

Sensitization of Mice with Olive Pollen Allergen Ole e 1 Induces a Th2 Response

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Key Words

Type 1 allergy · Olive pollen · Murine model · Ole e 1

Abstract

Background: Olive pollen is one of the main causes of allergy in Mediterranean countries, where it is widely distributed. One inconvenience in studying new immunotherapies for olive pollen allergy is the lack of suitable animal models. The aim of this study was to develop a murine model of IgE sensitization to Ole e 1, the major allergen of olive pollen, which mimics the immunological features of olive pollinosis in humans and to investigate the in vivo antigenicity of the recombinant form of the allergen. **Methods:** BALB/c mice were sensitized by intraperitoneal administration of natural Ole e 1 (nOle e 1) and recombinant Ole e 1 (rOle e 1) in Al(OH)₃, respectively. Serum levels of specific IgE, IgG1 and IgG2a, cytokine production and the proliferative response of splenocytes after in vitro stimulation with nOle e 1 were analyzed by ELISA and flow cytometry. The binding capacity of rOle e 1-specific IgG1 was examined by ELISA and immunoblotting. **Results:** Sensitization with nOle e 1 or rOle e 1 induced high levels of specific IgE and IgG1 versus low IgG2a antibody levels. Splenocytes from sensitized mice exhibited a proliferative response to nOle e 1. In vitro stimulated splenic cells from nOle e 1-primed mice produced IL-4 and low or nondetectable levels of IFN- γ . Spe-

cific IgE and IgG1 antibodies of immunized mice bound to the same Ole e 1 isoforms and showed a similar degree of cross-reactivity as observed for human IgE. Mouse specific nOle e 1 IgG1 was strongly inhibited by IgE from allergic patients. The IgG1 antibodies elicited by rOle e 1 reacted with both the recombinant and natural forms of the allergen. **Conclusions:** A murine model of Ole e 1 sensitization has been established. rOle e 1 shows similar allergenicity and antigenicity to its natural form. This model should provide a useful tool for evaluating antigenic molecules and exploring new therapeutic approaches in order to treat IgE-mediated olive pollinosis.

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Introduction

Pollinosis to olive pollen is one of the most important causes of allergy in the Mediterranean countries, with more than 20% of the population being affected [1]. The major allergen of olive pollen is Ole e 1, a polymorphic glycoprotein of 145 amino acids which is recognized by more than 70% of patients allergic to this pollen. Ole e 1 seems to be the most important allergenic protein of the olive pollen, since its depletion from the pollen extract has been shown to lead to a reduction in the allergenic activity of the extract for an important population of olive pollen-allergic individuals [2]. Its amino acid sequence has

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been determined [3] and contains an N-glycan moiety with allergenic activity [4]. Ole e 1 belongs to a family of pollen-specific proteins which have been suggested to play a role in pollen hydration and/or pollen germination [5]. The cDNA of Ole e 1 has been cloned and expressed in *Escherichia coli* [6] as a fusion protein, and more recently, Ole e 1 has been produced in *Pichia pastoris* as a recombinant secretion protein which displays molecular and immunological properties equivalent to those of the natural allergen [7]. Moreover, the in vivo immunological activity of recombinant Ole e 1 (rOle e 1) has been proven by skin prick test [8]. Ole e 1-like proteins have been isolated from other members of the *Oleaceae* family (*Fraxinus excelsior*, *Ligustrum vulgare* and *Syringa vulgaris*) [9–11]. The structural and immunological similarity exhibited by these proteins could explain the cross-reactivity observed among these pollens, and indicates the existence of a common main allergen in the *Oleaceae* family [9–12].

Current treatments of allergic patients are based on pharmacotherapy by antiallergic, antihistamine and steroid drugs, as well as specific immunotherapy by injection of increasing doses of allergenic extract. However, an effective and safe therapy has essentially not been established and the design of new strategies for the prevention and treatment of allergic disorders, whose prevalence has increased over recent years, has become one of the main goals of the allergy research field.

One of the major difficulties in the development of alternative immunotherapy methods is the lack of suitable animal models which can mimic the clinical situation. The use of BALB/c mice as an animal model of olive pollinosis would provide a valuable preclinical test system for evaluating the potential use of new prophylactic and therapeutic approaches in humans, as well as for studying the events involved in olive allergy. It has been reported that the intraperitoneal administration of allergens in aluminum hydroxide [Al(OH)₃] induces IgE synthesis (Th2 response) in mice, an immunological state comparable to that of patients with type 1 allergy [13–15].

