

Prophylactic Intranasal Treatment with Fragments of 1,3- β -Glucanase Olive Pollen Allergen Prevents Airway Inflammation in a Murine Model of Type I Allergy

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Key Words

Animal model · 1,3- β -Glucanase · Olive pollen allergy · Prophylactic intranasal treatment · Recombinant allergen

Abstract

Background: Olive pollen is an important cause of allergy in Mediterranean countries. More than 50% of olive-pollen-allergic patients are sensitized against the 1,3- β -glucanase Ole e 9. To date, prophylactic and therapeutic treatments using purified recombinant allergens have not been studied in animal models of olive pollen allergy.

Methods: BALB/c mice were immunized against Ole e 9 combining intraperitoneal injections of the allergen in Al(OH)₃ with airway allergen challenges. A prophylactic treatment was performed by intranasal administration of a mixture of the recombinant fragments of the allergen prior to Ole e 9 sensitization. Serum levels of specific IgE, IgG1, IgG2a and IgG2b were measured by ELISA, and total IgE levels by sandwich ELISA. Bronchoalveolar lavage and lungs from mice were collected to study airway inflammation by light microscopy. **Results:** BALB/c mice immunized against Ole e 9 developed a predominantly Th2-like immune response with allergen-specific immunoglobulin induction and airway inflammation accompanied by the infiltration of eosinophils, lymphocytes, and neutrophils in the lung. Prophylactic

lactic treatment by intranasal application of the recombinant fragments of Ole e 9 avoids airway inflammation induced by sensitization with this allergen although the levels of Ole e 9-specific antibodies remain unchanged.

Conclusions: Prophylactic intranasal treatment with recombinant fragments of Ole e 9 prevents airway inflammation triggered by immunization to this allergen in a murine model of type I allergy.

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Introduction

Allergy to olive pollen is an important disease of increased incidence in Mediterranean countries, and areas of America, Africa and Australia, where this tree is intensively cultivated [1, 2]. In Spain, olive pollen is the second most important cause of pollinosis after grass pollen [3]. The epidemiological and clinical impact of olive pollen is very dependent on the geographical area. Thus, it has been described that patients allergic to olive pollen living in areas with high atmospheric pollen concentration present a more complex allergogram and exacerbation of clinical symptoms than patients living in areas of low-level exposure [4, 5]. Olive pollen extracts contain a high number of allergenic components, from which up to ten (Ole e 1 to Ole e 10) have been characterized [5–7]. Among them, Ole e 9, a 1,3- β -glucanase that belongs to the PR-2 family of

the pathogenesis-related proteins, is a major allergen [8]. Recently, the N- and C-terminal domains of Ole e 9 have been produced as two independent recombinant molecules in the yeast *Pichia pastoris* and they retained the IgE-binding capability of the whole allergen [9, 10].

Specific immunotherapy (SIT) against olive pollen allergy is currently being performed by injections of increasing amounts of allergen extracts to induce hyporesponsiveness [11, 12]. Although SIT with standardized olive pollen extracts has been described as an effective treatment in many cases, it presents several disadvantages such as allergic side reactions, the long duration of the treatment and the necessity of frequent injections [13]. These drawbacks have also been reported for other biological sources. To increase patient compliance, a patient-tailored therapy using the disease-eliciting allergens in recombinant form, as well as the use of less invasive routes have been suggested as alternative approaches [14, 15]. In this respect, the establishment of murine models that mimic the features of human type I allergic diseases constitutes a powerful tool to validate new prophylactic and therapeutic strategies before clinical application [16, 17]. Regarding olive pollen, it has been previously reported that sensitization of mice with Ole e 1, a major olive pollen allergen, induces a Th2 response [18]. However, prophylactic and therapeutic treatments using recombinant allergens have not been assayed in animal models of olive pollen allergy so far. In this work, we have established an Ole e 9-allergic mouse model to determine the effect of prophylactic treatment by intranasal application of recombinant fragments of the allergen.

