

Quantification of the Major Allergen from Cypress (*Cupressus arizonica*) Pollen, Cup a 1, by Monoclonal Antibody-Based ELISA

M.C. Arilla^a I. Ibarrola^a R. García^b B. de la Hoz^b A. Martínez^a
J.A. Asturias^a

^aResearch and Development Department, Bial-Aristegui, Bilbao, and ^bAllergy Department, Ramón y Cajal Hospital, Madrid, Spain

Key Words

Allergen standardization · Cross-reactivity · Cypress pollen · Immunoassay · Monoclonal antibodies

Abstract

Background: Cypress pollen allergy is an important cause of rhinoconjunctivitis and asthma in Mediterranean countries. Cypress allergenic extracts are difficult to produce since they have low protein and high carbohydrate content, thus accurate standardization of them is essential to guarantee their quality. The aim of this study is to develop a sandwich ELISA for the quantification of Cup a 1, the major allergen of cypress (*Cupressus arizonica*) pollen extract. **Methods:** Monoclonal antibodies directed to purified Cup a 1 were produced. Two of them (9C7 as capture antibody and 3D2 as the tracer) were selected to develop a quantitative sandwich ELISA. This ELISA was subsequently evaluated and compared with other techniques. **Results:** The described ELISA is very sensitive with a detection limit of 8.7 ng/ml and a practical working range of 62.5–1,000 ng/ml. The assay is also highly reproducible with intra-assay and interassay coefficients of variation of less than 10%. The purified Cup a 1, used as standard, presents pectate lyase enzymatic

activity. The assay also detected Cup a 1-like proteins in pollen from other Cupressaceae. A good correlation was obtained between Cup a 1 content of 12 *C. arizonica* pollen extracts and their IgE-binding activity. **Conclusions:** The described Cup a 1 ELISA is sensitive, specific and reproducible and can be used for the quantification of Cup a 1 in *C. arizonica* and other related pollen extracts. It also provides a reliable indication of the allergenic activity of the whole cypress pollen extract.

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Introduction

Cupressaceae pollen allergy, which has been reported to cause winter conjunctivitis, rhinitis and asthma in various areas worldwide, has had an increasing trend over the last years in Mediterranean countries, reaching up to 30% among pollinosis cases [1–4]. The reasons for such an increase could be explained by the detection of high Cupressaceae pollen counts, in particular *Cupressus arizonica* that is widely used in reforestation, for ornamental purposes in gardens and parks, and also as wind and noise barriers. In addition, this pollinosis has been underestimated due to the symptoms coincident with those of

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Correspondence to: Dr. Juan A. Asturias
Bial-Aristegui, R&D; Alameda Urquijo, 27
ES-48008-Bilbao (Spain)
Tel. +34 944 438 000, Fax +34 944 438 016
E-Mail juan.asturias@bial.com

generic winter respiratory diseases (common cold, influenza syndrome) and the poor quality of available diagnostic extracts [5].

Pollen extracts from Cupressaceae species have a low protein and high carbohydrate content [6, 7]. *C. arizonica* pollen extracts contain at least six allergenic components with a predominant glycoprotein of 43 kD (Cup a 1) [6] having an incidence of 82% among Arizona cypress-allergic patients [8]. Cloned Cup a 1 from *C. arizonica* displayed a high sequence identity to Jun a 1 (91%) and Cha o 1 (81%) from the taxonomically related *Juniperus ashei* and *Chamaecyparis obtusa* pollens, respectively, and a significant identity to the family of pectate lyase-like proteins [9]. The pectate lyase-active site of Cup a 1, also found in Cry j 1 and Jun a 1, suggests that this allergen may have a similar enzymatic activity [10] but no experimental results have been found.

The low protein and high carbohydrate content found in Cupressaceae pollen extracts have hindered for a long time the availability of standardized extracts for the diagnosis and immunotherapy of cypress pollinosis [9]. Nowadays, the development of techniques to measure major allergen levels in diagnostic and therapeutic allergen products is a considerable improvement and of great interest. Although there is evidence that specific immunotherapy with maintenance doses of 5–20 µg of major allergen per injection is associated with significant improvement in patient symptom records [11], more clinical trials using specific immunotherapy with allergen-quantified extracts should be performed.