In the present study, a murine model of IgE sensitization to Ole e 1 was established, since the sensitization of BALB/c mice with Ole e 1 induced an immune response comparable to that of human olive pollen allergy.

Materials and Methods

Animals

Female 6-week-old BALB/c mice were obtained from Harlan Interfauna Ibérica SA (Barcelona, Spain). Animals were maintained at the Animal Care Services of the Faculty of Biology of the Universi-

dad Complutense (Madrid, Spain), according to the local guidelines for animal care.

Antigens and Pollen Extracts

Natural Ole e 1 (nOle e 1) was purified from olive pollen (Allergon AB, Ängelholm, Sweden) as previously described [3]. rOle e 1 allergen was produced in *P. pastoris* and purified as described elsewhere [7].

Pollen from olive (*Olea europaea*), ash (*F. excelsior*), privet (*L. vulgare*) and lilac (*S. vulgaris*) were purchased from Allergon AB. Pollen proteins (2% w/v) were extracted [10], analyzed by SDS-PAGE [16] and Coomassie brilliant blue (Serva, Heidelberg, Germany) staining and stored after lyophilizing at –20°C until use. Protein concentration was determined by the method of Lowry et al. [17], with bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, Mo., USA) as standard.

Immunizations

BALB/c mice were intraperitoneally immunized with 1 µg of nOle e 1 or rOle e 1 adsorbed to 2 mg of Al(OH)₃ (Sigma Chemical Co.) in 150 µl of PBS on days 0, 7, 14 and 21. Control groups received either nOle e 1, rOle e 1, PBS or Al(OH)₃ by the intraperitoneal route at the same time points. Blood samples were collected from the retro-orbital plexus at weekly intervals. Sera were stored at –20°C until analysis.

ELISA and Competitive ELISA

Microtiter plates (Costar, Cambridge, Mass., USA) were coated with nOle e 1 (1 µg/ml) in PBS, pH 7.2, overnight at 4°C. Unspecific binding sites were blocked with PBS containing 1% BSA. Serum samples diluted in the blocking buffer (1/20,000 for IgG1, 1/400 for IgG2a and 1/10 for IgE antibodies) were incubated for 2 h at room temperature. Bound mouse immunoglobulins were detected using either peroxidase-conjugated rabbit anti-mouse IgG1 (diluted 1/5,000), anti-mouse IgG2a (diluted 1/5,000) or goat anti-mouse IgE (diluted 1/5,000) antibodies (Rockland, Gilbertsville, Pa., USA) and ortho-phenylenediamine chloride (Merck & Co. Inc., Whitehouse Station, N.J., USA) as substrate. Optical density (OD) was measured at 492 nm.

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.

In the competitive ELISA procedure, nOle e 1-coated plates were incubated simultaneously with mice nOle e 1-sensitive sera (1/20,000) and different dilutions of pooled sera from olive-allergic patients, or from nonallergic individuals for control purposes. Binding of IgG1 was detected as described above. The percentage of inhibition of IgG1 binding to nOle e 1 after coincubation with human allergic sera versus nonallergic sera was determined according to the following formula: percentage inhibition of IgG1 binding = $(1 - \text{OD}_{492 \text{ nm}} \text{ with sera from allergic patients} / \text{OD}_{492 \text{ nm}} \text{ with sera from nonallergic individuals}) \times 100$.

Proliferation Assays

Seven days after the last immunization, the spleen was aseptically removed from the animal, minced and forced through a stainless steel mesh. After erythrocytes were lysed, cells were washed and resuspended in complete medium (RPMI containing 10% fetal bovine serum, 25 mM HEPES, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 mg/ml gentamicin, 2 mM glutamine and

50 μ M 2-mercaptoethanol). Spleen cells (2×10^6 /ml, 0.2 ml/well) were plated into 96-well round-bottomed plates and stimulated with and without concanavalin A (2 μ g/ml) and nOle e 1 (0–5 μ g/ml) for 4 days. Thereafter, cultures were pulsed with 2 μ l/well bromodeoxyuridine (BrdU Flow kit, PharMingen) for 1 h and then harvested, and the incorporated reagent was measured with the fluorescein isothiocyanate-labeled antibromodeoxyuridine antibody following the manufacturer's instructions. The proliferative response was analyzed with a flow cytometer (FACScan, Becton Dickinson, USA). The stimulation index was calculated as the ratio between the mean of the fluorescent signal of cells with allergen and the mean of the fluorescent signal of cells without allergen.

