

Comparison between the native glycosylated and the recombinant Cup a1 allergen: role of carbohydrates in the histamine release from basophils

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Summary

Background Cypress pollinosis is an important cause of respiratory allergies. Recently, the *Cupressus arizonica* major allergen, Cup a1, has been cloned and expressed. The native counterpart of this allergen has been purified and characterized by our group. It has been suggested that sugar moieties play a role in the *in vitro* IgE binding on *Cupressus arizonica* pollen extract.

Objective To characterize the immunoreactivity of the recombinant major allergen in comparison with its native counterpart. To evaluate the role of carbohydrate moieties in the IgE-mediated *in vitro* histamine release from basophils by using the native glycosylated Cup a1 as compared with the recombinant one.

Methods Recombinant Cup a1 was expressed in *E. coli*. IgE reactivity of Cupressaceae-allergic patients on the native as well as the recombinant molecule was investigated by immunoblotting, ELISA experiments and histamine release test from passively sensitized basophils.

Results Fourteen out of 17 Cup a1-positive sera had IgE antibodies reactive with the native molecule only and lost their reactivity after periodate deglycosylation of the allergen. Moreover, only native molecule was capable of inducing histamine release by this group of sera. Both the recombinant and the native molecules were recognized by three out of the 17 sera and were equally capable of triggering degranulation.

Conclusion A large number of sera reactive with the major allergen recognize carbohydrate epitopes only. IgE from these sera are able to induce histamine release from basophils and they might play a functional role in the clinical symptoms of allergy.

Keywords allergy, carbohydrates, Cupressaceae, histamine-release test, IgE, major allergens, recombinant allergens

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Introduction

It is now well established that common epitopes shared by various components belonging to different allergenic extracts are responsible for an increasing number of cross-reactions [1], which have been associated with both protein and carbohydrate epitopes. Although the actual role of cross-reactive carbohydrate determinants (CCDs) in allergic diseases is still a debated question, their presence in a wide range of allergens of plant and invertebrate origin [2–4] lead to unexpected cross-reactivities and to false-positive or clinically irrelevant *in vitro* results [5, 6]. In particular, evidence that CCDs could be biologically active in triggering effector cell degranulation is still preliminary [2, 7]. Divergent opinions have been expressed on their ability to

trigger histamine release and to play any clinical role [8], taking also into account that recombinant non-glycosylated allergens have been in many cases proposed to replace the native one for diagnostic and therapeutic purposes [9, 10]. These controversial findings prompt further investigations on carbohydrate epitopes at the diagnostic level as well as in the induction of clinical symptoms.

We have recently reported that *in vitro* IgE binding from Cupressaceae-allergic subjects on *Cupressus arizonica* (*C. arizonica*) components [11, 12] is strongly influenced by the presence of carbohydrate moieties, thus suggesting that a large number of patients suffering from Cupressaceae pollinosis display IgE specific for carbohydrate epitopes. This feature characterizes this pollinosis as an appropriate model to investigate the phenomenon in more detail.

In the present paper, we have evaluated the role of carbohydrates in the induction of histamine release from basophils passively sensitized with sera from well-characterized subjects allergic to cypress. In particular, we compared the reactivity of the native major allergen of *C. arizonica* (nCup a1) purified from pollen extract [13] with that of its recombinant

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¹The nucleotide and deduced amino acid sequences of the molecule DNA have been submitted to the GenBank database with the accession number AJ278498.

non-glycosylated counterpart. To achieve this, we cloned recombinant Cup a1.02 (rCup a1.02) in *E. coli* and identified it as a Cup a1 isoform on the basis of sequence comparison analysis with the allergen recently cloned by Aceituno et al. [14].

Materials and methods

Total RNA isolation, cDNA synthesis and cloning

The full-length cDNA of *C. arizonica* pollen was obtained by a PCR from total RNA isolated from pollen by the guanidinium thiocyanate method of Chomczynski and Sacchi [15]. Primers used in PCR (5'-ATG GAT CCA TGG CTT CCC CAT GCT T-3' and 5'-CCG GAA TTC TTA GGT TAC AAC TCC AG-3') were based on the Jun a1 cDNA sequence [16] (accession number AF106662) and Bam HI and Eco RI restriction sites were introduced. PCR reaction was performed using the Takara kit (Takara shuzo Co., Otsu, Shiga, Japan) according to the manufacturer's instructions. PCR product was inserted into the p-Gex-6P (Stratagene, Gebouw, CA, USA) vector and positive clones were identified. In order to remove the leader sequence of 21 amino acids, which is absent in the native molecule, a PCR was performed on the full-length selected clone using the primers 5'-ATG GAT CCG ATA ATC CCA TCG AC-3' and 5'-CCG GAA TTC TTA GGT TAC AAC TCC AG-3' and a mature product (lacking in 63 bp) was obtained.