In this work, we demonstrate that the allergen Cup a 1 is an enzymatically active pectate lyase, and describe, for the first time, a solid phase ELISA based on the use of two different monoclonal antibodies (mAbs) recognizing non-overlapping epitopes of Cup a 1. The developed assay provides a specific and sensitive estimation of the content of the major allergen in cypress extracts.

Materials and Methods

Allergenic Extracts and Cup a 1 Purification

C. arizonica pollen was purchased from Iber-Polen (Málaga, Spain). Defatted pollen was extracted by magnetic stirring (4 h at 4°C) in 50 mM of phosphate buffer plus 65 mM NaCl pH 8.0 at 6.6% (w/v). The extract was clarified by centrifugation at 5,000 g for 30 min, filtered through AP glass fiber prefilters (Millipore, Bedford, Mass., USA) and dialyzed exhaustively by ultrafiltration in a Pelli-con System (Millipore) with a 5,000-dalton cutoff. For the Cup a 1 purification, the pollen was rapidly washed in acidic buffer (pH 4.3) at low ionic strength to solubilize carbohydrates, followed by an extraction of the proteins with 40 mM NH₄HCO₃ at 4°C [8]. The

clear supernatant of pollen extract was applied to a High Flow Phenyl-Sepharose 16/20 column equilibrated with 2 M (NH₄)₂SO₄ in an A KTA-prime system (Amersham Biosciences, Buckinghamshire, UK), and eluted with 20 mM phosphate buffer pH 7.0. Cup a 1-containing fractions were pooled, concentrated and applied to a molecular permeation chromatography column, Superdex S-200 16/60 (Amersham Biosciences), equilibrated with phosphate-buffered saline (PBS). The fractions corresponding to Cup a 1 were pooled and concentrated. The protein content was measured by the Bradford method using Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif., USA) and bovine serum albumin as standard. Recombinant Cup a 1, expressed in pTrc-HisA vector (Invitrogen, Groningen, The Netherlands), was produced in *Escherichia coli* and purified by metal-chelating chromatography.

Analysis of Pectate Lyase Enzyme Activity

The pectate lyase activity was analyzed by the method of Keen et al. [12]. The reaction mixture contained 0.33% (w/v) of poly-*D*-galacturonic acid methyl ester (Fluka, Buchs, Switzerland), 50 mM Tris-HCl buffer pH 9.0, 5 mM CaCl₂ and 5 µg of Cup a 1 in a total volume of 1 ml. One unit of pectate lyase activity was defined as the amount necessary to form 1 µM of unsaturated uronide product per minute with an extinction coefficient of 4,600 at 235 nm.

Human Sera

Serum samples were obtained from 11 subjects monosensitized to *Arizonica* cypress according to clinical histories of seasonal rhinoconjunctivitis and/or bronchial asthma, with positive skin prick test using biologically standardized *C. arizonica* pollen extract (Bial-Aristegui, Bilbao, Spain) and specific IgE levels to *C. arizonica* pollen extract greater than class 2 score (CAP System, Pharmacia Diagnostics, Uppsala, Sweden).

Production, Purification and Biotinylation of mAbs

BALB/c mice were immunized intraperitoneally with 20 µg of Cup a 1 emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., USA). The same amount of antigen with incomplete Freund's adjuvant was given every 2 weeks for 2 months. Detailed procedures for the fusion and hybridoma maintenance have been previously described [13]. Isotype detection was done with the IsoStrip kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. mAbs were purified from hybridoma culture supernatants with an HiTrap Protein G (Amersham) and labeled with biotin using the ECL protein biotinylation module (Amersham).

Competitive ELISA Inhibition

Polystyrene 96-microwell plates (Greiner, Frickenhausen, Germany) were coated with 2 µg/ml of Cup a 1 in 0.1 M bicarbonate buffer pH 9.6, and blocked with 1% bovine serum albumin, 0.05% Tween 20 in PBS (PBS-B-T). Afterwards, different concentrations of unlabeled purified mAbs (3D2 and 9C7) were added to the wells and incubated for 1 h at 37°C, then purified biotin-labeled mAbs (3D2 or 9C7) were added and incubated for one more hour, and developed as previously described [14]. The inhibition binding percentage of the biotin-labeled mAb to Cup a 1 was calculated according to the following formula: $[1 - (\text{OD in presence of mAb competitor} / \text{OD in absence of competitor})] \times 100$.

