



Influence of the 3D-conformation, glycan component and micro-heterogeneity on the epitope structure of Ole e 1, the major olive allergen

Use of recombinant isoforms and specific monoclonal antibodies as immunological tools

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Abstract

Ole e 1 is the main allergen of olive pollen, which is a major cause of pollinosis in countries of the Mediterranean area. Nine Ole e 1-specific murine monoclonal antibodies (mAbs), as well as two Ole e 1-isoforms and two Ole e 1-like allergens from lilac and privet, all of them obtained in *Pichia pastoris* by recombinant methods, have been used as tools to determine the role of the three-dimensional (3D)-folding, the glycan component and several point changes of the amino acid sequence in the binding of murine IgG mAbs and human IgE to the olive allergen. Seven mAb families (F1–F7) were established, two of which (F1 and F2) recognize continuous epitopes. The carbohydrate moiety of Ole e 1 was involved in the binding to F2 and F4, whereas F3 and F7 were able to bind to all Ole e 1 variants. The remaining families of IgG murine antibodies exhibited different affinities for the antigens assayed in a native or denatured conformation. Although the binding of human IgE to Ole e 1 was not affected by heat treatment, it was shown to be strongly dependent on the integrity of the disulfide bridges and was partially inhibited by F3–F7 IgG antibodies, their individual values ranging from 12 to 31% and reaching 53% with their mixture. The IgE from sera of olive-allergic patients showed a significant diversity of binding capacity to the members of the Ole e 1-like family due to the microheterogeneity of their polypeptide sequences, in spite of their highly conserved primary structures. Whereas one of the isoforms of Ole e 1 exhibits a highly similar behavior to the natural form, being a putative molecule for diagnostic purposes, other ones can be considered as hypoallergenic variants of this allergen and, thus, potential candidates to be used in immunotherapy.

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1. Introduction

Allergy to olive (*Olea europaea*) pollen is an important cause of hypersensitivity in the Mediterranean countries and

in some areas of North America (Liccardi et al., 1996), and is particularly relevant in regions where the olive tree is extensively cultivated, such as the south of Spain and Italy, Greece and Israel.

The identification and complete characterization of allergens, especially major allergens, are essential requirements for an accurate diagnosis and treatment of allergic diseases, as well as for the study of their molecular bases. To date, nine allergens have been isolated from the olive pollen and characterized (Rodríguez et al., 2001). Of these, Ole e 1 has been recognized as the most prevalent allergenic molecule of this source (Lauzurica et al., 1988; Wheeler et al., 1990; Villalba et al., 1993), since more than 70% of the patients allergic to olive pollen are sensitized by this allergen, which

Abbreviations: ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; nOle e 1, natural Ole e 1 allergen; OD, optical density; PBS, phosphate-buffered saline; RAST, radioallergosorbent test; rLig v 1, recombinant Lig v 1; rOle e 1/3c, recombinant Ole e 1, clone OLE3c; rOle e 1/5c, recombinant Ole e 1, clone OLE5c; rSyr v 1, recombinant Syr v 1; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 3D, three-dimensional

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elicits both humoral and cellular responses. Ole e 1 is the most abundant component of olive pollen and the best structurally known allergen of this biological source. This allergen is a polymorphic glycoprotein of 145 amino acid residues, of which more than 10 isomeric forms were analyzed (Villalba et al., 1993; Villalba et al., 1994). The glycan moiety of the allergen, the structure of which has been recently elucidated (van Ree et al., 2000b), has been shown to contribute to the allergenicity of the molecule (Batanero et al., 1994a; Batanero et al., 1996a). Ole e 1 has been efficiently produced in the yeast *Pichia pastoris*, which is able to secrete high amounts of properly-folded recombinant allergen (Huecas et al., 1999). Besides Ole e 1, the homologous proteins Syr v 1 and Lig v 1 from lilac and privet have been isolated and characterized (Batanero et al., 1994b; Batanero et al., 1996b), and further produced as homogeneous and well defined recombinant forms in the same heterologous system (González et al., 2001). Since their polypeptide sequences are similar but not identical to that of Ole e 1, these molecules may be considered as variants of the olive allergen and, therefore, as useful tools for epitope analyses.

In spite of the exhaustive molecular characterization of Ole e 1, little is known about the structural organization of its antigenic and allergenic epitopes, which are critical in B- and T-cell recognition. Immunodominant T-cell epitopes were located by Cárdena et al. (1998), but they were not recognized by IgE antibodies. The detailed knowledge of these IgG and IgE-binding regions would be valuable for designing hypoallergenic mutants of this allergen to be used as therapeutic reagents in allergy treatment, thus decreasing the possibility of side effects associated to current therapy.

