
Allergenic contribution of the IgE-reactive domains of the 1,3- β -glucanase Ole e 9: diagnostic value in olive pollen allergy

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Background: Designing of methods for an accurate diagnosis is a main goal of allergy research. Olive pollen allergy is currently diagnosed using commercially available pollen extracts that do not allow identification of the molecules that elicit the disease.

Objective: To analyze the suitability of using the N- and C-terminal domains (NtD and CtD, respectively) of the 1,3- β -glucanase Ole e 9, a major allergen from olive pollen, for in vitro diagnosis.

Methods: Serum samples from 55 olive-allergic patients were assayed using enzyme-linked immunosorbent assay to study hypersensitive patients with IgE reactivity to Ole e 9. The specific IgEs to NtD and CtD, obtained by recombinant technology, were determined by means of immunoblotting, enzyme-linked immunosorbent assay, and inhibition assays.

Results: Thirty-one of 33 serum samples from Ole e 9-allergic patients were IgE reactive to recombinant NtD (rNtD) ($n = 26$ [79%]), recombinant CtD (rCtD) ($n = 22$ [67%]), or both ($n = 17$ [52%]). Nine patients (27%) were exclusively reactive to rNtD and 5 (15%) to rCtD. Inhibition assays of IgE binding to Ole e 9 with a mixture of both domains abolished 90% of the binding, whereas 44% and 45% were abolished when rNtD and rCtD were used, respectively.

Conclusions: Because sensitization to NtD or CtD of Ole e 9 could be correlated to vegetable food-latex-pollen cross-reactivity processes or to the exacerbation and persistence of asthma, respectively, these molecules could be used in vitro as markers of disease to classify patients and to design a patient-tailored immunotherapy approach.

Ann Allergy Asthma Immunol. 2006;97:61–65.

INTRODUCTION

Olea europaea pollen is an important cause of seasonal respiratory allergy in Mediterranean countries and in areas of America, Africa, and Australia, where this tree is intensively cultivated.^{1,2} Patients allergic to olive pollen are being diagnosed using commercially available protein extracts obtained from the pollen. These preparations are usually standardized in biological units or on the basis of the level of Ole e 1, which is a major allergen and constitutes up to 20% of the total protein.^{3–5} In addition to Ole e 1, other olive pollen allergens have been characterized,^{6–9} and some of them, such as Ole e 9 and Ole e 10, have been described as major allergens in populations exposed to high levels of olive pollen.^{8–10} However, most of those allergens are present in very low amounts in the pollen cell^{8,9,11} and, therefore, in the extracts used for clinical purposes.

Ole e 9 is a major inducer of IgE-mediated hypersensitivity, affecting more than 50% of olive pollen-allergic patients from Mediterranean areas.⁸ However, this allergen represents less than 0.3% of the total protein content of the pollen.⁸ Ole

e 9 exhibits 1,3- β -glucanase activity and, thus, belongs to group 2 of the pathogenesis-related protein family (PR-2).⁸ Ole e 9 is composed of an N-terminal domain (NtD) (36-kDa molecular mass, approximately 330 amino acid residues) and a C-terminal domain (CtD) (10.6-kDa molecular mass, approximately 100 amino acid residues), which are connected by a segment 10 to 15 amino acids in length.^{12,13} The NtD, which contains the 1,3- β -glucanase activity of the protein, has been reported to show cross-reactivity to pollens, vegetable foods, and latex protein extracts,¹³ and the CtD is homologous to Ole e 10, an allergen that has been associated with bronchial asthma with increased severity.¹⁴ Purified Ole e 9 is not available for diagnosis and specific immunotherapy protocols because it is obtained at very low yield from olive pollen. Furthermore, the recombinant production of Ole e 9 has not been achieved to date. An alternative approach to having the allergen available consists of the production of NtD and CtD by recombinant DNA technology in the yeast *Pichia pastoris*, which has been reported to yield high levels of these molecules with the proper conformation and retaining the capability to bind IgE from serum samples from olive pollen-allergic patients.^{12,13}

In the present study, we explore the potential use of recombinant NtD (rNtD) and recombinant CtD (rCtD) of Ole e 9 in diagnosis protocols in vitro to identify Ole e 9-allergic patients and classify them according to their sensitization profiles, which could help design a more precise and efficient specific immunotherapy for olive pollen-allergic patients.