Periodate Treatment on Solid-Phase Adsorbed Cup a 1

Cup a 1 was subjected to periodate oxidation after adsorption on ELISA plates according to the method described by Woodward et al. [15]. Cup a 1 adsorbed to the plate was washed with 50 mM sodium acetate (pH 4.5) prior to exposure to 5 mM sodium periodate for 1 h at room temperature in the dark. Following a brief rinse with 50 mM sodium acetate, the plates were incubated with 1% glycine in PBS for 30 min at 23°C. After five washes with PBS-0.05% Tween 20, the plates were incubated with mAb and developed as described above.

Gel Scanning Densitometry and Immunodetection Assay

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole extracts (8 µg per lane) and purified Cup a 1 (2 µg per lane) were performed with 12.5% acrylamide gel under reducing conditions [16]. Protein bands were detected with Coomassie Blue R-250, and Cup a 1 was quantified by densitometry as previously described [17]. For immunodetection assays, extracts and purified Cup a 1 were loaded at 0.5 and 0.125 µg per lane, respectively. Separated protein bands were electrophoretically transferred onto polyvinylidene difluoride [18] and membranes were blocked for 1 h at room temperature with 0.1% Tween 20 in PBS. Membranes were incubated for 1 h with mAbs followed by anti-mouse IgG-horseradish peroxidase conjugate incubation and detected by the ECL chemiluminescent method as recommended by the manufacturer (Amersham Biosciences).

Quantification of Cup a 1 by mAb-Based Sandwich ELISA

Microwell plates were coated overnight at 4°C with 100 µl of mAb 9C7 at 5 µg/ml in PBS. After blocking with 200 µl of PBS-B-T for 1 h at 37°C, 100 µl of serially diluted Cup a 1 in PBS-B-T were added and incubated under the same conditions. After washing, 100 µl of biotinylated 3D2 (0.3 µg/ml in PBS-B-T) was added to each well and incubated for 1 h at 37°C. Wells were washed 3 times with PBS-T and bound complexes were detected by the incubation for 1 h at 37°C with streptavidin-horseradish peroxidase conjugate (Sigma Chemical, St. Louis, Mo., USA) at 250 ng/ml in PBS-B-T. After three washes, wells were incubated at room temperature in the dark with a solution of *o*-phenylenediamine (Sigma). The reaction was stopped after 30 min by adding 50 µl/well of 3 M H₂SO₄, and the optical density was measured at 492 nm. A standard curve was generated with dilutions of purified Cup a 1 between 4,000 and 4 ng/ml. The analytical sensitivity or detection limit was calculated as the amount of Cup a 1 that corresponded to the mean plus 3.29 times the standard deviation obtained after 30 measurements of the zero standard. Crude extracts were assayed as dilution series on 3 different days and the concentrations were interpolated from the linear portion of the standard curve. For evaluation of intra-assay variations, three Cup a 1 concentrations (62.5, 250, and 500 ng/ml) were measured 20 times in the same assay, and for interassay variations ten curves were done on different days.

Cup a 1-Depleted Extracts

In order to obtain Cup a 1-depleted extracts, polyclonal antibodies were obtained from New Zealand rabbits by immunizing with 5 boosts of 200 µg of natural Cup a 1 every 2 weeks, emulsified in complete Freund's adjuvant (Difco) and purified with a HiTrap Protein G (Amersham). Purified anti-Cup a 1 polyclonal antibodies were bound to protein G-Sepharose beads (Amersham) according to the manufacturer's instructions. After extensive washing, 50 µl of a 50% beads suspension were incubated with *C. arizonica* extract (0.2 mg/

