

Variability of Ole e 9 Allergen in Olive Pollen Extracts: Relevance of Minor Allergens in Immunotherapy Treatments

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Key Words

Allergen · *Olea europaea* · 1,3-β-Glucanase · Ole e 9 quantitation · Olive pollen allergy · Immunotherapy

Abstract

Background: Clustered severe adverse reactions to immunotherapy with olive pollen extracts have been occasionally reported in areas where olive trees are extensively grown. Allergic patients from these areas, in addition to the major olive pollen allergen Ole e 1, frequently recognize a recently described allergen, Ole e 9. **Objective:** We aimed to develop an immunoassay to measure Ole e 9 concentration and to study the variability of this allergen in olive pollen extracts. **Methods:** Monoclonal antibodies (mAb) to Ole e 9 were produced from mice immunized with the pure allergen. One of these mAbs was used to develop a sandwich ELISA with an anti-olive pollen extract rabbit serum as the tracer. Olive pollen batches from several suppliers were analyzed using this method. These batches were also analyzed for Ole e 1 content and biological activity. **Results:** A 10-fold variation between the extreme values was found for the biological activity of the batches analyzed. Ole e 1 concentration showed a 25-fold variation. Variability of Ole e 9 concentration was extremely high, up to 161 times. The ratio Ole e 1/Ole e 9 varied in a range

from 0.6 to 390.4. **Conclusion:** The availability of a mAb-based ELISA for Ole e 9 made it possible for us to detect an important source of variability in olive pollen batches. This variability may be the cause of outbreaks of adverse reactions in the course of immunotherapy treatments, which have sometimes been observed among olive-allergic patients living in areas with very high levels of airborne olive pollen.

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Introduction

Standardization methods currently enforced for allergen vaccines are based on the measurement of the total allergenic potency and the concentration of major allergens. However, the term ‘major allergen’ is based on a statistical estimation of the prevalence of sensitization to a particular allergen within a general population of patients allergic to a determined allergen source. Since each allergic patient mounts a unique immune response against the offending allergen source, the proteins to which the patient reacts and the concentration and affinities of IgE antibodies produced may be quite different. Thus, some patients can react strongly to allergens statistically considered as minor allergens. As the concentration of these allergens in allergen products is not controlled with the

current standardization methods, these patients could potentially experience severe adverse reactions when subjected to immunotherapy treatment.

Olive (*Olea europaea*) pollen is one of the most important causes of respiratory allergies in the Mediterranean region and some areas of North America, where the olive tree is widely cultivated [1–4]. The prevalence of olive pollinosis depends on the cultivation intensity. Thus, 15.2% of patients suffering from pollinosis, investigated over a period of 1 year, were shown to be skin test positive to olive pollen extract in the Montpellier area [1], and the prevalence in the region of Bari is estimated between 30 and 40% of pollen-sensitized patients [2]. In a study conducted in the southern Spanish province of Jaén, including 294 pollen-allergic patients, over 80% were found to be sensitized to olive pollen [3]. In this province, more than 500,000 hectares are cultivated and atmospheric pollen concentrations during the pollen season reach weekly averages of 500 grains/m³, with exceptional daily peaks higher than 5,000 grains/m³. In past years, we have observed periodical clusters of severe adverse reactions to immunotherapy treatment with olive pollen extracts in areas where this tree is extensively grown. These outbreaks were apparently linked to determined batches of allergen extracts. However, upon extensive analysis, no association with the quality of the extract, analyzed with the established methods for quality control, could be proved, which suggests that adverse reactions might have been caused by a noncontrolled parameter.