The mAbs against well-defined epitopes are probably the best tools to assess the presence of specific and common antigenic determinants borne by different molecules, and they can help to delimitate antigenic regions in the protein. Furthermore, the use of murine mAb to block the binding of human IgE to specific allergens has allowed the localization of allergenic epitopes (Chapman et al., 1984).

In this work, we evaluate the relevance of the allergen conformation in the binding of Ole e 1 to IgG and IgE antibodies, the implication of its carbohydrate moiety in the antigenicity and the role of point alterations of the amino acid sequence of the allergen in its IgG- and IgE-binding capacity.

2. Materials and methods

2.1. Antibodies and sera

The mAbs OL2–OL9 against Ole e 1 were obtained in mice as indicated (Lombardero et al., 1992). The murine mAb 19G6 was prepared as described (Martín-Orozco et al., 1994) and mAbs 5C1, 10A12 and 3H8 were produced also in mice as described (van Ree et al., 2000a). Coupling of mAbs to horseradish peroxidase type VI (Sigma–Aldrich

Química S.A., Spain) was performed as reported (Nakane and Kawaoi, 1974).

Sera from 20 untreated hypersensitive individuals, which exhibited a positive reaction in immunoblotting to Ole e 1, were selected from a population with a clinical history of allergy to olive pollen and a positive skin-prick test and CAP–FEIA System (Pharmacia AB, Uppsala, Sweden) classes 3–6 to olive pollen extract.

2.2. Natural, chemically-modified and recombinant allergens

The nOle e 1 was purified from olive pollen (Allergon AB, Ångelholm, Sweden) as described (Villalba et al., 1993). Reduction and alkylation of nOle e 1 was performed by incubating the protein (10 mg/ml) in 1 M Tris–HCl, pH 8.5, 6 M guanidine chloride, 2 mM EDTA and 100 mM dithiothreitol at 37 °C for 90 min. Iodoacetamide was added at a final concentration of 150 mM and incubated for 90 min at room temperature in the dark. Chemically modified protein was purified by reverse-phase HPLC on a Nucleosil C18 column with an acetonitrile gradient in 0.1% trifluoroacetic acid.

Enzymatic deglycosylation of the allergen was performed with PNGase F (EC 3.2.2.18, Boehringer Mannheim GmbH, Mannheim, Germany) as described (Batanero et al., 1994a). The nOle e 1 (2 mg/ml) was dissolved in 100 mM Tris–HCl, pH 8.0, containing 1 mM phenylmethylsulphonyl fluoride and 1% (w/v) *n*-octylglucoside. PNGase F (20 units/mg of Ole e 1) was added and the mixture was incubated for 18 h at 37 °C. Deglycosylated protein was purified on a Sephadex G-25 superfine column (1 cm × 28 cm) with 0.2 M ammonium bicarbonate, pH 8.0.

Recombinant proteins (clones OLE3c and OLE5c from Ole e 1 called rOle e 1/3c and rOle e 1/5c respectively; clone S46 from Syr v 1 called rSyr v 1, and clone L10 from Lig v 1 called rLig v 1) (Fig. 1) were produced as secretion proteins in the heterologous expression system *P. pastoris*, and purified from the extracellular medium according to Huecas et al. (1999) and González et al. (2001).

2.3. Complementations assays

Simultaneous binding of mAb to nOle e 1 was tested by coating high-affinity polystyrene plates with 100 µl of purified antibody (10 µg/ml) in PBS. After blocking with PBS containing 3% non-fat milk and 0.1% Tween 20, nOle e 1 was added at 1 µg/ml (0.1 µg per well) in blocking buffer and incubated at 37 °C for 2 h. Once the plates were washed, peroxidase-labeled mAb at different dilutions in 1.5% non-fat milk and 0.05% Tween 20 was added and plates were kept at 37 °C for 2 h. The peroxidase reaction was developed by adding freshly prepared enzyme substrate (0.3% H₂O₂ and 0.63 mg/ml *o*-phenylenediamine in 0.1 M sodium citrate, pH 5.0) and it was quantified by measuring the OD at 492 nm. Triplicate assays were performed for each determination.