Expression, production and purification of the recombinant molecule

Plasmid DNA of the mature product was purified by the Qiagen kit (Qiagen Genomics Inc., Washington DC, USA). The recombinant protein was expressed in *E. coli* BL-21 strain. The molecule was purified using a matrix of Glutathione Sepharose 4B (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The GST-Cup a1.02 fusion protein was treated with PreScission Protease (Amersham Pharmacia Biotech Inc.) to remove the GST portion from the molecule. The enzyme was applied according to the manufacturer's instructions. The yield of purified recombinant allergen was 2 mg per litre of bacterial culture. Such a recombinant molecule, together with the native major allergen, was used throughout the present study.

Sequence analysis

The DNA sequences of both the full-length¹ and the mature product were determined using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and run on ABI 310 DNA sequencer (Applied Biosystems, Perkin-Elmer Italia, Milano, Italy). The deduced amino acid sequences were analysed by using the PROSITE database (Release 17.1). The alignment between proteins was performed by using the Clustal W (1.74 and 1.81) program.

Human sera

Human sera were obtained from 30 patients allergic to Cupressaceae pollen (polysensitized or monosensitized), as proven by clinical history, skin prick test (SPT) with commercial extracts and specific IgE *in vitro* detection (CAP System, Pharmacia Upjohn, Uppsala, Sweden) (Table 1). The subjects were free from allergen-specific immunotherapy, corticosteroids and

Table 1. Features of the 30 cypress-allergic patients (SPT and cypress CAP results)

Sera number	Cypress reactivity		
	CAP (class)	SPT (positivity)	Other sensitizations (SPT)
1	4	++++	Not available
2	4	++	GRA; OLEA; PAR
3	2	++++	Monospecific
4	3	++	BET; DER; PAR
5	3	++	BET; GRA; OLEA; PAR
6	3	++	DER; LATEX; PAR
7	3	++++	Monospecific
8	3	++	BET; COMP; DER
9	3	+++	DER; GRA; PAR
10	2	++	GRA; PAR
11	2	++	DER
12	3	++++	DER; GRA; OLEA; PAR
13	3	++	OLEA
14	4	++++	Monospecific
15	4	++++	Monospecific
16	2	++	DER; PAR
17	4	+++	DER
18	2	++	DER; GRA; PAR
19	2	+++	GRA; PAR
20	3	++++	ALT; DER
21	3	+++	Monospecific
22	3	+++	DER
23	3	+++	PAR
24	3	+++	ALT; DER; MOS
25	4	+++	COMP; DER; PAR
26	3	+++	DER; GRA; PAR
27	2	++	GRA; PAR
28	4	++++	OLEA
29	4	++++	COMP; GRA
30	3	+++	DER; PAR

ALT = *Alternaria alternata*; BET = *Betula verrucosa*; COMP = compositae mixtures; DER = mite mixture; GRA = grass mixture; LATEX; MOS = mosquito; OLEA = *Olea europaea*; PAR = *Parietaria judaica*.

antihistaminic therapy. Their informed consent to participate in the study was obtained.

Serum samples from non-atopic donors were collected to be used as controls. Sera were stored at -20°C until use.

Native Cup a1 (nCup a1)

The purified major allergen nCup a1 was prepared as previously described [13].

Monoclonal antibodies

Monoclonal antibodies (MAbs) 5E6, 2D5 and 4A6 were prepared and characterized as reported in Barletta et al. [17]. MAb 5E6 is specific for a periodate-sensitive allergenic epitope [12] on nCup a1, whereas MAbs 2D5 and 4A6 recognize a periodate-resistant epitope on nCup a1 [17].

SDS-PAGE and immunoblotting

The molecules were analysed in 15% SDS-PAGE under reducing condition. SDS-PAGE and immunoblotting experiments were carried out as previously described [17, 18]. The amount of

both native and recombinant proteins loaded on the gel was 1 µg for each lane (4 mm × 7 cm). MAbs reactivity was evaluated in immunoblotting on the recombinant and native protein. Monoclonal antibodies were diluted 1 : 1000 in phosphate buffered saline pH 7.2 (PBS) – 0.05% Tween (PBS-T).

Sera from the 30 Cupressaceae-allergic subjects were tested in immunoblotting to evaluate their reactivity on the native and recombinant molecule. Human sera were diluted 1 : 10 in PBS-T. Bound IgE were detected by ¹²⁵I-labelled antihuman IgE (Bio-Allergy, Rome, Italy). Results were quantitatively analysed by densitometric scanning of the autoradiographed films on a Multi-Analyst/PC-Densitometer (Bio-Rad, Hercules, CA, USA).