Cytokine Assays

Spleen cells were plated in 48-well plates at 10^7 cells/ml (0.5 ml/well) and stimulated with and without concanavalin A (2 μ g/ml) or nOle e 1 (0.01–10 μ g/ml). After 48 h, supernatants were taken and stored at -20°C until analysis. Then, 96-well plates were coated with rat anti-murine IL-4 (PharMingen) or rat anti-murine IFN- γ (PharMingen) antibodies at 2 μ g/ml in 0.1 M sodium carbonate buffer, pH 9.6, overnight at 4°C . Undiluted cultured supernatants and standard solutions were incubated for 2 h at room temperature. Bound IL-4 and IFN- γ were detected by incubation with biotinylated rat anti-murine IL-4 (PharMingen) or rat anti-murine IFN- γ (PharMingen) antibodies at 1 μ g/ml in PBS-1% BSA for 1 h. Bound antibodies were detected using peroxidase-conjugated streptavidin (diluted 1/1,000, Pierce Chemical Co., Rockford, Ill., USA) and ortho-phenylenediamine chloride as substrate. Values were calculated according to standard curves obtained with recombinant mouse IL-4 and IFN- γ (PharMingen). The sensitivity of the IL-4 assay was 5 pg/ml and that of the IFN- γ ELISA was 25 pg/ml. Cells stimulated with concanavalin A were used as positive control.

Western Blot Analysis

nOle e 1 (0.5 μ g/lane), rOle e 1 (0.5 μ g/lane) and pollen extracts (40 μ g of total protein/lane) were separated by 15% SDS-PAGE according to the method of Laemmli [16]. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham, Buckinghamshire, UK) according to the method of Towbin et al. [18]. After blocking with 3% nonfat milk in PBS-0.1% Tween 20 (blocking buffer), the membranes were incubated with a pool of sera from mice allergic to nOle e 1 or rOle e 1 (diluted 1/10 in blocking buffer for IgE and 1/5,000 for IgG1) at room temperature for 2 h. Bound mouse immunoglobulin was detected with goat anti-mouse IgE (diluted 1/1,000, Rockland) or rabbit anti-mouse IgG1 (diluted 1/5,000, Rockland), both labeled with peroxidase. The blotted allergen was visualized using ECL Western blotting reagent and ECL hyperfilm (Amersham).

Immunodetection of blotted proteins with IgE (diluted 1/10) from patients allergic to olive pollen was performed as previously described [3].

Two-Dimensional Electrophoresis Blot

In the first dimension, Ole e 1 isoforms (5 μ g) were separated by isoelectric focusing with strip gels (4% T, 3% C, BioRad Laboratories, Hercules, Calif., USA) containing an immobilized pH gradient from 4 to 7, with a Protean IEF system (BioRad) according to the manufacturer's protocol. In the second dimension, SDS-PAGE was performed to separate the proteins according to their molecular mass [16]. For immunological analysis, electroblotting was performed

onto nitrocellulose membranes as mentioned above. The determination of pI was achieved using standard proteins (pI range 3.6–6.6, Sigma).

Results

High Levels of Specific IgE and IgG1 Antibodies Were Induced by Intraperitoneal Sensitization with nOle e 1 in Al(OH)₃

BALB/c mice were intraperitoneally immunized with nOle e 1 in Al(OH)₃ 4 times at weekly intervals and their allergic state was determined. nOle e 1-sensitized mice developed high levels of specific IgE and IgG1 antibodies (fig. 1) in sera, starting on the 13th and 20th day, respectively, after the first immunization and peaking on the 28th day. The achieved antibody levels were maintained for 8 weeks after the first immunization. In contrast, very low or even no nOle e 1-specific IgG2a production was observed (fig. 1). No specific antibody production was observed in control mice receiving nOle e 1, PBS or Al(OH)₃. The enhancement of anti-nOle e 1 IgE antibody levels was parallel to the increase in total IgE concentration in the animals' sera, at around 27 times that of the nonsensitized mice (fig. 2).

The significantly increased production of IgE and IgG1 and the low levels of IgG2a in nOle e 1-sensitized mice are compatible with the idea that intraperitoneal immunization of nOle e 1 in Al(OH)₃ causes a Th2 response to this allergen.