Olive pollen extracts show a great complexity in allergen composition. Ten different allergens have been identified in olive pollen up to date [5, 6]. All the authors agree on the identification of Ole e 1 as the most important allergen [5, 7, 8]. However, for the rest of the allergens, prevalence data are variable, since a number of factors, such as the population selected, analytical methods and reagents used to perform the tests, can influence the results obtained [5]. Recently, a new olive pollen allergen, Ole e 9, has been described. Ole e 9, which belongs to the group 2 pathogenesis-related protein family, consists of a single glycosylated polypeptide chain (434 amino acid residues, 46 kD) with 1,3- β -glucanase activity [9]. Structurally, Ole e 9 is organized in two well-defined domains: an N-terminal portion (around 340 residues), containing the catalytic site, and a C-terminal domain (around 100 residues). Both domains have been produced, separately, as recombinant proteins in the yeast *Pichia pastoris*. These recombinant proteins were correctly folded and displayed immunochemical properties similar to their natural counterparts [10, 11]. In a recent study, we ob-

served that patients living in geographical areas with extensive olive tree cultivation produce IgE antibodies to Ole e 9, whereas this allergen is rarely recognized by IgE from patients living outside these areas [Rodríguez et al., unpubl. results].

The objective of this work was to study the variability of the concentration of Ole e 9 in olive pollen extracts, since this variability can represent a risk factor for adverse reactions in some populations of allergic patients. For this purpose, we produced mouse monoclonal antibodies (mAbs) to Ole e 9 and developed a specific immunoassay to measure its concentration.

Materials and Methods

Preparation of Olive Pollen Extracts

Different batches of olive pollen were purchased from qualified collectors licensed to supply pollen source materials to allergen vaccine manufacturers (Allergon AB, Ängelholm, Sweden; Greer Laboratories, Lenoir, N.C., USA; Biopol Laboratory, Spokane, Wash., USA, and Crystal Laboratory, Luther, Okla., USA). Olive pollen was extracted at a 1:10 (w/v) ratio in phosphate buffer, pH 6.5, with magnetic stirring, at 4°C for 90 min. The soluble fraction was separated by centrifugation at 22,000 g for 20 min at 4°C and filtered through 0.22- μ m filters (Sartorius, Göttingen, Germany).

Production of Anti-Ole e 9 mAbs

A preparation of Ole e 9 allergen, purified from olive pollen as previously published [9], was used for mice immunization. Female BALB/c mice (CRIFFA, Barcelona, Spain) were injected intraperitoneally with 16 μ g of Ole e 9 in Freund's complete adjuvant. On day 15 and day 30 after the injection, mice were boosted with an identical amount of antigen in Freund's incomplete adjuvant. On day 42, mice were boosted intravenously with the same dose of antigen in phosphate-buffered saline (PBS) and, 3 days later, spleen cells from immunized mice were fused with P3.X63.Ag8.653 myeloma cells according to the method of Galfré and Milstein [12]. Ten days after the fusion, cell culture supernatants were screened for anti-Ole e 9-specific antibodies by an enzyme-linked immunosorbent assay (ELISA) with the allergen on the solid phase. The P3.X63.Ag8 mouse myeloma culture supernatant was used as a negative control. The positive hybridomas were cloned and subcloned by limiting dilution. The specificity of these hybridomas to the N- and C-terminal domains of Ole e 9 and to Ole e 10 was tested by ELISAs with these proteins, purified following previously reported methods [6, 10, 11], on the solid phase. Selected mAbs were purified from hybridoma culture supernatants using a Protein G Sepharose affinity column (Amersham Biosciences, Uppsala, Sweden), following the manufacturer's instructions. The isotype of the antibodies was determined by ELISA with anti-mouse subclass antisera (Nordic, Tilburg, The Netherlands).

Both mAb and polyclonal antibody production (described below) were carried out in accordance with the OECD principles of good laboratory practice (as revised in 1997) and the European Council Directive No. 609 (1986).

Anti-Olive Pollen Polyclonal Rabbit Serum

Polyclonal antibodies against olive pollen extract were produced in New Zealand rabbits. Each rabbit was injected intramuscularly with pollen extract, 2 mg of protein dissolved in 500 µl of PBS and mixed with the same volume of Freund's adjuvant (complete for the first injection and incomplete for the rest). Injections were repeated every 15 days and sera were collected 10 days after each injection. A rabbit serum pool from 4 animals was made up of three successive bleedings.