To further characterize the nature of the epitope recognized by specific IgE on nCup a1, human sera were tested with nCup a1 before and after chemical deglycosylation with sodium metaperiodate in mild conditions (10 mM). The molecule was subjected to sodium metaperiodate oxidation after electrotransfer onto nitrocellulose membranes according to the method described by Woodward et al. [19]. After washing in PBS-T, strips were rinsed with 50 mM sodium acetate buffer (pH 4.5); treated strips ('deg') were exposed to 10 mM sodium metaperiodate (NaIO₄) in sodium acetate buffer for 1 h at room temperature in the dark, whereas control strips were treated with the buffer only. Treated and control strips were rinsed again with sodium acetate buffer followed by 50 mM sodium borohydride in PBS for 30 min. After washing in PBS-T, strips were incubated with the sera.

Direct ELISA and ELISA inhibition

ELISA and ELISA inhibition experiments were carried out as previously described [12, 18]. The amount of nCup a1 and rCup a1.02 coated on the ELISA plates (polystyrene microtitre 96-well, Greiner, Frickenhausen, Germany) was 5 µg/mL. The amount of antigens to be used in plates coating was determined on the basis of preliminary titration experiments. Human sera were diluted 1 : 10 in PBS-T. Inhibitors were used at the concentration of 20, 10, 5, 1, 0.1 µg/mL.

Basophils histamine release assay

Heparinized blood from a non-atopic donor was collected and mixed with 0.25 volumes of 6% w/v dextran. Leucocytes were isolated as described by Okayama et al. [20] and were washed in

Ca²⁺, Mg²⁺-free-PBS. Passive sensitization of basophils with specific IgE from allergic sera was performed after non-specific IgE stripping according to the method of Pruzansky et al. [21]. Three representative sera from Cupressaceae-allergic patients and two control sera (one from a non-atopic subject and one from a mite-allergic patient) diluted 1 : 2, were used in this test. The samples passively sensitized were diluted 1 : 7 in the buffer provided by the kit utilized to measure histamine release (Histamine kit: Immunotech Pharmaceuticals, San Diego, CA, USA). The procedure was carried out according to kit instructions as described by Batanero et al. [7]. The concentration of allergens used in the test was 10, 5, 2, 0.5, 0.05 µg allergen/mL, both for samples from Cupressaceae-allergic patients as well as for negative controls.

Results

Sequence analysis of the recombinant allergen

Sequence analysis of the mature recombinant allergen (lacking in 63 bp) cDNA revealed a 1041-bp clone. The deduced amino acid sequence corresponds to a protein of 346 aa with two potential sites of N-glycosylation (positions 127–130: NTSV, and 157–160: NVTN) and an estimated molecular weight of 37.6 kDa. The nucleotide sequence of the recombinant allergen was compared with the recombinant Cup a1 cloned by Aceituno et al. [14] (accession number AJ 243570). This analysis revealed 24 nucleotide differences leading to 11 amino acid substitutions. The homologies observed (96%) suggest that the molecule we have cloned might represent an isoallergen of Cup a1, hereafter designated as Cup a1.02. The amino acidic sequence alignment of the two molecules is shown in Fig. 1.

The predicted sequence showed a high degree of identity (94%) to the recombinant Jun a1 (*Juniperus ashei*) [16]. Moreover, the identity to the sequence of the recombinant Cha o1 (*Chamaecyparis obtusa*, accession number D45404) [22], Cry j1 (*Cryptomeria japonica*, accession number P18632) [23], Jun v1 (*Juniperus virginiana*, accession number AF 151427) [24] was very high, with a percentage ranging from 79% to 93% (data not shown).

Cup a1.02	DNPIDSCWRGDSNWDQNRMKLADCVVGFGSLTMGGKGGEIYTVTSSDDNPVNPTPGTLRY
Cup a1	DNPIDSCWRGDSNWDQNRMKLADCVVGFGSSTMGGKGGEIYTVTSSDDNPVNPTPGTLRY

Cup a1.02	GATREKALWIIIFSQNMNIKLQMPYVAGYKTIDGRGADVHLGNGGPGCLFMRKTASHVILHG
Cup a1	GATREKALWIIIFSQNMNIKLQMPYVAGYKTIDGRGAVVHLGNGGPGCLFMRKASHVILHG

Cup a1.02	LHIHGCNTSVLGDVLVSESIGVEPVHAQDGAITMRNVNTAWIDHNSLSDCSDGLIDVTL
Cup a1	LHIHGCNTSVLGDVLVSESIGVEPVHAQDGAITMRNVNTAWIDHNSLSDCSDGLIDVTL