Mouse B Cell Responses to nOle e 1 Are Equivalent to Those from Patients Allergic to Olive Pollen

To confirm the allergen-specific binding of IgE and IgG1 and to evaluate the antibody reactivity pattern, Western blotting was performed with sera from nOle e 1-sensitized mice. The specific IgE and IgG1 antibodies from sera of sensitized mice reacted to blotted nOle e 1 (fig. 3a) and showed a similar binding pattern to that previously characterized for IgE from patients allergic to olive pollen [3]. Control sera did not show any immunoglobulin binding to the natural allergen (data not shown). In addition, binding of IgE and IgG1 antibodies from mice and human subjects allergic to Ole e 1 was compared by using two-dimensional SDS-PAGE and immunoblotting (fig. 3b). IgE and IgG1 from nOle e 1-sensitized mice recognized the same pattern of Ole e 1 isoforms as IgE from olive pollen-allergic patients, i.e. two strong bands of pI 5.4 and 5.9 and one weak band of 5.0.

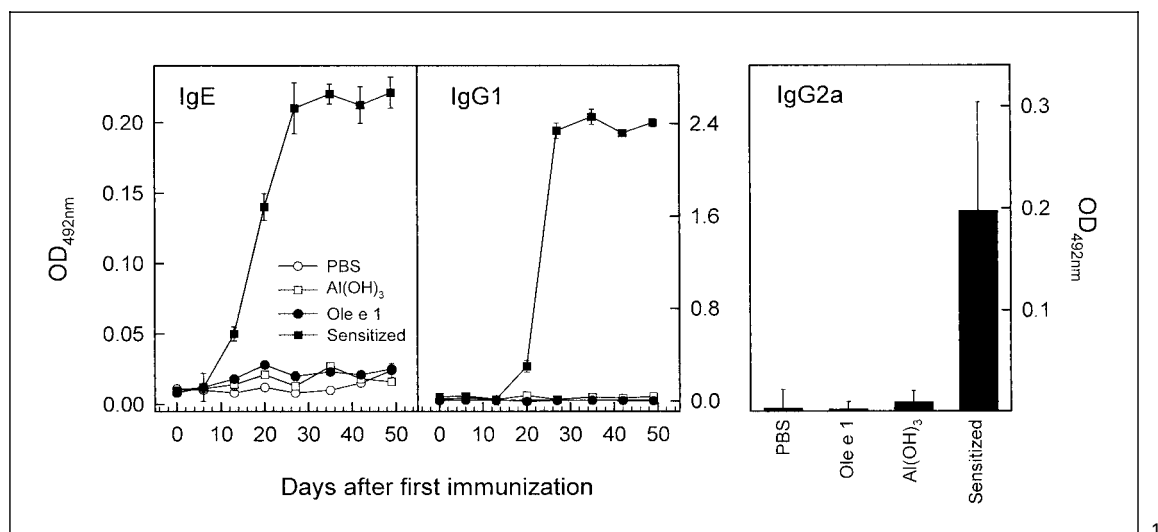


Fig. 1. Time course and levels of specific IgE and IgG1 responses to nOle e 1 in mice after intraperitoneal sensitization with nOle e 1 in Al(OH)₃. Sera were taken at weekly intervals. IgE and IgG1 levels were determined by ELISA and their titers are shown as OD at 492 nm. Results show the mean of individual sera from 4 mice. Data are representative of 7 separate experiments. nOle e 1-specific IgG2a levels of serum from individual animals obtained on the 28th day after immunization were measured by ELISA. Results show the mean of duplicate wells. Data represent 5 independent experiments. Control mice were administered with intraperitoneal doses of PBS, nOle e 1 or Al(OH)₃.

Fig. 2. Total IgE production in sera of nOle e 1-sensitized mice. Levels of total IgE were estimated by a sandwich ELISA assay. Sera were diluted 1/40. The OD value was correlated with the protein concentration with standard curves derived from purified mouse IgE. Total IgE levels of normal serum were 28 ± 2 ng/ml for BALB/c mice. Results show mean values from 4 mice. Data represent 5 separate experiments. Inset: enhancement of total IgE concentration in response to nOle e 1 sensitization on the 28th day after immunization.