SDS-PAGE and IgE Immunoblotting

SDS-PAGE was carried out using nonreducing conditions on a 10–20% tricine-polyacrylamide running gel (Novex, San Diego, Calif., USA). The proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (0.4 × 7 cm) as described by Towbin et al. [13]. After blocking with 1% (w/v) bovine serum albumin (BSA), 0.1% (v/v) Tween 20 in PBS (PBS-BSA-Tween), immunodetection of IgE-binding proteins was achieved by incubating with a 1/3 dilution of a pool of sera from 20 olive pollen-allergic patients, followed by sequential incubations with a 1/3,000 dilution of mouse anti-human IgE mAb HE-2 ascites fluid [14] and then with rabbit anti-mouse immunoglobulin antibodies conjugated with horseradish peroxidase (1/5,000 dilution, Dako, Glostrup, Denmark). IgE-binding proteins were detected by enhanced chemiluminescence following the manufacturer's instructions (ECL, Amersham Biosciences). As a negative control, a membrane blotted with transferred proteins was incubated with PBS-BSA-Tween instead of the human serum pool.

Allergenic Activity of Olive Pollen Extracts

Allergenic activity of olive pollen extracts was determined by RAST inhibition. Paper disks were activated with CNBr and sensitized with an olive pollen in-house reference extract following the procedures described [15]. A pool of human sera as described above was used throughout the experiments. A volume of 50 µl of a 1/3 dilution of the serum pool was added per well to a 96-well microtiter plate (Costar, Cambridge, Mass., USA) and incubated together with the same volume of 3-fold serial dilutions of samples (olive pollen extracts) and in-house reference for 30 min at 37°C. One allergen disk per well was then added and incubated for another 3–4 h at room temperature. After washing the disks 3 times with 0.1% Tween 20 in PBS, about 125,000 cpm/well of ¹²⁵I-labelled anti-human IgE mAb HE-2 [14] was added and incubated overnight at room temperature. Finally, the disks were washed and the bound radioactivity was measured in a gamma counter. The allergenic activity of olive extracts was expressed in BU/ml by comparison with the in-house reference, which previously had been calibrated in BU/ml by skin prick test [16].

Ole e 1 ELISA

Quantitation of Ole e 1 was achieved by a 2-site solid-phase ELISA, adapted from the radioimmunoassay described by Lombardero et al. [8]. Briefly, ELISA plates (Costar) were coated overnight at 4°C with 100 µl of anti-Ole e 1 mAb OL7 at 5 µg/ml in PBS. After blocking with PBS-BSA-Tween for 30 min at room temperature, wells were sequentially incubated with samples and references, biotin-labelled anti-Ole e 1 mAb OL2 (1/1,000 dilution) and streptavidin-peroxidase (1/1,000 dilution, Amersham Biosciences). All incubations were carried out for 1 h at room temperature with intermediate washes with 0.1% Tween 20 in PBS between suc-

cessive steps. Finally, the wells were incubated in the dark at room temperature with peroxidase substrate buffer (0.012% H₂O₂, 0.66 mg/ml *o*-phenylenediamine, OPD; Dako) for 30 min. The reaction was then stopped with 50 µl of 2 M H₂SO₄, and the optical density was measured at 490 nm with a 650-nm reference filter. Assays were performed in duplicate. PBS-BSA-Tween was used as a negative control. The Ole e 1 content of the samples was obtained by interpolating from a standard curve (range 0.435–0.006 µg/ml) constructed with serial 2-fold dilutions of a reference with known Ole e 1 concentration.

Purification of Ole e 9 by Affinity Chromatography

An immunosorbent column was prepared by coupling anti-Ole e 9 mAb 1.18 to CNBr-activated Sepharose 4B gel (Amersham Biosciences), following the manufacturer's recommendations. Olive pollen extract was passed through the column and, after extensive washing with PBS, Ole e 9 was eluted using 100 mM glycine, pH 2.5. 1-ml fractions were collected in tubes containing 50 µl of neutralizing buffer (1 M Tris buffer, pH 9.0). Fractions were pooled on the basis of protein content, dialyzed against distilled water and stored in aliquot samples at –40°C until use. The purity of affinity-purified Ole e 9 was assessed by SDS-PAGE, and the IgE-binding capacity by SDS-PAGE/Immunoblot with a pool of sera from olive pollen-allergic patients. Amino acid analysis was carried out using reagents and equipment from Biochrom (Cambridge, UK).