Materials and Methods

Animals. Female 6-week-old BALB/c mice were obtained from Harlan Interfauna Ibérica (Barcelona, Spain). Animals were maintained at the Animal Care Services of the Universidad Complutense (Madrid, Spain), according to the local guidelines for animal care.

Antigens. Ole e 9 was purified from olive pollen as described [8]. Recombinant N-terminal (rNtD) and recombinant C-terminal domains (rCtD) of Ole e 9 were obtained in *P. pastoris* and purified as described [9, 10].

Allergic Sensitization and Airway Inflammation. Five micrograms of Ole e 9 adsorbed to 2 mg of aluminum hydroxide [Al(OH)₃] in 150 µl of PBS were intraperitoneally injected to BALB/c mice (n = 5) on days 0 and 7. On day 14, mice were intranasally challenged for 3 consecutive days with 10 µg of Ole e 9 in 20 µl of PBS under slight anesthesia. Mice from the control group (n = 5) received Al(OH)₃ by intraperitoneal route or PBS by intranasal route at the same time points.

Prophylactic Treatment. Mice were intranasally treated with a mixture of rNtD and rCtD (50 µg of each domain in 20 µl of PBS)

for 3 consecutive days. This dose was selected based on a previous dose-dependent study in which 5 µg of each recombinant fragments were also administered in the same conditions. Seven days after starting the treatment mice were sensitized to Ole e 9 following the protocol described above (group IT, n = 5). A group of animals was sham treated with PBS and sensitized against Ole e 9 (group AL, n = 5), and another group of animals received PBS by the intranasal route or Al(OH)₃ by the intraperitoneal route at the same time points (group Co, n = 5).

Blood Sampling. Blood samples were collected from the retro-orbital plexus 5 days after the last challenge. Sera were stored at -20°C until used.

Determination of Allergen-Specific Antibodies in Sera. Microtiter plates were coated with 0.1 µg of Ole e 9 in PBS, pH 7.2, overnight at 4°C. Non-specific binding sites were blocked with PBS containing 1% BSA. Serum samples (n = 3 per group and per experiment) diluted in the blocking buffer (1/20,000 for IgG1, 1/400 for IgG2a and IgG2b and 1/10 for IgE antibodies) were incubated for 2 h at room temperature. Mouse immunoglobulin binding was detected using either peroxidase-conjugated rabbit anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b or goat anti-mouse IgE antibodies (diluted 1/5,000 in all the cases; Rockland, Gilbertsville, Pa., USA) and o-phenylenediamine as substrate. Optical density (OD) was measured at 492 nm. Each OD was calculated as the mean of two determinations. Total IgE antibodies were measured by a sandwich ELISA using the OptEIA mouse IgE set (PharMingen, San Diego, Calif., USA) according to the manufacturer's instructions.

Determination of Cellular Infiltration within the Lung after Bronchoalveolar Lavage. Bronchoalveolar lavage (BAL) fluids were collected 24 h after the last intranasal Ole e 9 challenge (n = 2 per group and per experiment). The trachea was cannulated and 1 ml of sterile PBS instilled and gently harvested. Lavage fluid was centrifuged at 1,500 g for 5 min at 4°C. A total of 2×10^5 cells were washed twice with 1 ml of PBS, resuspended in 200 µl of PBS, spun onto microscope slides, air dried and stained with Giemsa solution. At least 200 cells were counted and differentiated by light microscopy according to standard morphological criteria in a blinded manner. The total number of counted cells was set to 100%, and the relative percentage of macrophages, eosinophils, lymphocytes and neutrophils was calculated.

Histopathologic Analysis of Mouse Lungs. After BAL fluid collection, the lungs were carefully removed, separated from heart and thymus, fixed in 10% (w/v) formaldehyde and embedded in paraffin. Longitudinal sections (5 µm) were stained with hematoxylin-eosin solution and analyzed by light microscopy by investigators blinded to the protocol design.