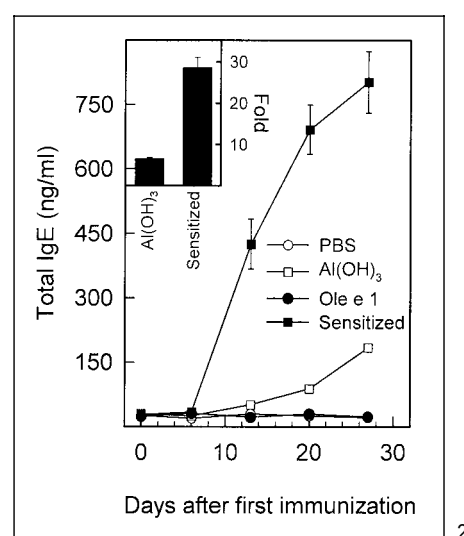


Fig. 3. Immunoblotting of IgE and IgG1 binding to nOle e 1 after SDS-PAGE (a) and two-dimensional SDS-PAGE (b) analyses. A sera pool from nOle e 1-sensitized mice was tested for IgE (mIgE) and IgG1 (mIgG1) reactivities to purified allergen (0.5 µg/lane) in comparison to that of human IgE (hIgE). The arrow indicates the glycosylated form of Ole e 1 (a) and the lines represent the pI values of the allergen (b).

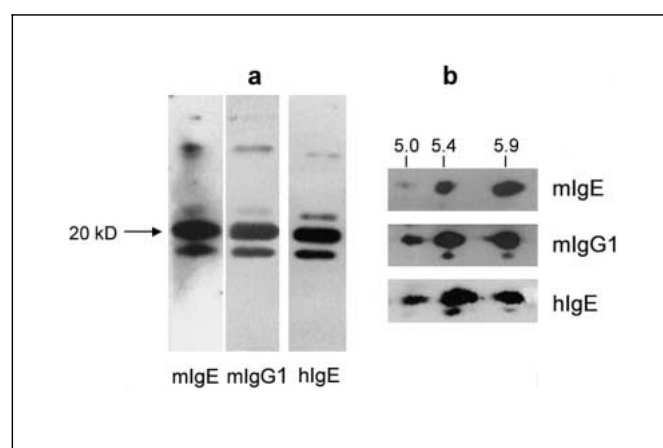
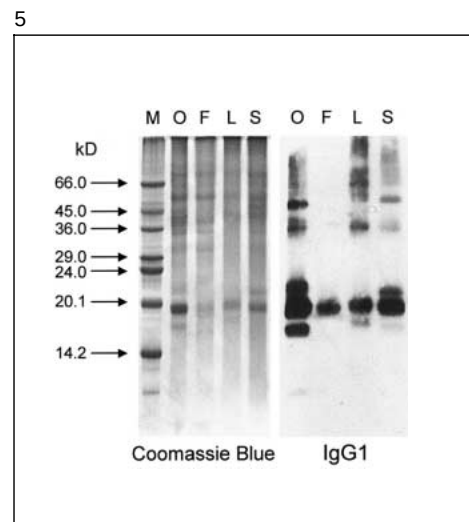
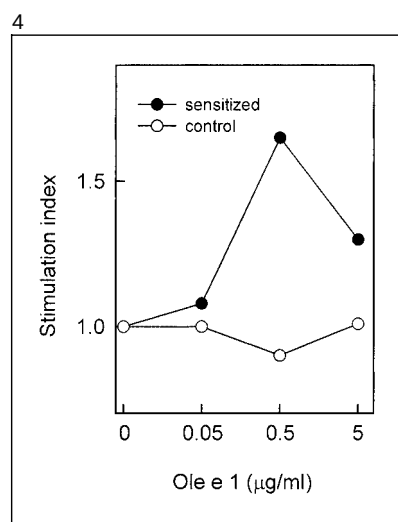


Fig. 4. Proliferation of splenocytes following stimulation with nOle e 1. Splenocytes were cultured in duplicate at 2×10^6 cells/ml. Results are expressed as a stimulation index, which was calculated as the ratio of the fluorescent signal of the allergen to the fluorescent signal of the medium.

Fig. 5. SDS-PAGE analysis of protein extracts (40 μ g of total protein/lane) from *O. europaea* (O), *F. excelsior* (F), *L. vulgare* (L) and *S. vulgaris* (S) pollens stained with Coomassie blue or blotted to nitrocellulose membranes and immunostained with IgG1 from a pool of sera from nOle e 1-sensitized mice. M = Molecular weight markers.