Ole e 9 ELISA

ELISA plates (Costar) were coated overnight at 4°C with 100 µl of anti-Ole e 9 mAb 18.1 at 10 µg/ml in PBS. After blocking with PBS-BSA-Tween, wells were sequentially incubated with samples and references, anti-olive pollen extract rabbit serum (1/1,000 dilution), and goat anti-rabbit immunoglobulin antibodies conjugated with horseradish peroxidase (1/10,000 dilution; Calbiochem, San Diego, Calif., USA). Samples, controls and reagents were diluted in PBS-BSA-Tween, and all incubations were carried out for 1 h at room temperature with intermediate washes with 0.1% (v/v) Tween 20 in PBS between successive steps. Detection was accomplished with OPD as described for Ole e 1 ELISA. Assays were performed in triplicate. PBS-BSA-Tween was used as a negative control. The Ole e 9 content of the samples was obtained by interpolating from a standard curve constructed with twelve serial 2-fold dilutions of affinity-purified Ole e 9, starting from 5.2 µg/ml.

Results

Production of Anti-Ole e 9 mAbs and Purification of Ole e 9 by Affinity Chromatography

Forty mAbs of IgG class, specific for Ole e 9, were obtained from the fusion of spleen cells of mice immunized with Ole e 9 and P3.X63.Ag8.653 myeloma cells. The specificity of the mAbs for the N- and C-terminal domains of Ole e 9 was tested by antibody capture ELISAs with the recombinant domains on the solid phase. Twenty-four out of the 40 mAbs were directed specifically to the C-terminal domain, 6 to the N-terminal domain, 2 recognized both domains, and 8 did not bind to any of

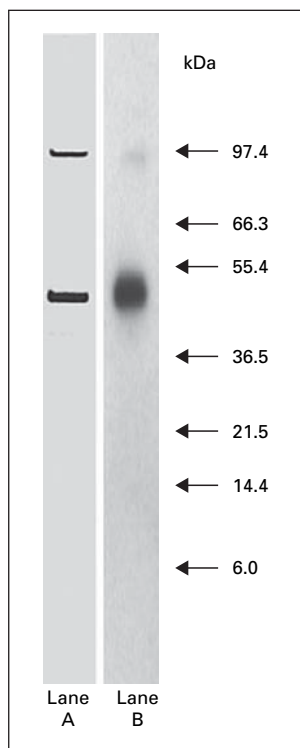


Fig. 1. SDS-PAGE electrophoretic analysis of Ole e 9 purified by immunoaffinity chromatography. Lane A represents Coomassie blue-stained SDS-PAGE pattern and lane B immunodetection with a serum pool from olive-allergic patients.

the separate domains. Since the final objective was to develop a quantitative ELISA for Ole e 9, we took into consideration the possible interferences of other olive pollen allergens to select the most adequate antibody. Thus, to avoid the potential recognition of putative short 1,3- β -glucanases (approximately 340 amino acid residues and equivalent to the N-terminal domain of Ole e 9) that cannot be discarded in olive pollen, mAbs positive to the N-terminal domain of Ole e 9 were rejected for their use in the quantitative ELISA. Moreover, in a similar experiment the 24 anti-C-terminal domain mAbs were tested for their ability to bind Ole e 10, since this allergen shows 53% sequence identity with this domain of Ole e 9 [6]. Nineteen mAbs gave a strong response to Ole e 10, 4 gave a low response, and 1 of them did not recognize Ole e 10. Then, this mAb (18.1) was selected to be used for immunoaffinity chromatography and to develop a specific ELISA to quantify Ole e 9. Affinity chromatography enabled us to purify Ole e 9 from an olive pollen extract. This method rendered Ole e 9 at a high purity level. The electrophoretic pattern of immunoaffinity-purified Ole e 9 (fig. 1, lane A) displayed two bands at 46 and 92 kD, which correspond to the monomer and dimer forms of the protein, respectively [9]. Both bands were immunostained

