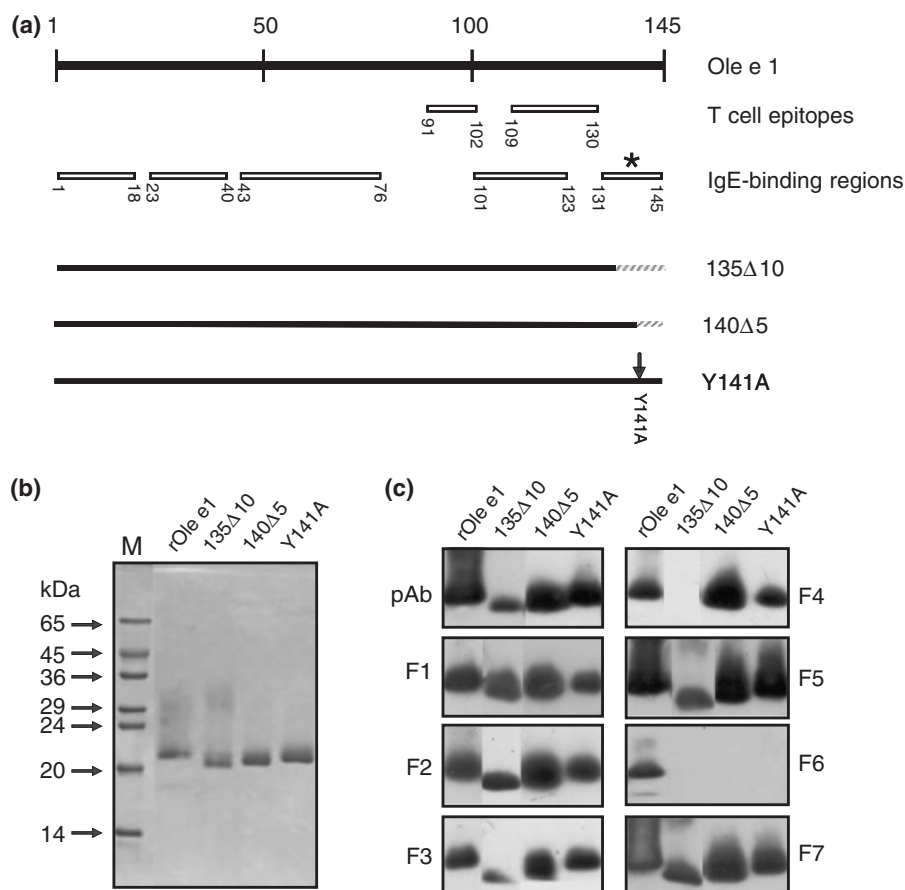


Fig. 1. (a) Schematic representation of the known epitopes (T cell epitopes [19] and Immunoglobulin E (IgE)-binding regions [21], white boxes) of Ole e 1 and the recombinant variants generated by site-directed mutagenesis. 135Δ10 and 140Δ5, deletion mutants; Y141A, point mutant. Point mutation is indicated by an arrow and deleted regions by hatched boxes. (*) Immunodominant IgE-epitope. Analysis of rOle e 1 and Ole e 1 mutants by (b) Coomassie Blue-stained gel after Sodium dodecyl sulphate polyacrylamide gel electrophoresis under nonreducing conditions, and (c) immunoblotting with a polyclonal antiserum (pAb) and seven mAbs (F1–F7) against Ole e 1. M, molecular weight markers.