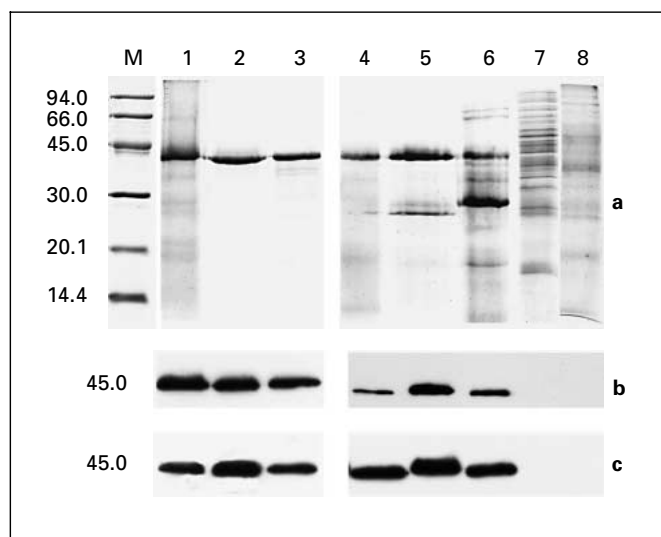


Fig. 1. Coomassie Brilliant Blue-stained SDS-PAGE (**a**) and immunoblots incubated with mAb 9C7 (**b**) or mAb 3D2 (**c**) of pollen extracts from *C. arizonica* (lane 1), *C. sempervirens* (lane 4), *T. plicata* (lane 5), *J. communis* (lane 6), *P. radiata* (lane 7), and *C. atlantica* (lane 8), natural Cup a 1 (lane 2), and recombinant Cup a 1 (lane 3). M = Molecular-weight markers.

ml) at 20°C for 2 h. Beads were removed for centrifugation and the process repeated until no Cup a 1 was detected.

Enzyme Allergosorbent Test Inhibition

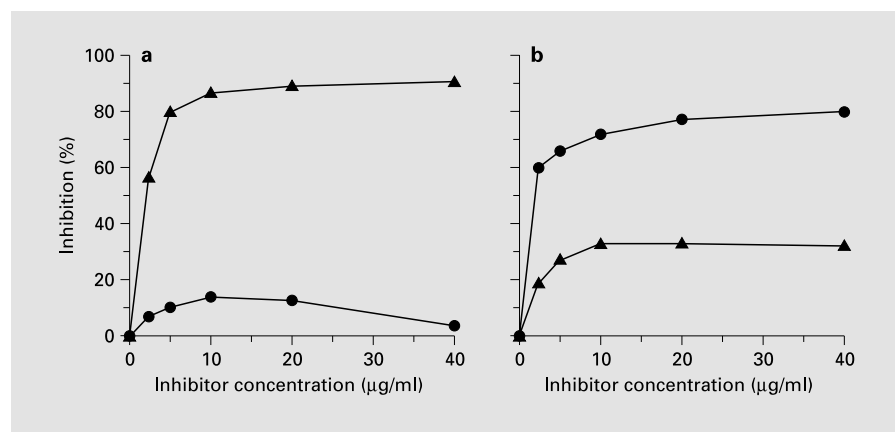
The IgE-binding activity of *C. arizonica* pollen extract was evaluated by enzyme allergosorbent test (EAST) inhibition (Hytec; Hyco Biomedical, Kassel, Germany) using paper discs coupled to a *C. arizonica* pollen extract as solid phase and a serum pool from cypress pollen-sensitive patients. Relative allergenic activity of the extracts was estimated on the basis of the 50% inhibition EAST values and expressed in relative allergenic units.

Results

Purification and Pectate Lyase Activity of Cup a 1

Natural Cup a 1 was isolated from *C. arizonica* pollen extract by a combination of hydrophobic interaction and gel permeation chromatographies. The purified protein was analyzed by imaging densitometry of SDS-PAGE gel and consisted of a 43-kD band with an estimated purity of 95% (fig. 1). Purification yield was 10 mg of protein per 20 g of pollen. Cup a 1-containing fraction showed a maximal pectate lyase activity of 1,200 U/mg at pH 9.0 and 37°C, with Ca²⁺ as the required cation.

Fig. 2. Competitive inhibition of biotinylated mAb to solid-phase Cup a 1 by unlabeled mAb. Cup a 1-coated plates were incubated with only buffer (no inhibition) or different concentrations of unlabeled mAb: 3D2 (▲) and 9C7 (●), and then biotinylated mAb 3D2 (a) or 9C7 (b) was added.