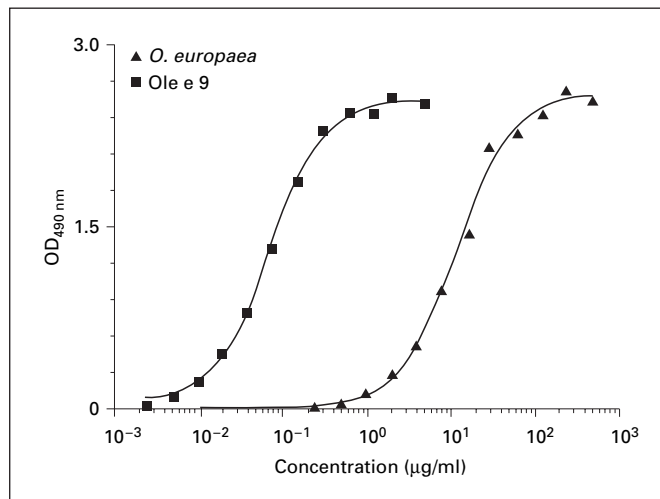


Fig. 2. Dose-response curves obtained in the Ole e 9 ELISA for an olive pollen extract and affinity-purified Ole e 9. Determinations were carried out in triplicate.

with the sera from olive-allergic patients in Western blot (fig. 1, lane B), showing that the allergen maintained its IgE-binding ability after the purification process.

ELISA for Ole e 9 Quantitation

The method we have developed to quantify Ole e 9 consists of a sandwich ELISA with the mAb 18.1 on the solid phase and an anti-olive pollen extract rabbit serum as the second antibody. Detection of the rabbit antibodies bound is accomplished with goat anti-rabbit immunoglobulin antibodies conjugated with horseradish peroxidase.

The dose-response curves obtained in the ELISA with affinity-purified Ole e 9 and olive pollen extract were parallel (fig. 2), showing that the allergen had not been antigenically altered during the purification process and that the assay was measuring the same molecule in the different preparations. The concentration of affinity-purified Ole e 9 was accurately measured by quantitative amino acid analysis, and this preparation was used as the primary standard in the ELISA method. The assay developed in this work is very sensitive, with a detection limit below 10 ng/ml and a practical working range between 20 and 200 ng/ml. Moreover, the assay is highly reproducible. The precision was determined by analyzing six replicates of the same sample in eight independent assays. The mean intra-assay coefficient of variation in these assays was 6.6%, and the interassay coefficient of variation was 11.0%.

Table 1. Specificity of Ole e 9 ELISA

Extract	OD ₄₉₂
<i>Olea europaea</i>	3.637
<i>Syringa vulgaris</i>	3.581
<i>Ligustrum vulgare</i>	3.409
<i>Fraxinus excelsior</i>	0.886
<i>Cupressus arizonica</i>	0.056
<i>Cynodon dactylon</i>	0.016
<i>Plantago lanceolata</i>	0.052
<i>Prunus persica</i>	0.073
Latex	0.082
<i>Dermatophagoides pteronyssinus</i>	0.008
<i>Felis domesticus</i>	0.002

Values of absorbance at 490 nm in Ole e 9 ELISA. Extracts from different allergen sources were tested at a total protein concentration of 100 µg/ml.

The specificity of the method was evaluated against a large battery of extracts from pollen of grasses, trees, weeds, and from other allergen sources, such as latex, mites, mammal epithelia and fruits. Only the extracts from pollens belonging to the Oleaceae family (*Ligustrum vulgare*, *Syringa vulgaris* and *Fraxinus excelsior*) gave a significant response with the ELISA method, but the rest were negative in the assay (table 1).

Variability of Olive Pollen

The variability of olive pollen was studied in twelve different batches using the ELISA described above for Ole e 9 quantitation, an ELISA for Ole e 1 quantitation and RAST inhibition for allergenic activity.

A high variability was observed in the allergenic activity. The activity of the most potent extract was almost 10 times the activity of the extract with the lowest activity. This one (batch sample 1) should be discarded for production of an olive allergy product intended for clinical use because of this low activity (fig. 3).