	1	10	20	30	40	50	60	70		
OLE3c	EDVPQPPVSQ	FHIQGQVY	CDTRCAGFIT	ELSEFIPGAS	LRLOCKDK	ENGDDVTF	TEVGYT	RAEGLYSML	VERDH	
OLE5c	-----	-----	-----R-----	-----V-----	-----G-----	-----SI-----	-----I-----	-----	-----	
S46	-----P-----	-----R-----	-----I-----	-----G-----	-----KI-----	-----I-----	-----G-----	-----	-----	
L10	-----Y-----	-----R-----	-----GV-----	-----G-----	-----KI-----	-----I-----	-----I-----	-----	-----	
	80	90	100	110	120	130	140			
OLE3c	KNEFCEITL	LISSGRKDC	NEIPTG	WVKPSLK	FILNTV	NGTTRTVN	PLGFFFK	EALPKCAQ	VYNKLGMYPPNM	% Identity
OLE5c	-D-----	-S-----	-D-----	-V-----	-M-----	-I-----	-----	-P-----	-F-----	90
S46	-----	-----	-D-----	-V-----	-K-----	-I-----	-I-----	-T-----	-----	90
L10	-----	-L-----	-S-----	-D-----	-I-----	-M-----	-I-----	-P-----	-F-----	89

Fig. 1. Amino acid sequence of the Ole e 1 clones (OLE3c, OLE5c), Syr v 1 (S46) and Lig v 1 (L10), which were used for recombinant expression in the yeast *P. pastoris*. Identity (%) of the amino acid sequences is referred to OLE3c.

2.4. ELISA inhibition assays

ELISA inhibition experiments were carried out as described by [Batanero et al. \(1994a\)](#) with minor modifications. Microtiter plates were coated with 100 μ l per well of the antigen (1 μ g/ml) in PBS and blocked. Afterwards, mAbs were co-incubated with different concentrations of the inhibitor in blocking solution and were added to the wells. Binding of IgG was detected by horseradish peroxidase-labeled goat anti-mouse IgG antibody (Pierce, Rockford, IL, USA) (diluted 1/2500). The reaction was developed as described above.

The inhibitory effect produced by mAbs on the IgE binding was determined by coating the microtiter plates with 100 μ l per well of Ole e 1 at 1 μ g/ml. After blocking unoccupied sites on the plates, a pool of 10 atopic sera (diluted 1/10) was co-incubated with Ole e 1-specific mAbs, transferred to the coated wells and incubated for 2 h. Then, plates were washed and biotin-labeled mouse anti-human IgE (ALK-Abelló, Madrid, Spain) at a 1/5000 dilution was allowed to react for 1 h at room temperature. After 1 h of incubation with horseradish peroxidase-labeled streptavidin (BioRad Lab., Richmond, CA, USA), the enzymatic reaction was developed. Duplicate assays were performed for each determination.

2.5. Electrophoresis, dot blot and immunostaining

SDS-PAGE was performed by the method of [Laemmli \(1970\)](#) in 15% polyacrylamide gels under non-reducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes or directly applied to membranes (1 µg/spot), and equilibrated in PBS-0.1% Tween 20 for 10 min ([Villalba et al., 1994](#)). Immunostaining was performed according to [Batanero et al. \(1996b\)](#). After blocking with 3% non-fat milk in the same buffer, the membranes were incubated with Ole e 1-specific mAbs or sera from olive-allergic patients. For IgE-binding detection, membranes were incubated with mouse anti-human IgE (diluted 1/5000). Horseradish peroxidase-labeled goat anti-mouse IgG (diluted 1/5000) was used for final immunodetection. The peroxidase reaction was developed with ECL Western

blotting reagent (Amersham Corp., Buckinghamshire, UK), according to the manufacturer's instructions.

2.6. IgE binding by individual allergic sera

Relative affinities of the individual allergic sera to the recombinant allergens were estimated by IgE-binding of solid phase-bound antigen as described (Batanero et al., 1996b). One hundred microliters aliquots of the antigen (1 μ g/ml) in PBS was added to individual wells and incubated at 4°C overnight. After blocking, plates were incubated with individual sera (diluted 1/10) from 20 olive-allergic patients. Incubations with mouse anti-human IgE (diluted 1/5000) (ALK-Abelló, Madrid, Spain) and horseradish peroxidase-labeled goat anti-mouse IgG were carried out, and the peroxidase reaction was developed as described above. Results are expressed as the mean value of two different determinations.