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This work was supported by grant SAF2002–02711 from the Ministerio de Ciencia y Tecnología (Spain).

Received for publication September 1, 2005.

Accepted for publication in revised form November 28, 2005.

METHODS

Patients

This study included 55 patients who fulfilled the following criteria: (1) seasonal rhinitis, asthma, or both between April and June; (2) a positive skin prick test reaction to *O europaea* pollen extract (ALK-Abelló, Madrid, Spain); and (3) no previous *O europaea* immunotherapy. The Ole e 1 content of olive pollen extract was 180 µg/mL. Written informed consent from the patients (or their parents if they were younger than 18 years) was necessary for participation in the study, and the protocol was approved by the Ethical and Research Committee of Complejo Hospitalario de Jaén.

Skin prick tests with *O europaea* extract and purified Ole e 9 were performed on the volar side of the forearm by puncture using a standard lancet with a 1-mm tip, following the recommendations of the European Academy of Allergology and Clinical Immunology.¹⁵ Histamine dihydrochloride (10 mg/mL) and phosphate-buffered solution glycerol were used as positive and negative controls, respectively. Skin test sites were evaluated after 15 minutes, and a wheal 3 mm or larger than that of the negative control was considered positive.

Olive Pollen Allergens

Ole e 9, rNtD, and rCtD were produced and purified as described elsewhere.^{8,12,13} Recombinant NtD contained the amino acids 1 to 327 and rCtD those from 334 to 434 of mature Ole e 9.

Indirect Enzyme-Linked Immunosorbent Assay and Inhibition Experiments

Microtiter plates were coated with 2.17 pmol per well of Ole e 9, rNtD, or rCtD as described elsewhere.¹⁶ Coated wells were incubated with serum samples from olive pollen-allergic patients diluted 1:10. Binding of human IgE was detected by mouse anti-human IgE antibodies (kindly donated by ALK-Abelló) diluted 1:5,000, followed by horseradish peroxidase-labeled goat anti-mouse IgG (Pierce, Rockford, IL) diluted 1:5,000. Peroxidase reaction was developed using fresh enzyme substrate and measuring optical density (OD) at 492 nm.⁸ Each OD value was calculated as the mean of 2 determinations and after subtraction of the blank values. Negative controls were achieved using serum samples from a patient allergic to mustard seeds and from a nonallergic individual and 2 unrelated proteins, such as bovine serum albumin (BSA) and Sin a 1 mustard allergen. Values less than 0.1 were considered negative responses.

For enzyme-linked immunosorbent assay (ELISA) inhibitions, the pool of serum samples (diluted 1:10) was preadsorbed with different amounts of Ole e 9, rNtD, or rCtD or an equimolecular mixture of rNtD and rCtD (0.0217–217 pmol) for 2 hours before incubation of the Ole e 9-coated wells. For determination of inhibition of individual serum samples, they were preincubated with a mixture of rNtD and rCtD (108 pmol of each) or with BSA for control purposes. The percentage of inhibition of the binding after preincubation with

the different inhibitors was calculated as $[(OD - OD_{inh})/OD] \times 100$, where OD and OD_{inh} indicate the absorbance values obtained without and with inhibitors before incubation, respectively. Each value is the mean of 2 determinations.

IgE Immunoblot Inhibition Experiments

Purified Ole e 9 (0.5 µg) underwent electrophoresis in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was transferred to nitrocellulose membranes and then immunostained as described elsewhere.¹⁶ Briefly, membranes were incubated with serum samples from patients allergic to olive pollen (diluted 1:10), which were preadsorbed with an equimolecular mixture of rNtD and rCtD (108 pmol of each) for 2 hours before incubation of the membrane. Afterward, mouse anti-human IgE (diluted 1:5,000) was added, and blots were incubated with horseradish peroxidase-labeled goat anti-mouse IgG diluted 1:5,000. The peroxidase reaction was developed using Western blotting reagent (ECL; Amersham Pharmacia Biotech, Uppsala, Sweden) as described elsewhere.¹⁶

RESULTS

Patients

All the allergic patients (n = 55) showed significant IgE antibody levels against olive pollen crude extract. Thirty-three of them (60%) displayed a significant IgE antibody response against Ole e 9. The IgE values against Ole e 9, and the clinical and serologic features of these patients, are given in Table 1. Skin prick tests were performed in 7 patients with purified Ole e 9, and the mean wheal diameter was 7.2 mm (range, 5–11 mm).