A higher variability was observed in Ole e 1 content (fig. 4a). Thus, a ratio of 25 was estimated between the sample with the highest Ole e 1 content (batch sample 11) and the one with the lowest content (batch sample 1). As a concentration of 60 µg of Ole e 1/ml has been established for an activity of 100 BU/ml, with an acceptance range between 30 and 120 µg/ml, four additional batches (samples 2, 4, 5 and 8) were found to be out of specifications (fig. 4b).

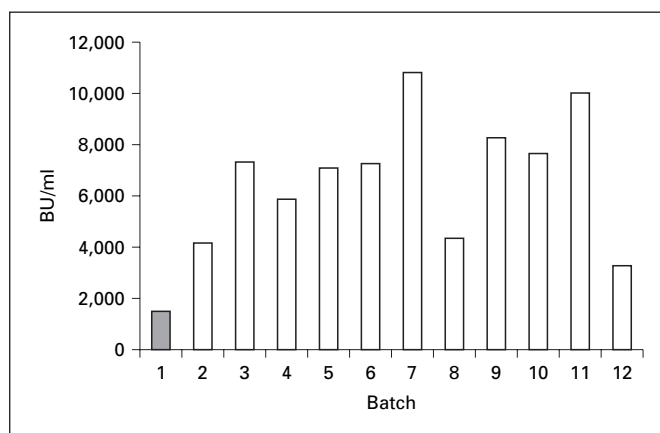


Fig. 3. Allergenic activity of twelve different batches of olive pollen, measured as described in Methods. Shaded bar indicates batch that should be rejected for not fulfilling specifications on allergenic activity.

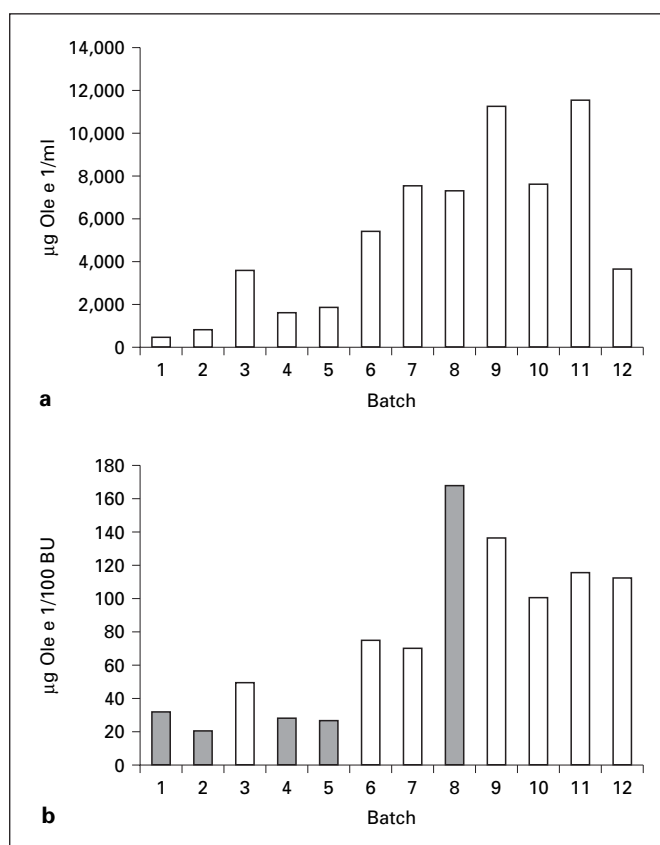


Fig. 4. a Major olive allergen Ole e 1 content in twelve batches of olive pollen. Values were determined by an ELISA method, as described in Methods. **b** Ratio between the concentration of Ole e 1 and activity. Shaded bars indicate batches that should be rejected for not fulfilling this criterion.