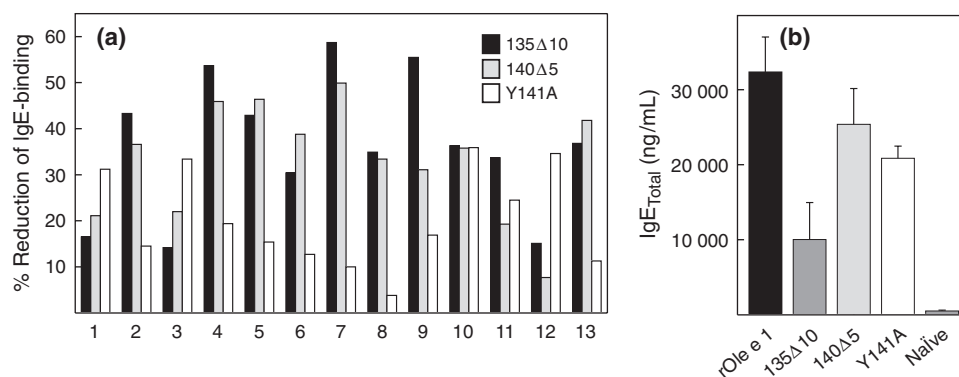


Fig. 2. (a) Immunoglobulin E (IgE)-binding capacity of the Ole e 1 mutants in comparison with the wild-type (WT) using 13 individual patients' sera analysed by ELISA. The results are shown as relative percentages of reduction of IgE binding for each patient in ELISA taking rOle e 1 as a reference (100% IgE binding). (b) Comparison of allergic activities of WT rOle e 1 and mutants in BALB/c mice. Total IgE concentration in sera of rOle e 1 and Ole e 1 mutant-sensitized mice. Results shown are means from three independent experiments with five animals per experiment. Error bars indicate SD.

was reduced by 60–12% compared with that of the WT rOle e 1 depending on the serum samples used (Fig. 2a). The deletion mutants 140Δ5 and 135Δ10 showed a mean reduction of 33% (SD, $\pm 12\%$) and 37% (SD, $\pm 12\%$), respectively, and the mutant Y141A of 20% (SD, $\pm 10\%$). The three mutants were considered to be potentially significant as more than 30% of the patients showed a reduction $> 30\%$. In addition, IgE reactivity to 135Δ10 was reduced more than 30% for 77% of the patients. Although the reactivity of the mutants *in vitro* varied considerably with either patient or mutants, the most consistent and strongest reduction in IgE binding was obtained for the 135Δ10 mutant. The results of the ELISA demonstrated that disruption of the C-terminal IgE-epitope of Ole e 1 decreased the IgE-binding capacity of the allergen.

Mice sensitized with mutants of Ole e 1 produce reduced levels of IgE

The *in vivo* allergenic activity of the Ole e 1 mutants was investigated in a mouse model of sensitization. Groups of five mice were sensitized with WT rOle e 1 or with the mutants. The IgE response induced by the mutants was lower than that induced by WT rOle e 1 (Fig. 2b). Again, the 135Δ10 mutant induced the lowest IgE levels in comparison with WT rOle e 1, 140Δ5 and Y141A.

Allergen-specific basophil degranulation experiments demonstrate reduction of allergenicity for Ole e 1 mutants

The allergenic activity of Ole e 1 mutants was further investigated by evaluating the activation of basophils using the rat basophil degranulation assay. Basophils preloaded with nOle e 1-specific mouse IgE from the seven sensitized mice were stimulated with WT rOle e 1 or the mutants. Stimulation with WT rOle e 1 resulted in activation of basophils in the seven animal sera tested, with

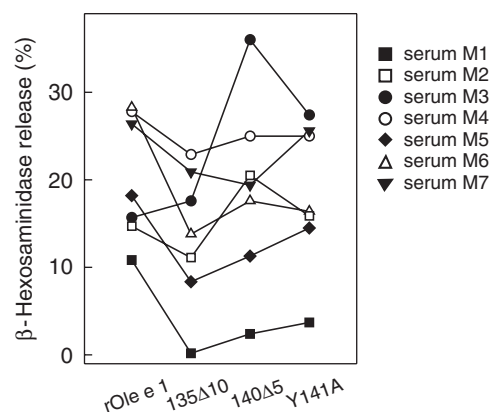


Fig. 3. Biological activity of Ole e 1 mutants vs. wild-type (WT). RBL-2H3 cells were sensitized with sera from nOle e 1-sensitized mice and stimulated with WT rOle e 1 and the three mutants. The percentage of β -hexosaminidase released from RBL-2H3 cells is given after subtraction of baseline release levels. Bars indicate the mean of the values from seven mouse sera.