Statistical Analysis. Data were analyzed using Student's t test, and p < 0.05 was considered statistically significant.

Results

Establishment of an Ole e 9-Allergic Mouse Model

Mice subjected to the sensitization protocol developed high levels of specific IgE and IgG1 in the sera on day 21 compared with mice from the control group (fig. 1a). Although an increase in the levels of specific IgG2a was

also observed, specific IgG2b were nearly undetectable (fig. 1a). These results indicate that the sensitization protocol induced a mixed Th2-/Th1-like immune response but with a predominant Th2 response against Ole e 9.

Airway inflammation was analyzed by differential counting of BAL cells. The percentage of eosinophils, lymphocytes and neutrophils was significantly higher in Ole e 9-sensitized mice than in the control group (fig. 1b).

Allergen-Specific Antibody Responses in Prophylactically Treated versus Non-Treated Mice

Prophylactic treatment based on the intranasal administration of a mixture of rNtD and rCtD domains of the allergen prior to sensitization was assayed. Firstly, we studied the effect of the treatment on the Ole e 9-specific antibody induction. Mice from the sham-treated group (AL) sensitized against Ole e 9 demonstrated significantly increased allergen-specific IgG1, IgE and IgG2a and also total IgE levels in sera compared to the control group (Co) (fig. 2). However, pre-treated mice (IT) did not show significant modifications in Ole e 9-specific IgG1, IgE, IgG2a and IgG2b levels in the sera compared to the sham-treated group. Although a decrease in total IgE levels in the sera of pre-treated mice was observed in comparison to those of the sham-treated group, differences were not statistically significant.

Effect of the Intranasal Prophylactic Treatment in Airway Inflammation

To analyze the effect of the prophylactic treatment with rNtD and rCtD on airway inflammation, the cellular infiltration was analyzed in BAL and lungs from mice belonging to the three groups.

As shown in figure 3a, the relative number of eosinophils, lymphocytes and neutrophils was significantly increased in BAL from sham-treated mice (AL) in comparison to mice from the control group (Co), indicating an infiltration of typical inflammatory cells in Ole e 9-sensitized mice. Histological evidence of this airway inflammation is shown in figure 3b, since a prominent perivascular and peribronchial cell infiltration was detected in lungs from sham-treated mice (AL). In contrast, the relative percentage of inflammatory cells was significantly decreased in BAL from treated mice (IT), and no infiltration of cells was detected in the lungs of these mice. These results showed that mice from the treated group displayed a similar cellular profile in BAL and the same lung histological pattern as mice from the control group.