3. Results

3.1. Ole e 1-specific mAbs and topographical distribution

The relative binding site of nine mAbs specific for Ole e 1 was determined by complementation assays (Fig. 2A). Previously, their relative affinities were analyzed by ELISA inhibition assays; the amount of nOle e 1 required to reach 50% binding inhibition of mAb to the solid phase-bound antigen was found to range from 0.04 ng/ml for OL2 to 50 ng/ml for 3H8. The different complementation patterns obtained allowed to classify Ole e 1-specific murine mAbs into seven topographic groups: F1 (OL2), F2 (OL3), F3 (OL6, OL7, OL9), F4 (19G6), F5 (5C1), F6 (10A12) and F7 (3H8) (Fig. 2B). Nevertheless, some overlapping could be detected between binding sites of different groups. The binding of OL3 to Ole e 1 inhibited the recognition of the antigen by the other groups; 19G6 inhibited the binding to 10A12; OL3, OL6, OL7, OL9 inhibited the binding to 3H8, and 5C1 partially inhibited the recognition of the antigen by 10A12. These results indicate that the epitopes recognized

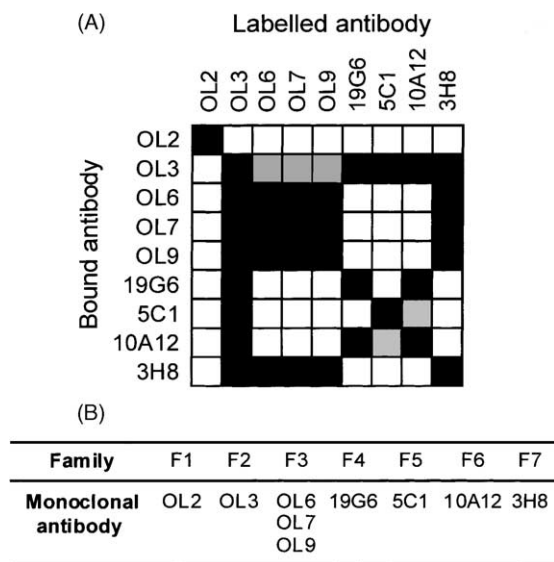


Fig. 2. Complementation profile of Ole e 1-specific mAb. (A) The analysis was performed by ELISA. The amount of peroxidase-labeled antibody bound to Ole e 1 was determined by measuring OD at 492 nm: white, OD > 1.0; clear hatching 1.0 > OD > 0.5; dark hatching 0.5 > OD > 0.075; black, 0.075 > OD. (B) Classification of Ole e 1-specific mAbs according to their complementation pattern.

by these mAbs could be adjacent or partially overlapping in the antigen structure, reducing the total number of antigenic regions in Ole e 1.

3.2. Implication of the native conformation and the glycan moiety in the mAb-binding capacity of Ole e 1

The effect of reduction and alkylation of cystine residues of Ole e 1 in the binding of specific anti-Ole e 1 mAb was evaluated by ELISA inhibition experiments (Fig. 3). Only F1 and F2 were able to bind carboxyamidomethylated Ole e

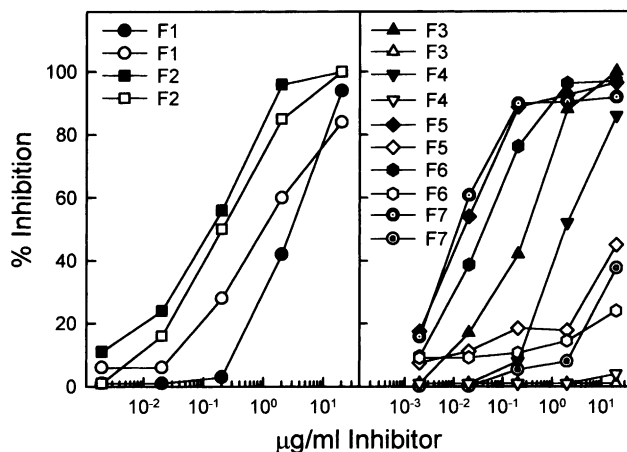


Fig. 3. Chemical stability of Ole e 1 epitopes. The inhibition of the binding of Ole e 1-specific mAbs to nOle e 1-coated wells of ELISA microtiter plates was assayed using nOle e 1 (dark symbols) and carboxyamidomethylated nOle e 1 (open symbols) as inhibitors. IgG-binding was measured colorimetrically at OD 492 nm.

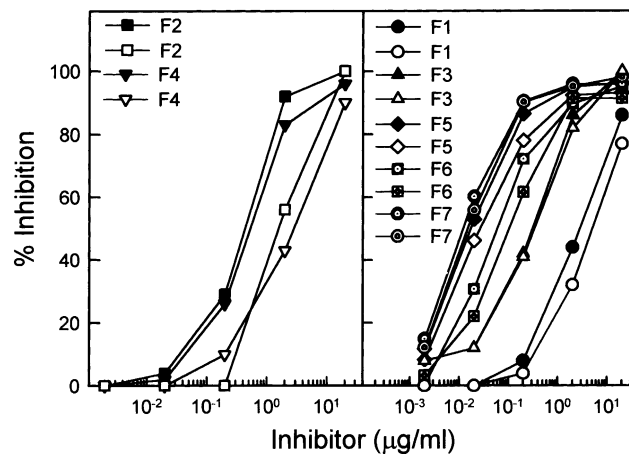


Fig. 4. Glycan involvement in Ole e 1-specific IgG epitopes. The inhibition of the binding of mAbs to nOle e 1-coated wells was assayed using nOle e 1 (dark symbols) and PNGase F-deglycosylated nOle e 1 (open symbols) as inhibitors.