Prevalence of rNtD and rCtD Among Olive Pollen-Allergic Patients With a Positive Response to Ole e 9

To determine the prevalence of rNtD and rCtD, the 33 serum samples with a positive response to Ole e 9 were tested by ELISA against rNtD and rCtD. The responses were compared with those obtained for the whole allergen (Fig 1A). Thirty-one (94%) of the 33 patients were reactive to rNtD or rCtD (Fig 1B). Only 2 patients had a negative response to both independent recombinant domains, which could be explained if their IgE antibodies were directed to (1) putative glycan epitopes that are not contained in the recombinant molecules but in the natural allergen or (2) conformational epitopes involving both domains. Twenty-six (79%) of the 33 tested patients had a positive response to rNtD, 22 (67%) to rCtD, and 17 (52%) to both. Interestingly, 9 patients (27%) were exclusively reactive to rNtD and 5 (15%) to rCtD, indicating that many Ole e 9-allergic patients (42%) can be specifically sensitized against NtD or CtD of the allergen.

rNtD and rCtD Contain Most of the IgE Epitopes of Ole e 9

Quantitative ELISA inhibition experiments were performed to determine the IgE binding capability of a mixture of the recombinant fragments of Ole e 9. Serum samples from 20 Ole e 9-allergic patients were preadsorbed with an equimo-

Table 1. Characteristics of Patients With Olive Pollen Allergy Sensitized to Ole e 9

Patient No./ sex/age, y	Symptoms	Skin test wheal diameter, mm*	Specific IgE† to	
			Olive pollen	Ole e 9
2/F/26	R/BA	8	2.23	0.40
3/F/14	R/BA	13	2.04	0.37
5/F/35	R/BA	9	2.50	0.81
7/F/22	R/BA	17	1.36	0.40
11/M/29	R/BA	8	1.38	0.35
12/M/22	R/BA	6	2.50	1.95
16/M/13	R	11	0.71	0.47
22/M/16	R/BA	10	1.13	0.42
26/F/28	R/BA	13	1.44	0.93
27/F/15	R	8	2.50	0.29
28/M/13	R/BA	8	2.50	0.87
30/M/13	R/BA	10	2.50	0.40
32/F/13	R/BA	6	2.50	0.73
33/F/18	R/BA	10	0.80	0.56
34/F/17	R/BA	8	2.50	1.80
35/F/23	R/BA	9	2.50	0.34
38/F/34	R/BA	7	2.50	0.35
39/F/18	R/BA	10	1.71	0.26
40/F/19	R/BA	7	2.40	0.77
42/M/21	R/BA	10	2.14	0.94
43/F/36	R/BA	14	2.50	1.14
44/F/19	R	9	0.26	0.37
45/M/14	R/BA	13	2.03	0.82
46/F/19	R/BA	13	2.50	2.86
47/F/11	R/BA	7	2.50	2.67
48/M/15	R	8	2.50	1.27
49/F/17	R/BA	7	2.50	1.89
50/F/30	R/BA	6	0.84	0.55
51/F/15	R/BA	5	2.50	0.99
52/M/10	R/BA	8	2.50	0.60
53/M/19	R/BA	7	0.93	0.51
54/M/19	R/BA	11	2.50	0.53
55/F/47	R/BA	10	1.37	0.62

Abbreviations: BA, bronchial asthma; R, rhinitis.

* Obtained with olive pollen extract.

† Determined by enzyme-linked immunosorbent assay as optical density at 492 nm.

lecular mixture of rNtD and rCtD, or with BSA for control purposes, and then added to Ole e 9–coated wells, and the remaining binding was measured. The results revealed that the mixture of rNtD and rCtD strongly inhibited IgE binding to Ole e 9 (range, 61%–87%; mean, 76%). The lack of complete inhibition could be for the same reasons mentioned in the previous paragraph.