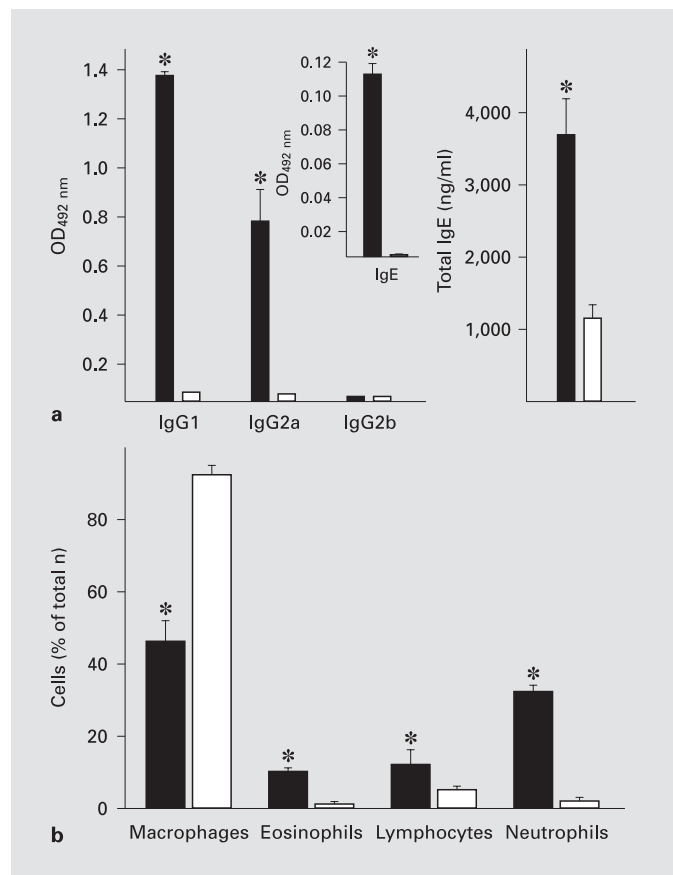
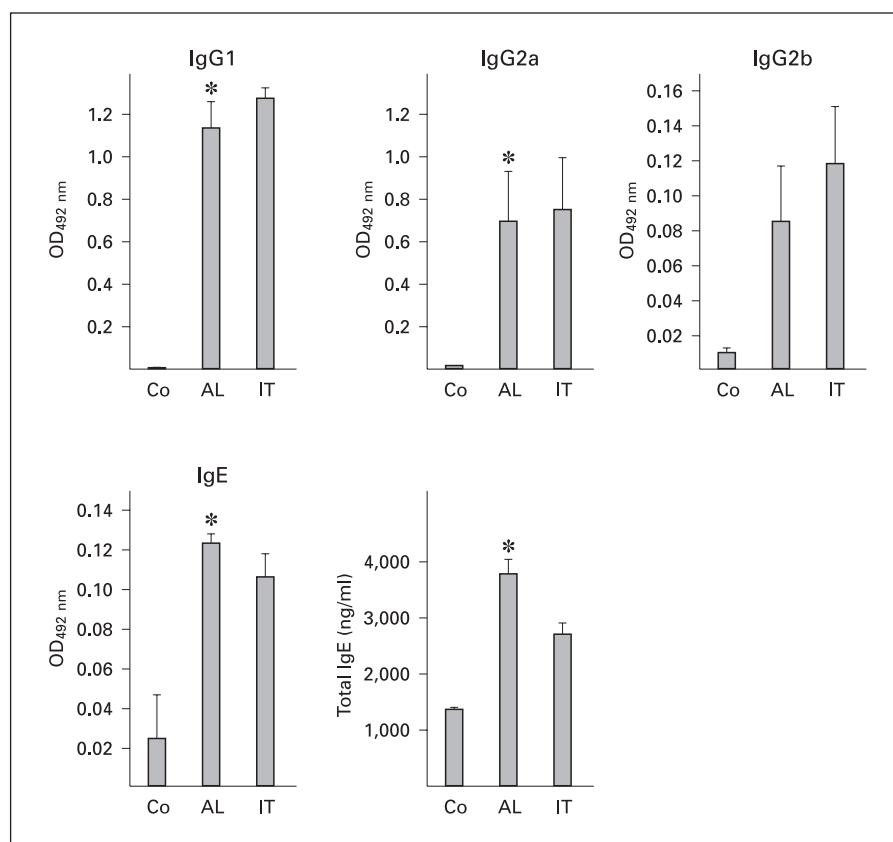


Fig. 1. **a** Total IgE levels and Ole e 9-specific IgG1, IgG2a, IgG2b and IgE in sera from Ole e 9-sensitized mice (■) and from controls (□). Error bars represent standard deviations. * $p < 0.05$. **b** Cell counts (% of the total number of cells counted) in BAL of Ole e 9-sensitized mice (■) and control mice (□). * $p < 0.05$.