values of β -hexosaminidase release ranging from 12% to 29% (Fig. 3). A mean decrease in basophil activation of 44% was observed with mutants Y141A, 140Δ5 and 135Δ10 for three (43%), four (57%) and six (86%) of the seven sera tested, respectively. The percentage of β -hexosaminidase release was inhibited by 20–98% depending on the mutant and the serum tested. The level of significant change of the mediator release was set at $> \pm 10\%$ when compared with those of the WT as a reference (100%). Again, the strongest reduction of allergenic activity was observed for the 135Δ10 mutant.

Ole e 1 mutants conserve the ability to stimulate allergen-specific T cells

To investigate whether Ole e 1 mutants conserved the relevant T cell epitopes of Ole e 1, proliferation and cytokine assays were performed with splenocytes from

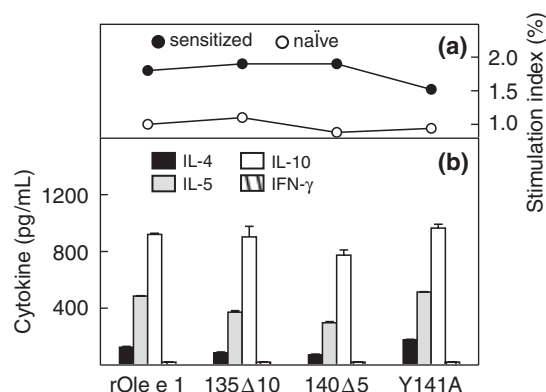


Fig. 4. T-cell reactivity in spleen cell cultures from nOle e 1-sensitized mice after *in vitro* stimulation with wild-type rOle e 1 or Ole e 1 mutants (135Δ10, 140Δ5 and Y141A). (a) Proliferative responses were measured by [³H]thymidine incorporation and indicated as stimulation indices. (b) Cytokine concentrations were determined in supernatants after antigen-specific stimulation by ELISA. Results are shown as mean ± standard deviation of two separate experiments in duplicate from five sensitized mice.

nOle e 1-sensitized mice that resemble the T cell epitope spectrum of Ole e 1. Figure 4a shows that the proliferative responses induced by the mutants were comparable with that induced by WT rOle e 1. Similarly, no significant differences were observed in the cytokine profiles obtained with the mutants in comparison with those induced by WT rOle e 1 (Fig. 4b). IFN-γ levels were not detected in the culture supernatants.

IgE from allergic patients block the binding to nOle e 1 of IgG1 Abs induced in mice by the mutants

To estimate whether the mutants induced *in vivo* IgG Abs capable of blocking the IgE-binding to Ole e 1 from allergic patients' sera, an experimental mouse model was used based on the immunization of animals with the mutants. Then, the ability of hIgE from 9 Ole e 1-allergic patients to inhibit the binding of the mouse anti-Y141A, anti-135Δ10 and anti-140Δ5 IgG1 Abs to nOle e 1 was analysed by ELISA competition (Fig. 5a). hIgE inhibited the binding of IgG1 to nOle e 1 between 56 and 92% (78.7% mean inhibition). No significant differences were detected among mutants.

IgG1 Abs induced in mice by the mutants cross-react with nOle e 1 and Ole e 1-related allergens

The immunization of mice with Ole e 1 mutants induced high levels of specific IgG1 Abs that reacted with rOle e 1, nOle e 1 and Ole e 1-homologous proteins from ash, privet and lilac pollens (Fig. 5b). It can be explained because Ole e 1-like allergens from *Oleaceae* exhibit a high degree of sequence identity of 85–92% with Ole e 1 [13–15]. No

reactivity was observed when blotted allergens were incubated with the corresponding preimmune sera.