Proliferative Response and Predominant Th2-Type Cytokine Production in Spleen Cells Induced by nOle e 1

To determine the role of T cells in the development of allergy to nOle e 1, the proliferative response and cytokine production of spleen cells from mice sensitized to this allergen were examined. Spleen cells from sensitized mice showed a significant dose-dependent proliferative response to rOle e 1 stimulation, but not those from control mice (fig. 4). Similarly, significant IL-4 levels were detected in nOle e 1-stimulated cultures (22 ± 6 pg/ml) when compared with nonstimulated cells (<5 pg/ml). In contrast, IFN- γ levels were very low or undetectable in Ole e 1-stimulated cells from sensitized mice. Neither IL-4 nor IFN- γ could be detected in the culture supernatant of mice in control groups. According to their cytokine secretion pattern, the immune response of the mice sensitized to nOle e 1 could be classified as a dominant Th2-like response.

Cross-Reactivity of Mouse IgG1 with Ole e 1-Homologous Allergens from Oleaceae Pollens

Anti-nOle e 1 IgG1 mouse antibodies reacted in immunoblotting with Ole e 1-homologous proteins from ash, privet and lilac pollen extracts. Reactive bands of olive pollen extract at 18.5, 20, 22 and 40 kD correspond respectively to the four previously described variants of Ole e 1, i.e. nonglycosylated, glycosylated, highly glycosylated and dimeric forms [3]. IgG1 mouse antibodies recognized similar patterns on ash, privet and lilac pollen extracts (fig. 5), the differences between them being due to the different degree of glycosylation of Ole e 1 counterparts from these *Oleaceae* pollens [3, 10, 11]. In addition,

these patterns were identical to those detected by immunostaining of Ole e 1-like proteins of ash, lilac and privet with human IgE and Ole e 1-specific monoclonal antibodies [10–12]. These results indicated that these allergens displayed common B epitopes for mouse antibodies, as occurred for human IgE.

Human IgE from Allergic Patients Blocks Binding of Mouse IgG1 to nOle e 1

To investigate whether the binding of mouse IgG1 to nOle e 1 can be blocked by human IgE, ELISA competition experiments were performed. Well-coupled nOle e 1 was incubated with sera from nOle e 1-sensitive mice and different dilutions of a sera pool from olive pollen-allergic patients or a sera pool from nonallergic individuals for control purposes (fig. 6). The binding of mouse IgG1 to nOle e 1 was inhibited (up to $51 \pm 2\%$) by the sera from allergic patients but not by nonallergic sera.

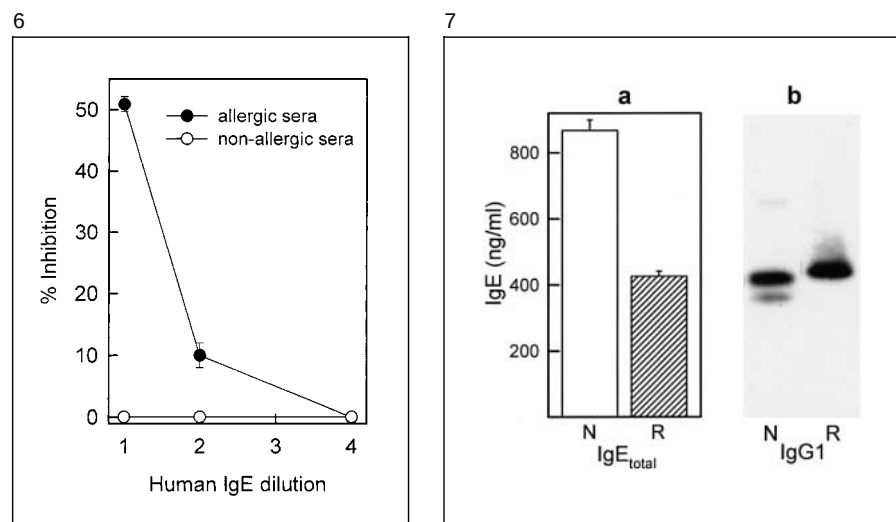
Induction of Ole e 1-Specific Antibodies with rOle e 1

Mice were immunized with rOle e 1 and serum samples were tested for IgE levels and for IgG1 cross-reactivity with nOle e 1 (fig. 7). Immunization with rOle e 1 induced IgE synthesis, and the overall levels were higher in nOle e 1-sensitized mice compared to mice immunized with rOle e 1 (fig. 7a).