1, suggesting the continuous nature of these epitopes. F3 and F4 did not recognize denatured Ole e 1, and reduced Ole e 1 led to only a scarce degree of inhibition of F5–F7 binding to nOle e 1, at large amounts of inhibitor. This indicates a strong dependence on the native Ole e 1 conformation, which is determined by disulfide bond formation, for the binding of these mAbs.

The role of the glycan moiety of Ole e 1 in the recognition by mAbs was analyzed by ELISA inhibition assays. The inhibitory capacity of PNGase F-deglycosylated allergen was similar to that of nOle e 1 for F1, F3, F5–F7 binding (Fig. 4). These data indicate that the Ole e 1 oligosaccharide is not implicated in epitopes detected by these antibodies. The reduction of the binding affinity of F2 and F4 to deglycosylated Ole e 1, in contrast to nOle e 1, points to a possible implication of the glycan moiety of Ole e 1 in the antigen-mAb interaction or in the structural stabilization of the epitopes recognized by these mAbs.

3.3. IgG binding of different Ole e 1 recombinant isoforms and homologous proteins

The recognition of two recombinant isoforms of Ole e 1 (clones 3c and 5c), as well as Syr v 1 and Lig v 1, by Ole e 1-specific mAbs was tested by dot blot (Fig. 5A) and Western blotting (Fig. 5B), in order to compare the binding capacities in the absence and the presence of denaturing agents. All the mAbs bound to rOle e 1/3c, which agrees with the fact that this is the most abundant isoform in the pollen (Villalba et al., 1993). F3 and F7 were able to recognize all of the variants, under both native and denaturing conditions, indicating their binding to conserved regions of the amino acid sequence of the allergen. F5 displays a weak recognition of rSyr v 1 and rLig v 1, which is enhanced in the presence of SDS. F4 failed to bind rOle e 1/5c, rSyr v 1 and rLig v 1, so common changes present in their sequences with respect

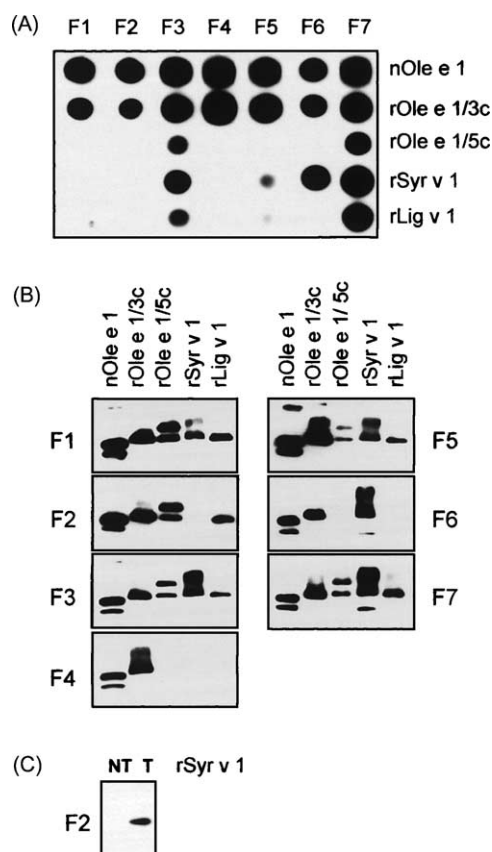


Fig. 5. Dot blot (A) and Western blotting (B) of nOle e 1 and recombinant variants rOle e 1/3c, rOle e 1/5c, rSyr v 1 and rLig v 1 with Ole e 1-specific mAbs. One microgram of protein was applied in each lane. Multiple bands obtained for nOle e 1, rOle e 1/5c, and rSyr v 1 are due to the glycosylation pattern of this family of proteins (see references in the text). (C) Immunoblotting of PNGase F-treated (T) and non-treated (NT) rSyr v 1; the immunostaining was performed with F2 mAb.

to that of rOle e 1/3c could be responsible for the lack of binding. F1 and F2 recognized rOle e 1/5c and rLig v 1 only after SDS-PAGE, emphasizing the continuous nature of these epitopes, as was suggested by their binding to the reduced and alkylated Ole e 1 (Fig. 3). Nevertheless, F2 did not bind at all to rSyr v 1, except when the protein was deglycosylated by treatment with PNGase F (Fig. 5C), which suggests a proximity between the F2 epitope and the glycosylation region in these allergens. Finally, F6 failed to recognize rOle e 1/5c and rLig v 1.