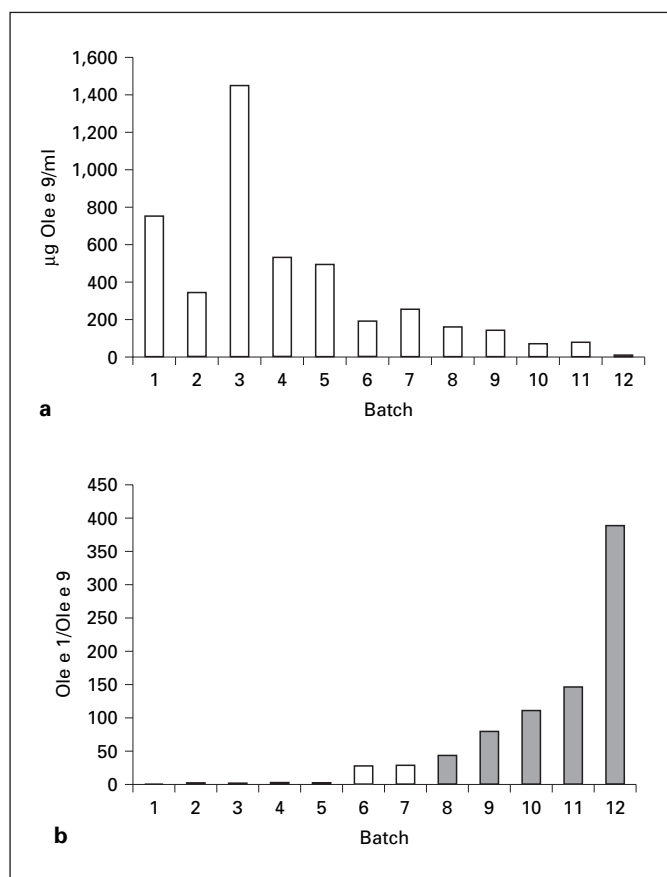


Fig. 5. **a** Olive allergen Ole e 9 content in twelve batches of olive pollen. Values were determined by an ELISA method, as described in Methods. **b** Ratio between the concentration of Ole e 1 and Ole e 9. Shaded bars indicate batches that should be rejected if a range of 50–200% of the median value found (30.0) was defined as a criterion for acceptance.

Moreover, an unexpected extremely high variability was found when the samples were analyzed for Ole e 9 content. Concentration of Ole e 9 in sample 3 was 161-fold higher than the concentration in batch sample 12 (fig. 5a). It should be noted that batches with differences in Ole e 9 content as high as 161 times could be released according to current standardization methods, since both batches were within specifications regarding the former criteria.

When the concentrations of Ole e 1 and Ole e 9 were compared, a significant inverse correlation between them was observed ($r_s = -0.7203$, $p = 0.0082$). The ratio between the concentration of both allergens showed a very high variability (fig. 5b), up to 650-fold, with values ranging from 0.6 (batch 1) to 390.4 (batch 12). The median

value for the Ole e 1/Ole e 9 ratio was 30.0. If acceptance criteria based on this value were established, with an accepted range between 15.0 and 60.0, five additional batches should be rejected (samples 3, 9, 10, 11 and 12). Therefore, only two batches (samples 6 and 7) would fulfill the acceptance criteria.

Discussion

Allergen extracts used therapeutically are crude mixtures of proteins and nonprotein components obtained from natural sources. The first objective of the standardization of allergen extracts is the definition of effective and safe doses for immunotherapy treatment. The combination of biological activity measurement, major allergen quantification and antigenic/allergenic qualitative profile is the most advanced standardization methodology. Nevertheless, in spite of the high standardization level, adverse reactions to immunotherapy treatments are sometimes reported. Since the effective and safe doses are established on the basis of results in averaged populations, mainly reacting to major allergens, patient subsets with different sensitization profiles could account for a significant part of adverse reactions. We had the opportunity to investigate a population overexposed to olive pollen, with a high frequency of sensitization to minor allergens, among which an abnormally high rate of adverse reactions to immunotherapy, clustered in time, had sometimes been recorded. It should be noted that the frequency of recognition of these minor allergens in populations with lower levels of exposition is about 5% [Rodríguez et al., unpubl. results]. Probably, the risk of adverse reactions in these patients is similar to those in highly exposed areas, but the rate of these reactions lie within acceptable limits and then they are not noticed as an outbreak. Therefore, it seems that an important deficiency in the current standardization methods is the fact that a clinically relevant factor, i.e. the amount and affinities of IgE antibodies directed against a particular allergen, either major or minor, that an individual patient can produce, is not taken into consideration. Consequently, the lack of quantitative control on the concentration of minor allergens might be the origin of a proportion of side effects observed when administering immunotherapy treatments, anaphylaxis being the major risk associated with these treatments.