Contribution of Each Domain to the Overall Binding of Ole e 9 Specific IgE Antibodies

The ELISA inhibition experiments with Ole e 9–coated wells were performed using a pool of serum samples and the 2 recombinant domains as inhibitors (Fig 2A). The IgE binding to Ole e 9 was nearly abolished (90%) when an equimolecular

mixture of rNtD and rCtD was used as inhibitor, and the extent of inhibition at high concentration was similar to that obtained after preincubation of the serum samples with the whole allergen Ole e 9. Inhibition reached values of 44% and 45% when rNtD and rCtD, respectively, were independently used as inhibitors. Immunoblotting inhibition experiments were also performed using the 5 individual serum samples from the pool. Preadsorption of each serum sample with an equimolecular mixture of rNtD and rCtD abolished IgE binding to Ole e 9 after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Fig 2B).

DISCUSSION

The allergenic pattern of olive pollen is complex and frequently differs among patients depending on the geographic area. We previously observed in Jaén (southern Spain) that the atmospheric olive pollen concentration varies between 500 and 1,000 grain/m³ during late April to early June, with peaks of more than 5,000 grain/m³ by mid-May. For this reason, patients have rhinitis or bronchial asthma of increased severity when they are exposed to these high levels of pollen,¹⁷ and the sensitivity against particular olive pollen allergens might affect the development and intensity of certain allergic diseases.¹⁴ Therefore, the use of purified olive allergens, obtained from the natural source or produced as recombinant proteins, would be necessary to identify the disease-eliciting allergen molecules, to classify patients in risk groups, and to design future patient-tailored immunotherapy protocols. In this regard, during the past few years, the use of well-defined recombinant allergens has notably increased for in vitro and in vivo assays.^{18,19}

Ole e 9 is a major allergen that belongs to the widespread PR-2 protein family. In this study, we show the functional reactivity of its epitopes as skin tests using purified Ole e 9 demonstrated that the allergen causes the release of inflammatory mediators in sensitized patients. To date, Ole e 9 is the single allergen identified in olive pollen with a molecular mass in the range of 40 to 60 kDa. Although most patients included in this study displayed IgE reactivity to high-molecular-weight proteins (data not shown), the ELISA experiments performed with purified Ole e 9 showed that only 60% of them were sensitized to this molecule, indicating that Ole e 9–allergic patients should be identified using the specific allergen. Because purified Ole e 9 is not available in the amount required for diagnosis protocols, we used the NtD and CtD of the allergen produced as 2 independent recombinant proteins to study and validate their potential use for in vitro diagnosis of allergy to olive pollen.

The results of this study show that most of the B-cell allergenic epitopes of Ole e 9 are represented in rNtD and rCtD because an equimolecular mixture of them accounts for a high percentage of IgE response to Ole e 9. In addition, 94% of the Ole e 9–allergic patients can be identified using both recombinant fragments. On the other hand, the particular use of these fragments also allows determination of the part of Ole e 9 that is responsible for patients' sensitization, and it