Production and Characterization of mAbs

Immunization of BALB/c mice with purified Cup a 1 resulted in 5 mAbs of IgG class with specificity for Cup a 1 as judged by ELISA. All possible pairs of mAbs were tested for the applicability in a sandwich ELISA or immunoenzymetric assay. Based on their reactivity two mAbs, namely 3D2 and 9C7, were chosen for further studies. Both of them were typed as IgG1 subclass with κ light chains. The specificity of mAbs 3D2 and 9C7 was demonstrated by immunodetection after SDS-PAGE of natural and recombinant Cup a 1 and *C. arizonica* extract (fig. 1). Western blot experiments were carried out in order to study the distribution of the antigenic determinants recognized by the mAbs in other conifer pollen extracts (*Cupressus*, *Juniperus*, *Thuja*, *Cedrus*, and *Pinus*). Both mAbs recognized a 43- to 42-kD band in the pollen extracts from members of the Cupressaceae family (*Cupressus*, *Thuja*, and *Juniperus*) although reactivity of 9C7 was weaker in the *Cupressus sempervirens* and *Juniperus* pollen extracts (fig. 1). No reactivity was found in *Pinus* and *Cedrus* pollen extracts.

Competitive inhibition studies were undertaken using unlabeled mAb in order to inhibit the binding of biotinylated mAb to the allergen (fig. 2). As expected binding inhibition was observed with the same unlabeled mAb (>80% when using mAb inhibitor concentrations of 20 μg/ml). In contrast, unlabeled mAb 9C7 inhibited less than 10% of the binding activity of the biotinylated mAb 3D2, while mAb 3D2 inhibited the 35% of the biotinylated 9C7 binding activity. These results demonstrated that both mAbs recognized different antigenic determinants in the protein.

Binding of mAb 3D2 and 9C7 to periodate-treated Cup a 1 showed a different behavior. 3D2 bound with

similar affinity to Cup a 1 and to deglycosylated Cup a 1, with an OD of 1.58 and 1.64, respectively, while 9C7 bound more strongly to treated Cup a 1 (OD of 1.51) than to Cup a 1 (OD of 0.81).

Cup a 1 ELISA

The mAb 9C7 was used as coating mAb and the biotinylated 3D2 as detector, because this combination rendered the highest optical densities in the standard curve of Cup a 1. A linear regression with a coefficient of regression of 0.995 was found in the range from 62.5 to 1,000 ng/ml. The coefficient of interassay variation was evaluated by measuring a standard curve per day on 10 different days and it was lower than 10% in the linear portion of the standard curve. The intra-assay coefficients of variation were 7, 6 and 4% at the concentrations of 62.5, 250 and 500 ng/ml ($n = 20$), respectively. The detection limit was calculated as 8.7 ng/ml. The dose-response curves obtained for purified recombinant Cup a 1, expressed in *E. coli* as a HisTag fusion protein, were compared with that obtained for natural Cup a 1 (fig. 3). Both curves were parallel, but that obtained with recombinant Cup a 1 was about 5-fold more sensitive.

The specificity of the assay was evaluated against a broad battery of extracts from tree pollen (*Olea europaea*, *Platanus acerifolia*, *Betula verrucosa*, *Pinus radiata*, *Cedrus atlantica*), grass pollen (*Dactylis glomerata*), weed pollen (*Parietaria judaica*, *Artemisia vulgaris*, *Ambrosia trifida*, *Plantago lanceolata*, *Helianthus annuus*), and other nonplant extracts (*Alternaria alternata*, *Dermatophagoides pteronyssinus*). No signal was found up to a concentration of 25 μg/ml (10 times the upper limit of the assay range for *C. arizonica* extract). Among the Cupressaceae family, proteins sharing common epitopes for