Discussion

SIT with olive pollen extracts has been proved as an effective treatment of rhinitis and asthma caused by this pollen in several cases [11, 12]. However, disadvantages such as allergic side reactions, the possibility of newly developing sensitizations, the long duration of treatment with frequent injections and the inherent difficulty of being used in already sensitized individuals have been associated with this conventional immunotherapy. For these reasons, the development of alternative strategies has become a major goal in allergy research. Thus, the possibility of using prophylactic treatments employing purified recombinant allergens has been discussed recently [19]. Prophylactic treatments might prevent the development of allergy by activating the immune system prior

Fig. 2. Ole e 9-specific antibody responses in control (Co), sham-treated (AL) and treated (IT) mice. The levels of IgG1, IgG2a, IgG2b and IgE specific to Ole e 9 were measured by ELISA, and total IgE by sandwich ELISA. Values are the mean of two independent experiments (total n = 6). Error bars represent standard deviations. * p < 0.05 vs. Co (Student's t test).



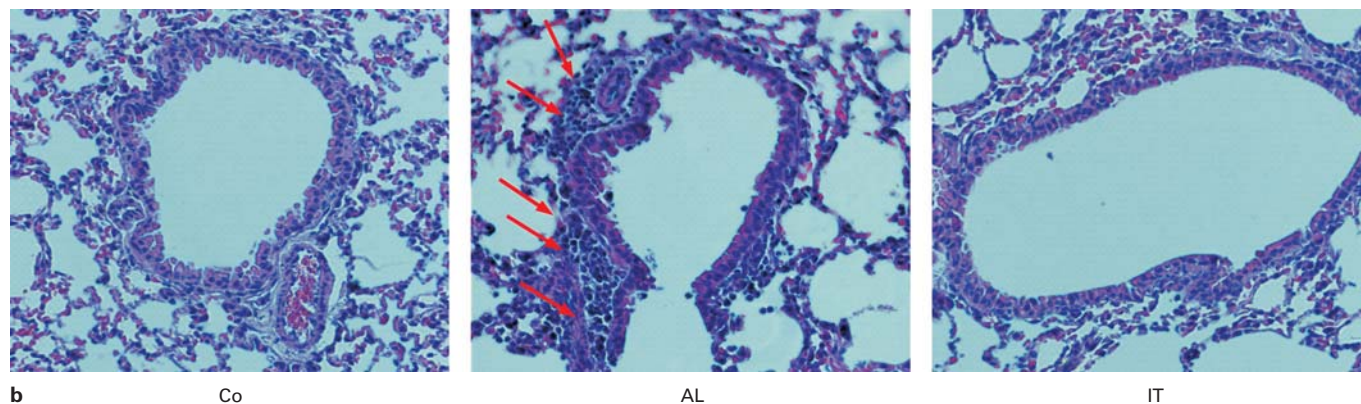
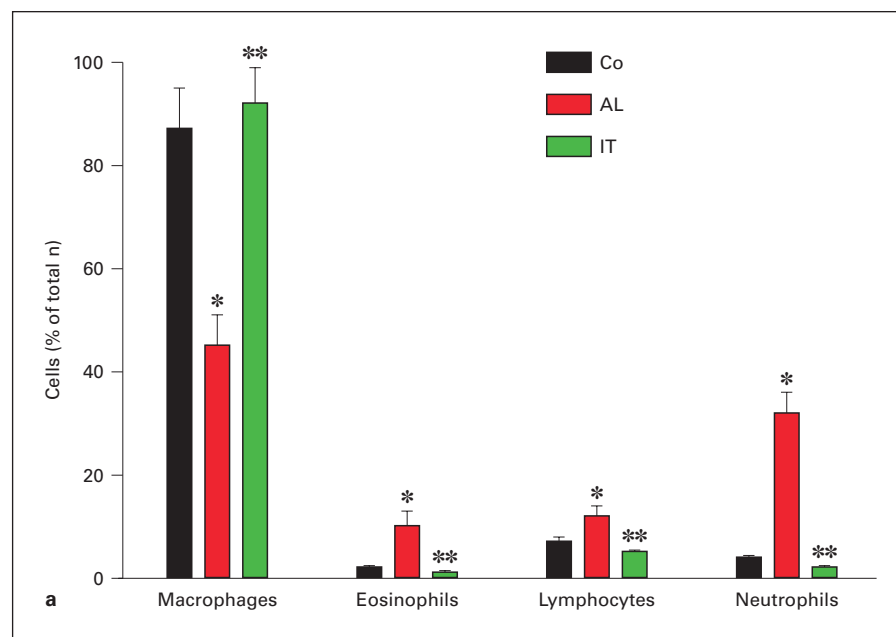
to allergen contact inducing a non-IgE-mediated response associated with IgG production or a mucosal tolerance state of specific non-responsiveness. However, before clinical application of prophylactic treatment in olive-pollen-allergic patients, the establishment of animal models, in which they could be assayed beforehand, could constitute a powerful tool.