3.4. IgG and IgE epitope relationships

The ability of mAbs to inhibit the reaction between IgE and the allergen was determined by ELISA (Fig. 6). IgE-binding to Ole e 1 was partially inhibited by F5, F3 and F6, and weakly affected by F7 and F4. No inhibition was detected by using F1 and F2 as inhibitors. To analyze if the IgE-binding site is partially shared by two or more mAbs, two pools of blocking mAbs were assayed as inhibitors: three mAbs that showed the highest inhibitory capacity

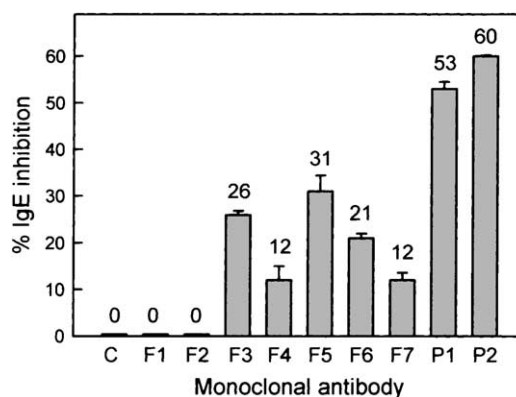


Fig. 6. Blocking of IgE binding to Ole e 1 by mAbs used alone or in combination. The concentration of each mAb used in this assay was 100 μ g/ml. The binding of a pool of olive-allergic sera to Ole e 1-coated wells was assayed. A non-related mAb (C) was used as negative control. P1 stands for F3 + F5 + F6 mAbs; P2, is F3 + F4 + F5 + F6 + F7 mAbs.

(P1: F3 + F5 + F6) and those that displayed any inhibitory activity (P2: F3 + F4 + F5 + F6 + F7) (Fig. 6). The former combination increased the IgE-binding inhibition to 53%, suggesting that epitopes recognized by these mAbs define distinct IgE-binding sites of Ole e 1. A weak increase of the inhibition percentage was obtained by the addition of F4 and F7 to the P1 pool, indicating a poor competition activity of these mAbs or the overlapping of IgE determinants recognized by them.

3.5. Conformation dependence of the IgE epitopes

The allergenic epitopes of Ole e 1 were analyzed by immunodetection of native, heat treated, and reduced and alkylated nOle e 1 with a pool of sera from allergic patients after SDS-PAGE and transference to nitrocellulose membranes (Fig. 7). Whereas the allergen showed a high thermostability, its reduction and carboxyamidomethylation nearly abolished the IgE-binding capacity, indicating a remarkable dependence on the disulfide bridges, which are disrupted by reduction and alkylation, and therefore a conformational nature for the IgE epitopes.

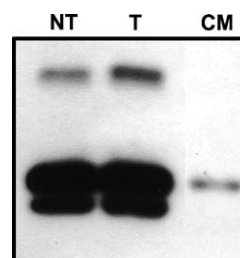


Fig. 7. IgE-binding to nOle e 1. The allergen was electrophorized in the presence of SDS, transferred to nitrocellulose membranes and immunostained with a pool of 10 allergic sera, after heating the sample at 100 $^{\circ}$ C during 20 min (T), non-heating (NT), or reducing and carboxyamidomethylation (CM).

Table 1
IgE reactivity of different Ole e 1 variants

	IgE binding ^a (%)																				Average
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14	Patient 15	Patient 16	Patient 17	Patient 18	Patient 19	Patient 20	
rOle e 1/3c	87	94	82	79	85	91	85	86	82	83	105	102	93	87	93	83	84	84	90	79	88
rOle e 1/5c	43	50	38	40	53	58	47	28	53	42	67	74	58	43	64	51	47	69	61	48	52
rSyr v 1	44	59	46	40	66	61	72	43	78	68	88	96	61	52	81	52	60	75	71	55	64
rLig v 1	43	54	17	23	44	52	59	26	59	36	56	65	57	35	73	49	62	65	32	43	47

^a Sera from 20 patients were assayed for their binding to rOle e 1/3c, rOle e 1/5c, rSyr v 1 and rLig v 1 in ELISA. Relative IgE percentage of binding was calculated taking the response of each sera to nOle e 1 as reference (100% IgE binding). The average for the 20 data was determined for each antigen.