To evaluate the IgG1 binding pattern to rOle e 1 and nOle e 1, immunoblotting with sera from mice sensitized with rOle e 1 was performed. As figure 7b shows, IgG1 antibodies induced by rOle e 1 reacted with both recombinant and natural allergen, suggesting that rOle e 1 has similar antigenicity to its natural counterpart in olive pollen.

Fig. 6. Inhibition of mouse IgG1 binding to nOle e 1 with human IgE from allergic patients determined by ELISA competition assay.

Fig. 7. Antibody response to rOle e 1. a IgE levels in sera of rOle e 1-sensitized mice (R) compared to those in animals immunized with the natural allergen (N). b Immunoblotting of rOle e 1 (R) and nOle e 1 (N) with IgG1 from sera of rOle e 1-sensitive mice.



Discussion

The increasing incidence of atopic diseases is a major public health problem in developed countries. The immune response in allergy is characterized by increased serum IgE levels after exposure to the allergen. This humoral response is considered to be a result of Th2 cell activation. In mice, the Th2 response results in IgE and IgG1 production, while the Th1 response leads to IgG2a synthesis [19]. Murine models are useful tools to analyze the sequence of steps that lead to the allergic response, as well as for the development of therapeutic strategies to prevent or minimize this immunological disorder. It is well known that coadministration of protein antigens with Al(OH)₃ preferentially induces an IgE response in mice [13–15]. In this respect, the murine allergic response has been shown to be influenced by the strain, but in general, BALB/c mice are good responders for IgE induction [13, 20]. On the basis of previous studies, we immunized BALB/c mice by intraperitoneal administration of Ole e 1 in Al(OH)₃ in order to establish a murine model of type 1 sensitization to this allergen. The high levels of specific IgE and IgG1 versus low levels of IgG2a induced in these sensitized mice suggested a predominant Th2 response. These results were similar to those observed by other groups [13–15]. Moreover, the IgE response to Ole e 1 induced in mice is long lasting, as observed in allergic patients.

To assess the suitability of this model for testing new approaches to immunotherapy, T and B cell reactivity to nOle e 1 was analyzed. nOle e 1 induced a large proliferative response and represented a predominant Th2-type

stimulating antigen with IL-4 production. Moreover, IgE and IgG1 from sensitized mice recognized the same Ole e 1 isoforms as IgE from patients allergic to olive pollen. Of particular significance is the finding that IgE from allergic patients inhibited the binding of mouse IgG1 to nOle e 1. These data indicate that the IgG1 of immunized mice recognized epitopes defined by human IgE. The immunization of mice with nOle e 1 induced IgG1 antibodies which cross-reacted with Ole e 1-homologous allergens from other members of the *Oleaceae* family. It is well known that Ole e 1 shares significant sequence similarity and immunological properties with the major allergens of ash, privet and lilac [9–12]. In this regard, deduced amino acid sequences of Lig v 1, Syr v 1 and Ole e 1 allergens share an average sequence identity of 85–92% [10, 11], which could be responsible for the recognition by mouse IgG1 antibodies. Our data indicated that B cell epitopes of these major allergens are conserved for mouse IgG1. Similar results have been described for mice models of sensitization using Bet v 1, Bet v 2, dog albumin, Phl p 5 and Ara h 2 [15, 21–23]. The present data support the idea, which was suggested by other groups [24], that a limited number of cross-reactive model allergens could be defined for allergy diagnosis and therapy. It can be concluded that immunization of mice with Ole e 1 induces IgG1 antibodies which display a similar degree of cross-reactivity to that observed for IgE from allergic patients [10, 11]. The immunization of mice with rOle e 1 induced high levels of IgE in serum and specific IgG1 that reacted not only with the recombinant allergen but also with the natural form. The differences observed in the IgE levels could be due to either the polymorphic nature of Ole e 1 or

the N-glycan group of the allergen. These data are in agreement with previous studies [7, 8] and confirm that rOle e 1 exhibits similar in vivo antigenicity to the natural protein, thus supporting the use of rOle e 1 for clinical purposes.

In summary, intraperitoneal administration of Ole e 1 in Al(OH)₃ to BALB/c mice causes a dominant Th2-type immune response against that olive allergen which is evidenced by high levels of specific IgE and IgG1 antibodies, a splenocyte proliferative response and production of IL-4. A mouse model of the IgE response to Ole e 1 which

mimics immunologic features of olive pollen allergy in humans provides a useful tool for exploring new prophylactic and therapeutic treatments for type 1 allergy.

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