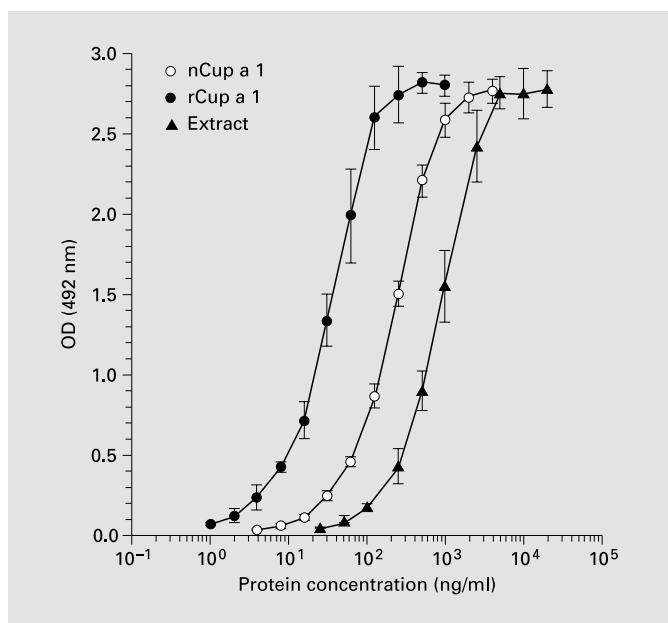


Fig. 3. Dose-response curves in sandwich ELISA of natural and recombinant Cup a 1 and *C. arizonica* pollen extract. Values are means of ten (Cup a 1) or five (extract) separate experiments $\pm$ SD.

mAbs 3D2 and 9C7 were found in *C. sempervirens*, *Thuja plicata* and *Juniperus communis* pollen extracts at 5, 12 and 16 μ g/mg, respectively. Nevertheless, for a more accurate determination of these Cup a 1-like proteins, their corresponding purified proteins should be used as standards in the dose-response curve.

Quantification of Cup a 1 in *C. arizonica* Pollen Extracts and Correlation with Allergenic Activity

The Cup a 1 content of 7 *C. arizonica* pollen extracts was evaluated by using the currently developed sandwich ELISA. The curves obtained showed good parallelism with the standard curve (fig. 3). Cup a 1 levels ranged from 16 to 41% of the total protein. An immunosorbent resin based on polyclonal antibodies was used to obtain Cup a 1-depleted extracts. The amount of allergen remaining after three rounds of Cup a 1 immunodepletion was less than 1% of the starting amount measured by sandwich ELISA.

Seven different unmodified *C. arizonica* pollen extracts and 5 *C. arizonica* extracts that had been experimentally reduced in their Cup a 1 content were assayed by ELISA and their IgE-binding activity was determined by EAST inhibition (fig. 4). A significant correlation ($r = 0.901$, $p < 0.001$) was found between the two methods,

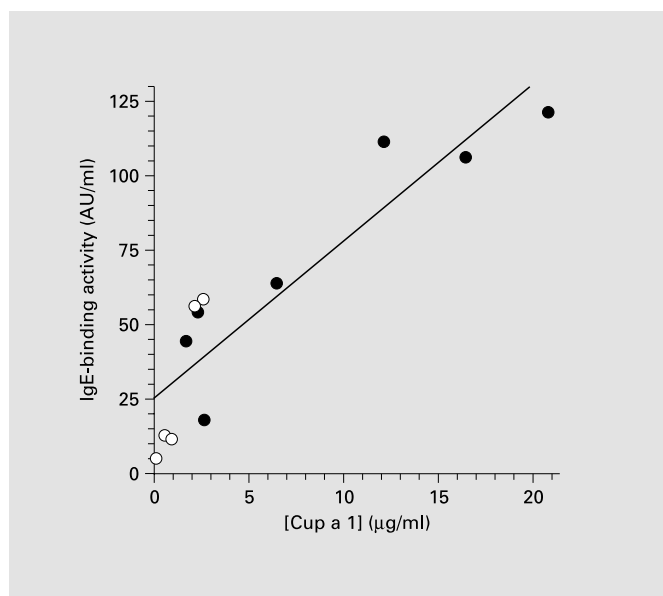


Fig. 4. Correlation between Cup a 1 content measured by ELISA and IgE-binding activity determined by EAST inhibition of 7 *C. arizonica* pollen extracts (●) and 5 *C. arizonica* extracts with Cup a 1 content reduced by adsorption to Cup a 1-specific rabbit antiserum (○).

demonstrating that the Cup a 1 is an important allergen in the extract, and is representative of the overall allergenic activity of the whole *C. arizonica* extract. In contrast, an insignificant correlation was found between the Cup a 1 content calculated by ELISA and the densitometry analysis of Coomassie-stained 43-kD band using known amounts of purified Cup a 1 as a reference (data not shown).