3.6. IgE-binding capacity of Ole e 1 isoforms and homologous proteins

The IgE-binding capacity of the four recombinant variants of Ole e 1 was analyzed by ELISA employing 20 sera from olive-allergic patients (Table 1). All tested sera recognised the four molecules, although significant quantitative differences were observed. The highest percentage of IgE-binding corresponded to rOle e 1/3c, indicating that this molecule might be the main sensitizing isoform of nOle e 1 in contrast to rOle e 1/5c, whose average IgE-binding represents only 52% of the IgE-binding by nOle e 1. Important differences were also observed in the recognition of rSyr v 1 and rLig v 1 by the different sera (i.e. sera 4 and 12 for rSyr v 1, or 3 and 15 for rLig v 1), but the binding of these allergens was always reduced against nOle e 1 in comparison to that of rOle e 1/3c isoform. The rLig v 1 was the variant with the smallest capacity of binding to IgE antibodies.

4. Discussion

The antigenic structure of the major olive pollen allergen, Ole e 1, has been investigated at the levels of both conformational and amino acid sequence. Two different tools have been used for this study: (i) specific mAbs directed against Ole e 1, and (ii) recombinant isoforms of the olive allergen and Ole e 1-like proteins from different but taxonomically related pollens.

Nine mAbs isolated from three different fusions, which were obtained by immunization with purified Ole e 1, have been used. The first objective was to group them into families by screening their reactivities. Seven families have been made after the complementation assays. However, not all the families can be assigned to strictly different antigenic regions because some of them, such as F3/F7 or F4/F6, overlapped. Therefore, these experiments suggest an antigenic relevance for a limited number of regions to which many of the antibodies are directed. The existence of four immunodominant regions in Ole e 1 was previously described by using six mAbs specific for Ole e 1 obtained from a single fusion experiment (Martín-Orozco et al., 1994). Studies on the epitope mapping of a number of allergenic proteins, which were also carried out with specific mAbs, showed the existence of immunodominant regions towards which most of the mAbs were directed. Thus, 70% of the mAbs obtained against Der p 1 recognized the same epitope (Chapman et al., 1987). A similar situation was described for 10 mAbs obtained against Sin a 1, the major allergen of mustard seeds, the antigenic targets of which were included in only two clearly defined immunodominant regions (Menéndez-Arias et al., 1990).

The relevance of the 3D-folding of Ole e 1 in its antigenicity has been evaluated. Studies carried out with a variety of allergens have indicated that significant changes in their antigenic and allergenic features occur after

denaturation or unfolding (King et al., 1987; Lombardero et al., 1990; Han et al., 1993). Our analyses revealed that most of the antigenic epitopes of Ole e 1 have a conformational nature, since the reduction and alkylation of the allergen results in a nearly complete loss of its binding capacity for most of the mAbs. Only two out of nine mAbs were directed against continuous epitopes of Ole e 1. The presence of conformational antigenic determinants is a common feature in allergenic molecules. Thus, the chemical modification of the cysteines involved in disulfide bonds or their substitution by site-directed mutagenesis have been useful strategies to alter the structural conformation and determine the existence of discontinuous IgG and IgE epitopes in Der f 1 and Der f 2 (Lombardero et al., 1990; Takai et al., 1997) as well as in Lep d 2 (Olsson et al., 1998). As a biotechnological alternative, gene fragmentation of cDNAs encoding allergens and recombinant expression of the fragments has been used as an approach to modify conformational IgE epitopes in order to diminish their reactivity and obtain hypoallergenic variants (Ferreira et al., 1998; Takai et al., 1999; Tang et al., 2000).

An immunological role has been attributed to the carbohydrate moiety of many glycoproteins, its epitope contribution being of special relevance in many cases. Thus, a set of IgG immunoglobulins of a Cry j 1-specific polyclonal antiserum was directed against the glycan components of the allergen (Hijikata et al., 1994). For Ole e 1, the antigenic nature of the glycan moiety was demonstrated, since IgG-binding to this component was detected by using an Ole e 1-specific polyclonal antiserum (Batanero et al., 1994a; Batanero et al., 1996a). Moreover, the ability of the glycan portion to induce histamine-release from basophils, as well as its capacity for binding IgE from a pool of sera of allergic patients, were also described (Batanero et al., 1999). The results herein presented show that the glycan component of Ole e 1 is not significantly involved in the recognition by murine mAbs, since only the families F2 and F4 seem to be partially affected by the PNGase-deglycosylation treatment of the allergen, and that the continuous epitope of Ole e 1 recognized by F2 could be located around the glycosylation site.