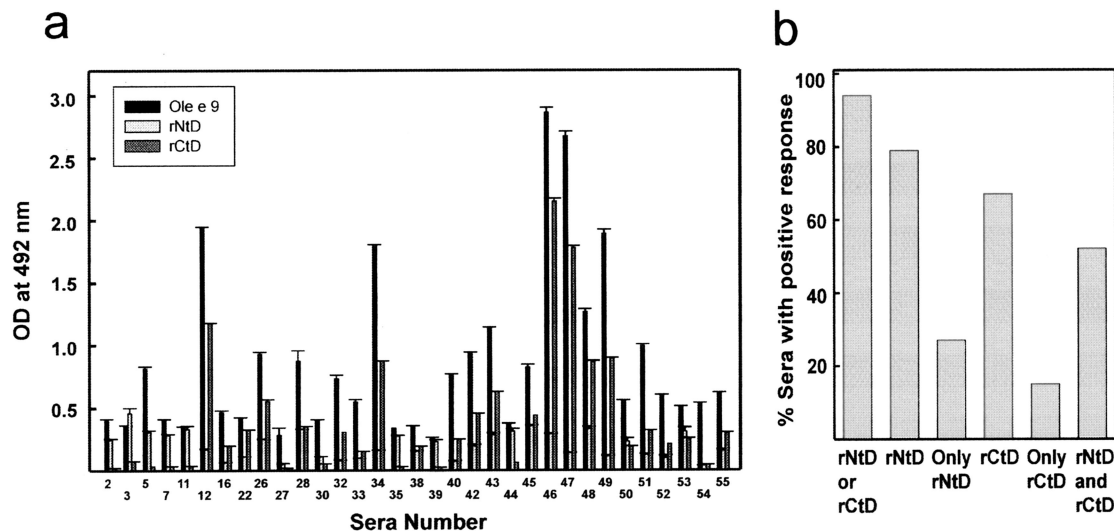


Figure 1. Determination of the prevalence of recombinant N-terminal domain (rNtD) and recombinant C-terminal domain (rCtD). A, IgE binding to Ole e 9, rNtD, and rCtD with serum samples from 33 Ole e 9-allergic patients. OD indicates optical density. Error bars represent SD. B, Prevalence of rNtD and rCtD obtained by enzyme-linked immunosorbent assay using the data obtained for the 33 Ole e 9-allergic patients. Absorbance values were calculated as the mean of 2 determinations, and values less than 0.1 were considered to be negative responses.

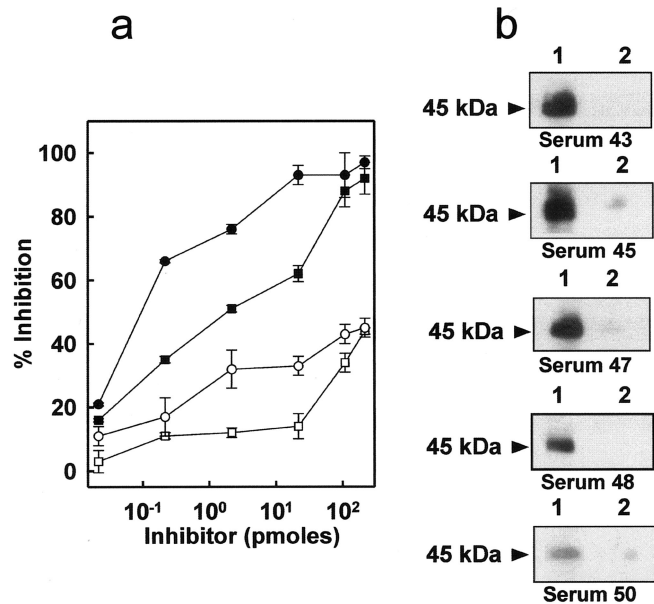


Figure 2. Contribution of each domain to the overall binding of Ole e 9 specific IgE antibodies. A, IgE binding of serum samples 43, 45, 47, 48, and 50 from allergic patients to Ole e 9-coated wells (2.17 pmol per well) was assayed using as inhibitors increasing amounts of Ole e 9 (●), recombinant N-terminal domain (rNtD) (□), recombinant C-terminal domain (rCtD) (○), and rNtD plus rCtD (■). Error bars represent SD. B, Nitrocellulose-blotted Ole e 9 was exposed to the individual serum samples, which had been preincubated with a mixture of rNtD and rCtD (108 pmol of each) (lane 2) or with bovine serum albumin (lane 1). The molecular mass of Ole e 9 is indicated in the left margin.

may be useful to classify patients according to their IgE reactivity profile.

Although it is not always possible to draw clinical conclusions from in vitro data because specific IgE binding does not always correlate with the disease, some evidence suggests that the IgE responses against each domain may be helpful markers for disease in, at least, 2 subsets of patients with olive pollen allergy: (1) individuals at risk of allergic symptoms to fruit, vegetables, and latex due to sensitization against rNtD, because this part of the molecule has been shown to be involved in latex-pollen-vegetable food cross-reactivity processes¹³ and (2) patients who could develop asthma due to sensitization against rCtD because this domain shows homology (53% identity) and high cross-reactivity with Ole e 10,⁹ which has been associated with an increased severity of bronchial asthma in olive pollen-allergic patients.¹⁴ The 1,3- β -glucanases have been found to be relevant allergens in latex,²⁰ but they have not been deeply explored in other allergenic sources. The ubiquity of these enzymes in plants and their inclusion as foreign genes in transgenic vegetables increase the interest in an accurate diagnosis for this family of PR-2 proteins.^{21,22}

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