In the particular case of olive pollen allergy, outbreaks of adverse events are almost exclusively confined to two southern Spanish provinces, Jaén and Córdoba, with very

high levels of exposure to airborne olive pollen. Among the allergens from olive pollen, a variability in the concentration of panallergens (profilin, Ole e 2 [17] and polcalcin, Ole e 3 [18]) is not very likely to provoke these outbreaks, as a more varied geographic pattern should have been observed. In contrast, we observed that sensitization to 1,3- β -glucanase (Ole e 9) is more frequent in patients living in those areas. Then, we decided to study the variability of this allergen in olive pollen as a potential cause of these adverse reactions. To this purpose, we developed a mAb-based sandwich ELISA to measure Ole e 9 content in solution. One out of forty Ole e 9-specific mAbs (mAb 1.18), which does not recognize Ole e 10 or putative short 1,3- β -glucanases, was selected for the quantitation ELISA. This mAb was also used to purify the allergen in an immunoreactive state using an efficient one-step procedure.

The quantitation ELISA method developed is specific for Ole e 9 and homologous proteins in pollen from the same Oleaceae family, and showed a high sensitivity. The presence of IgG- and IgE-reactive peptidic epitopes common to Ole e 9 has been shown in extracts from latex, some tree pollen and vegetable foods, using a polyclonal rabbit antiserum to the recombinant N-terminal domain of Ole e 9 and sera from olive pollen-allergic patients [11]. The epitope specificity of the mAb used as the capture antibody in the quantitation ELISA must account for the exclusive recognition of Ole e 9 homologues only in the taxonomically closely related Oleaceae plants.

The method described here allowed us to determine the concentration of Ole e 9 in twelve different batches of olive pollen. Moreover, we measured the Ole e 1 content and the biological activity of the extracts. Variability of Ole e 1 content in olive pollen had previously been reported [19], and these observations have been corroborated in this work, as variations of up to 25-fold were detected. Nevertheless, this value is low when compared to the extreme variability observed for Ole e 9 content. It is worth mentioning that most of the extracts analyzed could have been commercialized fulfilling the quality controls, established according to the current criteria of standardization, i.e. total allergenic potency and major allergen content. Thus, batches of allergen vaccines with 160-fold variations in the concentration of Ole e 9 could be released, implying a high risk of severe adverse reactions. Moreover, if the dosage were established considering only the Ole e 1 content, the ratio Ole e 1/Ole e 9 could vary up to 650-fold. It seems evident that treatment of highly exposed allergic patients, sensitized to Ole e 9, with an allergen vaccine in which the content of this allergen

was not adequately controlled could involve an elevated risk of anaphylactic reactions. Therefore, the new method developed enabled us to detect an unknown source of variability in batches of olive pollen. This high variability in Ole e 9 content, previously unnoticed, might have been the origin of the outbreaks of severe adverse reactions to olive immunotherapy treatment.

Apart from Ole e 9, we are investigating the variability of other minor allergens, Ole e 6 [20] and the lipid transfer protein Ole e 7 [21]. Moreover, it should be noted that, in addition to the variable amounts of minor allergens, patients sensitized to multiple allergens in a particular extract could simply not tolerate the cumulative allergen dose fixed for an average population. Preliminary data indicate that this could be the case [Rodríguez et al., unpubl. results]. These considerations could probably be extended to other allergen sources, and support the need for further improvement in allergen extract standardization.

In conclusion, to increase the safety of allergen vaccines, it is important to minimize the variations of minor allergen content. This can be achieved by using methods to control the content of minor allergens like the one reported here for the allergen Ole e 9. By using this method, the variability of olive allergen products can be significantly reduced, and the balance between major and minor allergens can be maintained within adequate limits to reduce the risk of adverse reactions.

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