Polymorphism, a common feature of many allergens, makes the characterization of the Ole e 1 epitopes difficult, since different isoforms can contribute in different ways to the antigenic and allergenic behavior of the molecule. The availability of individual isoforms of the allergen and rOle e 1-like proteins from related pollens, which represent well known variants of the olive allergen with a high similarity (Fig. 1), has allowed to identify putative key residues responsible for the IgG binding. As can be seen in Fig. 1, 21 positions of the sequence of rOle e 1/3c are occupied by different amino acid residues in the remaining molecules, their identity ranging from 89 to 90%. In the experiments performed with Ole e 1-specific mAbs, it has been possible to detect the existence of antigenic determinants located in conservative regions of Ole e 1, which are present in all the molecules analyzed and are recognized by two families (F3

and F7), as well as those assigned to heterogeneous segments. The latter involves epitopes recognized by F1, F2, and F4, which are present in nOle e 1 and its recombinant form rOle e 1/3c, but they completely lose their capacity for IgG binding on the remaining proteins. The selective binding of F5 and mainly F6 to rSyr v 1 and rOle e 1/3c leads us to suggest that some of the amino acid positions substituted in these isoforms and common to rOle e 1/5c and rLig v 1, such as Leu39, Val69, Gly86, Ile106, Ala132 and/or Tyr135, are implicated in the epitope recognized by these mAbs. This would agree with the fact that amino acid residues located at peripheral positions of the 3D-structure show less steric restrictions to be substituted as well as a high possibility of being involved in antigenic determinants.

The putative allergenic equivalence of the epitopes recognized by each family of mAbs was determined by analyzing the capacity of these to inhibit the binding to Ole e 1 of IgE antibodies of sera from olive-allergic patients. Interestingly, F1 and F2, which recognised continuous epitopes, did not block the IgE binding at all, which on the other hand is strongly dependent on the 3D-conformation of the allergen. IgG epitopes recognised by F3–F7 mAb families overlap to some extent with IgE epitopes, but the inhibitory activity of individual mAbs was partial, since it did not reach up to 50% of the IgE-binding. The overlapping of the IgE and IgG epitopes has been confirmed for other allergens such as Sin a 1, in which two of the families of mAbs blocked an allergenic epitope (Menéndez-Arias et al., 1990), or Der f 2, the IgE recognition of which is completely inhibited by two mAbs (Nishiyama et al., 1999). IgE-binding to Ole e 1 was markedly associated to the preservation of the disulfide bridges and, thus, to the correct folding of the allergen. This leads to difficulties in the study of the residues involved in the IgE epitopes, for which the production of recombinant forms of mutated Ole e 1-like clones is one of the scarce suitable tools. In this sense, the availability of *P. pastoris* as an efficient system to produce Ole e 1-like proteins (Huecas et al., 1999; González et al., 2001) will help to resolve the critical residues for antibody binding.

The importance of certain amino acid positions of the polypeptide chain of Ole e 1 for the recognition by antibodies is supported by the binding results obtained using sera of allergic patients. The isoform rOle e 1/3c showed the highest degree of IgE binding, indicating that it might be the main sensitizing form of Ole e 1, and suggesting that it is likely the main isoform component of the microheterogeneous Ole e 1. This would agree with the fact that this isoform was characterized as possessing the most similar amino acid sequence to that determined for the natural allergen by Edman degradation (Villalba et al., 1993, 1994). These data allow us to suggest that Ole e 1/3c would be the suitable recombinant candidate to be used for diagnosis of olive pollinosis. In contrast, rLig v 1 and rOle e 1/5c display a reduced affinity for IgE binding that make these molecules appropriate as hypoallergenic forms in immunotherapy protocols.

5. Conclusions

In this work, it has been shown that both IgE and IgG immunodominant regions of Ole e 1 preferentially exhibit a conformational nature, strongly dependent on the disulfide bond formation. The carbohydrate moiety of the allergen exhibited a moderate involvement in the binding to murine IgG mAbs. Our results suggest that the capacity for IgG- and IgE-binding of Ole e 1-like proteins depends on the conservation of the amino acid sequences of the corresponding epitopes, and that the antibodies are able to detect small differences, failing to bind to very similar but not identical homologous proteins present in related or non-related allergenic pollens.

In addition, this paper supports the fact that natural and genetic engineering-produced isoforms of allergens are useful tools for their immunologic characterization. An accurate knowledge of the antigenic sites of allergens should improve their use in both diagnostic and therapeutic applications.

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