Discussion

Allergy to Cupressaceae has been underestimated, partly due to the lack of characterized allergenic extracts for diagnosis. Cypress pollen extracts have low protein and high carbohydrate content because of the physicochemical properties of their pollen grains [19]. Efforts have been made to establish the optimal conditions for pollen extraction and to provide a qualitative definition of the relevant allergenic components in these extracts [6, 7, 20, 21]. Recent works have purified and cloned two IgE-reactive components of Arizona cypress pollen (Cup a 1 and Cup a 3) [10, 22, 23]. The IgE-based current standardization protocol faces two main problems. First, the techniques involve the use of human sera that are difficult

to standardize. Second, only an overall allergenic potency is determined on the basis of reactivity with IgE of pooled sera from allergic patients and, therefore, extracts with similar potency may present differences in major allergen ratios [24]. Standardization of cypress pollen extracts presents the additional difficulty of finding appropriate sera, since patients allergic to cypress pollen have a normal or subnormal total serum IgE level and a low titer in specific IgE for cypress extracts, making IgE-based techniques difficult to perform [19].

The quantification of Cup a 1 is an important goal for the standardization of cypress pollen extracts intended for clinical use since it allows an optimization of extraction procedures and comparison of batches with the biologically standardized inhouse reference extract. The described assay is based on two Cup a 1-specific mAbs with different epitope specificities and is highly specific for group 1 allergens from the Cupressaceae family, since it is unable to detect Cup a 1-like proteins in the Pinaceae family (*Cedrus* and *Pinus*). Cloned Cupressaceae group 1 allergens (Cup a 1, Cup s 1, Jun a 1 and Cha o 1) share more than 82% identical amino acid residues [9] and a primary structure of pectate lyase, enzymatic activity which has been experimentally demonstrated in this study for Cup a 1. The assay described has an adequate sensitivity (8.7 ng/ml) to be applicable to the routine measurement of Cup a 1 content in production batches of the low-protein-content cypress pollen extracts and prick test solutions. Prick test components (phenol and glycerol) had no effect on the assay since they can be diluted more than 1:2,000 due to the high sensitivity of the assay (data not shown). Further, the assay is highly reproducible, as demonstrated by both intra-assay and interassay coefficients of variation.

Recombinant allergens have been used as reference material for specific allergen quantification [25]. In the present assay, recombinant Cup a 1 cannot be used as standard since the stronger binding of 9C7 to recombinant allergen could underestimate Cup a 1 content in cypress pollen extracts. Overproduction of foreign proteins in the *E. coli* cytoplasm often goes together with misfolding of the recombinant proteins that could enhance antibody binding. The fact that 9C7 bound more strongly to the recombinant Cup a 1 or periodate-treated natural Cup a 1 than to the natural counterpart suggests that the relatively large glycan structures found in Cup a 1 [8] could interfere with the binding of 9C7 to the polypeptide chain of Cup a 1.

To our knowledge, this is the first assay based on specific mAbs that has been developed for the quantification

of major allergens in cypress pollen extract. A recent work has determined the Jun a 1 content of a *J. ashei* pollen extract by comparing the chemiluminescent signal of the extract with a standard using purified Jun a 1 [21]. In our hands, no significant correlation was found between the IgE-binding activity determined by EAST inhibition and the content of Cup a 1 calculated by densitometry of Coomassie-stained gels after SDS-PAGE. In contrast, a very significant correlation was obtained between the content of Cup a 1 in *C. arizonica* pollen extracts determined by ELISA and IgE-binding activity (fig. 4). This result confirms that this allergen is an important allergen in cypress extracts and indicates that the described assay could be very useful for the standardization of whole extracts in terms of their Cup a 1 allergen content and of their overall IgE-binding activity. Similarly, good correlations between mAb immunoassays and IgE-binding activity have been described in other extracts [14, 17, 25–27]. There is evidence that the optimal maintenance dose for specific immunotherapy with some allergens is 5–20 µg [11] but this range should not necessarily be followed as a general rule, especially for highly reactive allergens such as Alt a 1, Par j 1 or Cup a 1. Using the assay described, the average of Cup a 1 content in maintenance doses of specific immunotherapy with cypress pollen extracts is 0.7 µg. Clinical trials designed to validate the efficacy of specific immunotherapy using Cup a 1-quantified *C. arizonica* pollen extracts are currently underway.

In conclusion, we have described, for the first time, an ELISA procedure for the measurement of group 1 allergens in cypress pollen extracts. The assay is highly sensitive and specific, and could be very useful for quality control of preparations of cypress pollen allergen extracts intended for clinical use.

Acknowledgments

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