Discussion

The designing of hypoallergenic variants has been explored as a way to obtain alternative vaccines for safer and effective immunotherapy of allergy, in order to replace the generalized use of uncharacterized allergen extracts. Site-directed mutagenesis has been successfully applied to reduce or abolish the IgE reactivity of relevant allergens such as Bet v 1 [23–25], Der f 2 [26], Hev b 6 [27], Lol p 5 [28], Par j 1 [29], Phl p5b [30] and Phl p 7 [31]. Because Ole e 1 is the main allergen from olive pollen and represents a marker allergen for sensitization to this pollen and other members of the *Oleaceae* family [41], it was selected as a candidate molecule for vaccine development. The strategy for the design of Ole e 1 variants with reduced allergenic activity was based on the disruption of the immunodominant IgE epitope located at the C-terminal sequence of the molecule [22] by producing one point (Y141A) and two deletion mutants (135Δ10 and 140Δ5). To select the more suitable Ole e 1 mutant for immunotherapy, they were produced at high yield as soluble recombinant proteins in the yeast *P. pastoris* and evaluated by *in vivo* and *in vitro* immunological assays.

The antigenic structure of Ole e 1 mutants was studied by analysing in immunoblotting their reactivities to seven mAbs and one polyclonal antiserum raised against nOle e 1. As F1, F2, F3, F5 and F7 were able to bind all the mutants, and taking into account the conformational nature for most of the mAb epitopes of Ole e 1 [21], these results indicated that these mutations caused minimal perturbations on the overall folding of Ole e 1. The point mutation Y141A was sufficient to abolish the binding to F6 mAb, and suggested the involvement of this residue in the epitope for F6. The fact that 135Δ10 deletion also affected the binding to F4 confirmed our previous results concerning the epitope recognized by this mAb: it is adjacent or partially overlapping to the site for F6 binding [21]. All these results confirmed the immunodominant features of the C-terminal sequence of Ole e 1, which is involved in the IgG epitopes recognized by two mAb (F4 and F6). This is consistent with the notion that both N- and C-terminal segments of proteins often show high antigenic activity, possibly because they are predominantly surface oriented and have a high flexibility [42].

We analysed the IgE-binding capacity of the mutants to sera of Ole e 1-allergic patients and found that all of them exhibited reduced IgE reactivity. The substitution of one single amino acid in Ole e 1, tyrosine by alanine at position 141 (previously identified by epitope mapping as a critical amino acid for IgE binding [22]), was enough to achieve a 20% mean reduction of binding to patients' specific IgE compared with that of WT rOle e 1. The