In the present work, we have established a mouse model of Ole e 9 allergy combining intraperitoneal injections of the purified allergen adsorbed to the adjuvant Al(OH)₃ with several airway allergen challenges reflecting the pathology of the disease in humans. The results showed that BALB/c mice exposed to purified Ole e 9 developed a predominantly Th2-like immune response with allergen-specific immunoglobulin production and airway inflammation characterized by the infiltration of eosinophils, lymphocytes and neutrophils in the lung.

This model of allergy enabled to analyze the effect of a prophylactic treatment based on intranasal application of rNtD and rCtD prior to Ole e 9 sensitization. This treatment induced a remarkable decrease in the local airway inflammation triggered by sensitization to this major olive

pollen allergen, although a systemic blockade of the Ole e 9-specific antibodies was not observed. The latter may be explained by previous studies in mice models indicating that B cells (serum immunoglobulin levels) and the development of airway inflammation might be independently regulated [20, 21]. In addition, the route/type of sensitization can modify the effects of tolerance induction. Thus, reduced levels of Der p 1-specific IgE were only observed by peptide tolerance induction when mice were subjected to systemic immunization with the allergen but not after respiratory immunization [22]. On the other hand, tolerance induction by prophylactic treatments has also been assessed in several experimental animal studies using other pollen allergens. It has been demonstrated, for example, that intranasal treatment with a recombinant hypoallergenic derivative of Bet v 1 prevented allergic sensitization and airway inflammation in a murine model of birch pollen allergy [23]. Regarding grass pollen allergy, it has been shown that intranasal tolerance induction with Phl p 5 was less effective than that reported for Bet v 1 [24]. These results suggest that tolerogenic capabilities may differ among allergens, and they do not always correlate with the

Fig. 3. a The numbers of macrophages, eosinophils, lymphocytes and neutrophils in BAL were expressed as a percentage of the total number of cells counted. Results are the mean of two independent experiments (total n = 4). * $p < 0.05$ vs. Co; ** $p < 0.05$ vs. AL (Student's t test). **b** Lung micrographs from control (Co), sham-treated (AL) and treated (IT) mice. Lung sections were stained with HE. Red arrows show perivascular and peribronchiolar inflammatory cell infiltration.



immunogenic properties of the molecule. Moreover, it has been demonstrated that intranasal application of a peptide mixture of Bet v 1, Phl p 1 and Phl p 5 induced tolerance in a mouse model of allergic polysensitization while a mixture of the complete allergen molecules did not [25]. Although in the above mentioned studies prophylactic intranasal treatments prevented both airway inflammation and allergen-specific antibody induction, the underlying mechanisms operating in tolerance induction seem to be complex and not only dependent on the nature of the antigen but also on the dose of the antigen and the frequency of application [26].

To our knowledge, our results constitute the first approach to develop new alternatives to conventional SIT in olive pollen allergy in a mouse model. Taking into account that airway inflammation is one of the hallmarks

of allergic burst, impairments in this process could lead to attenuation in the main allergic symptoms. However, future studies based on this Ole e 9-allergic mouse model will be required to assess new strategies and to determine the underlying operating mechanism. In addition, studies to evaluate clinical efficacy and safety will be needed to determine whether prophylactic intranasal treatment using well-defined recombinant fragments of Ole e 9 could be a useful alternative to SIT in olive-pollen-allergic patients.

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