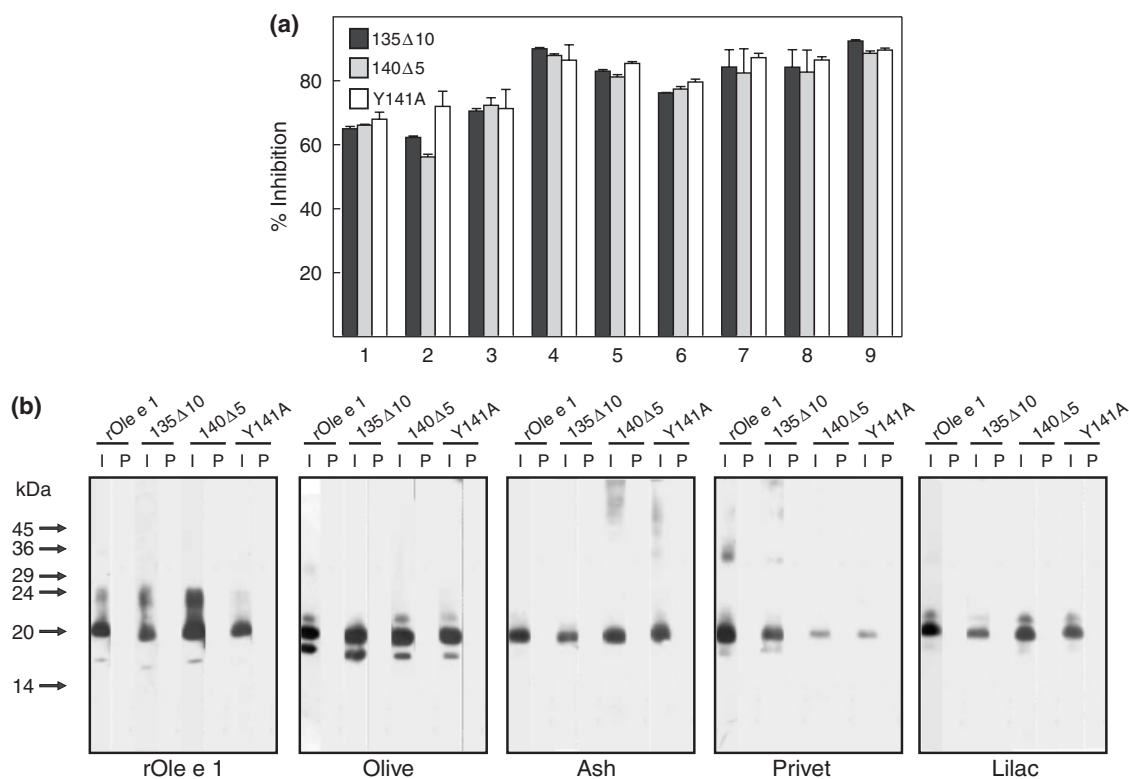


Fig. 5. (a) Inhibition of mouse Ole e 1 mutant-Immunoglobulin G1 (IgG1) binding to nOle e 1 with Immunoglobulin E from nine allergic patients' sera determined by ELISA competition assay. (b) Cross-reactivity of mouse IgG1 induced Abs by Ole e 1 mutant immunization to rOle e 1, nOle e 1 and Ole e 1-homologous allergens compared with rOle e 1. Nitrocellulose-blotted rOle e 1 and pollen extracts (olive, ash, privet, lilac) were incubated with mouse sera raised against purified rOle e 1, 135Δ10, 140Δ5 and Y141A mutants (I) or with the preimmune sera (P).

observation that a few residues from a predicted epitope area are critical for Ab binding has been reported for Ara h 3 [43], Bet v 1 [23], Hev b 6.02 [44], Lol p 5 [28] and Phl p 7 [31] allergens. On the other hand, the deletion of the ten C-terminal amino acids (135Δ10 mutant) decreased the IgE reactivity of about 77% of investigated patients (37% mean reduction). Considering the presence of multiple putative IgE epitopes on Ole e 1, it was expected that disruption of only one of them would not lead to a complete abolition of the IgE reactivity. Although a similar mean reduction of the IgE reactivity was observed for the two deletion mutants, the 135Δ10 mutant revealed a stronger and more consistent reduction of the IgE binding among different patients' sera. The variation of the IgE reactivity among patients could be explained by differences in individual patients' IgE repertoires, which would be determined by a combination of genetic and environmental factors.

The allergenic activity of allergenic molecules is usually evaluated by SPT [30, 31, 44, 45]. However, as the current legislation of several European countries – such as the Spanish one – does not allow the use of recombinant molecules on humans, the RBL-2H3 model system has been used in this study to compare the relative ability of the WT rOle e 1 and the mutants with active basophils as

an alternative to SPT. This system has been shown to be a very sensitive and specific method for evaluation of an allergen-induced immediate type reaction [31, 40]. The major concern of this method is that its specificity depends on mouse serum IgE that results from an artificial sensitization procedure. In this study, sera from Ole e 1-sensitized mice, which had been shown to be suitable with respect to their IgE response [38], were used. When analysed as a group, we found that disruption of the C-terminal IgE epitope of Ole e 1 resulted in a decrease in the allergen-induced degranulation of RBL-2H3 cells, however, with some mouse-to-mouse variation. The strongest reduction of the allergenic activity was observed for 135Δ10 mutant, which exhibited a 43% reduced allergenic activity compared with WT rOle e 1. As it was shown for human IgE, while a single alanine to tyrosine substitution reduced basophil activation, the deletion of the 10 C-terminal residues was necessary to decrease basophil activation in 86% of the mice tested. Our data supported the reliability of this system for testing the biologic activity of allergen and its derivatives without the involvement of allergic patients, as it has previously been shown [31, 40].

The *in vitro* results were confirmed in a mouse model of sensitization. Few studies have analysed the ability of

hypoallergens to induce an IgE response in an animal model [29]. Recently, we developed a mouse model of IgE sensitization to rOle e 1 mimicking the human B and T cell responses to its natural counterpart [38]. The immunization schedule based on repeated injections of low doses of antigen seems to be generally suitable to induce specific IgE in BALB/c mice. Thus, such a mouse model of sensitization could be useful for investigating the IgE responses elicited by the recombinant mutants themselves. Sensitization of mice with Ole e 1 mutants resulted in a lower IgE production when compared with that induced by WT rOle e 1. The deletion mutant 135Δ10 again revealed the lowest capacity to induce an IgE response *in vivo*, about a 4-fold reduction depending on the mouse tested. Our data demonstrated that the mouse model of IgE sensitization is a suitable system for the *in vivo* testing of mutant allergens. Both basophil mediator release and mouse sensitization experiments demonstrated the strongest reduction of allergenicity for the 135Δ10 mutant of Ole e 1. The good correlation of *in vitro* and *in vivo* results allowed us to consider the 135Δ10 mutant as the best hypoallergen variant of Ole e 1 tested.

The central role of T cell in the induction and maintenance of allergy response as well as in immunotherapy mechanisms is well established [46]. Thus, it is crucial that T cell epitopes of the mutants remain functional to be useful in immunotherapy. T cell reactivity of the mutants was analysed by stimulation of nOle e 1-specific spleen cells from nOle e 1-sensitized mice. The ability of the mutants to stimulate proliferation and cytokine release of splenocytes was equivalent to that of WT rOle e 1. These data showed that Ole e 1 mutants conserved the relevant T cell epitopes of Ole e 1 despite the disruption of the IgE reactivity; thus, they may be suitable candidates for alternative immunotherapy strategies aimed at the immunomodulation of T cell [46].

Ole e 1 mutants induced high titres of specific IgG1 Abs in mice that not only recognized nOle e 1 and Ole e 1-like allergens present in related species [13–15] but also competed with allergic patients' IgE for the binding to the natural allergen at significant levels. Similar observations have been made for derivatives of other important allergens such as Bet v 1 [25, 45], Par j 1 [29] and Phl p 7 [31]. The fact that immunization with Ole e 1 mutants induced blocking Abs *in vivo* that recognized similar epitopes as human IgE that are present on Ole e 1 and the homologous allergens from *Oleaceae* pollens may be of great clinical relevance for immunotherapy in humans. Increasing evidences support the importance of blocking Abs of the IgG isotype elicited through the course of the treatment for the successful outcome of the immunotherapy in patients [47, 48]. It must be reasonably expected that vaccination based on Ole e 1 mutants will induce IgG Abs that may contribute to clinical improvement by abrogation of IgE-mediated effects by at least two me-

chanisms: (1) suppression of allergen-induced activation of effector cells, thus reducing immediate symptoms [49], and (2) inhibition of IgE-mediated presentation of allergens to T cells, thus preventing chronic symptoms and disease progression [47].

Although the mouse model of sensitization used in our study has its limitations, this type of *in vivo* model has provided valuable information for preclinical testing of allergen and its derivatives. Moreover, mouse models of allergy represent unique tools for *in vivo* testing of new prophylactic and therapeutic strategies against allergy prior to application in human subjects as well as for understanding the immunologic mechanisms involved in the disease as it has rightly been pointed out by Herz et al. [50].

In conclusion, the decreased allergenic activity of the 135Δ10 mutant of Ole e 1, together with its ability to induce blocking IgG antibodies *in vivo* but preserving T cell epitopes, identified this molecule as the main candidate for immunotherapy of olive pollen allergy and, perhaps, allergies to pollen containing Ole e 1-like allergens highly similar to Ole e 1. Further studies are required to establish the efficiency and safety of 135Δ10 as an immunotherapeutic agent for treatment of allergic patients.

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