Cup a1.02	GSTGITISNNHFFNNHKKVMLLGHDDTYDDDISMKVTVAFNQFGPNAGQRMPRARYGLVHV
Cup a1	GSTGITISNNHFFNNHKKVMLLGHDDTYDDDKSMKVTVAFNQFGPNAGQRMPRARYGLVHV

Cup a1.02	ANNNDQWNIYAIGSSNPITLSEGNSTAPSESYKKEVTKRIGCESTSACANWVWRFR
Cup a1	ANNNDQWNIYAIGSSNPITLSEGNSTAPSESYKKEVTKRIGCESTSACSNWVWRSTR

Cup a1.02	DAFTNGAYFVSSGKAEETNIYNSNEAFKVENGNAAAPQLTQNAVVT
Cup a1	DAFTNGAYFVSSGKAEETNIYNSNEAFKVENGNAAAPQLTQNAVVA

Fig. 1. CLUSTAL W (1.74) multiple sequence alignment. Amino acidic sequence alignment of the two recombinant molecules. * = identical or conserved residues; : = conserved substitutions; . = semiconserved substitutions.

Reactivity of rCup a1.02 with specific IgE and monoclonal antibodies

The recombinant Cup a1.02 (rCup a1.02) was analysed by SDS-PAGE after Coomassie Brilliant Blue staining (Fig. 2, lane A) and IgE immunoblotting with a serum pool from 3 Cupressaceae-allergic patients (Fig. 2, lane B). To further evaluate the immunological reactivity of the recombinant molecule, the

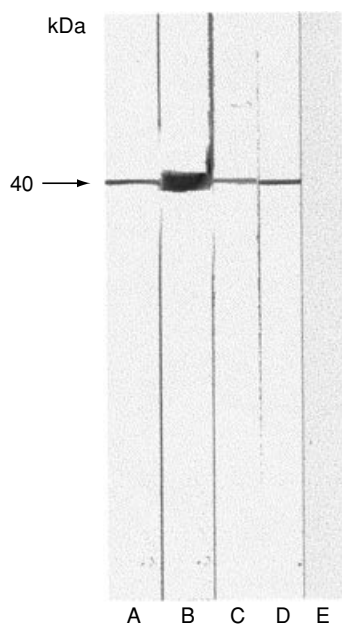


Fig. 2. SDS-PAGE analysis of rCup a1.02 (lane A) after Coomassie Brilliant Blue staining. Immunoblotting analysis was performed with a serum pool from three Cupressaceae-allergic subjects (sera n. 14, 15, 16; lane B) and with monoclonal antibodies, MAb 4A6 (lane C), MAb 2D5 (lane D) and MAb 5E6 (lane E).

following monoclonal antibodies were used. MABs 4A6 and 2D5, specific for different protein epitopes on nCup a1, were able to recognize rCup a1.02 (Fig. 2, lanes C and D, respectively). On the contrary, MAB 5E6, capable of recognizing a CCD-epitope on the native allergen, did not bind the recombinant protein (Fig. 2, lane E).

Native Cup a1 and rCup a1.02 were compared in terms of IgE reactivity, taking into account that IgE from some patients lose their reactivity after periodate deglycosylation of the pollen extract [11]. Thus, 30 sera from Cupressaceae-allergic patients were tested in immunoblotting and ELISA experiments with both nCup a1 and rCup a1.02. Seventeen out of 30 sera were positive to nCup a1 (57%). When tested on the recombinant allergen, three out of these 17 sera (18%) were positive. Deglycosylation of nCup a1 by periodate did not affect the binding of the three sera, but abrogated the IgE reactivity of the remaining 14 (82%) sera, which bound the native glycoprotein only. Table 2 shows the results of immunoblotting densitometric analysis as well as direct ELISA obtained by the 14 sera that recognized the native Cup a1 and by the three sera positive to both the molecules (n. 14, 15, 16). These 17 sera can be regarded as representative of two populations displaying different epitope(s) recognition, the first one restricted to carbohydrates only, the second one recognizing protein epitopes.

As shown in the Table 2, data obtained by immunoblotting are confirmed by ELISA experiments, indicating that IgE binding pattern of the sera tested is not dependent on the assay condition.

ELISA inhibition

To compare the equivalence of protein IgE epitopes represented on the recombinant and the native Cup a1, IgE ELISA inhibition experiments were carried out. To this aim, sera n. 14, 15

Table 2. IgE reactivity by immunoblotting (OD obtained by densitometric analysis) and direct ELISA (OD₄₉₀). The sera were tested with the nCup a1, the rCup a1.02 and the nCup a1 deglycosylated (nCup a1 deg)

Sera number	Immunoblotting nCup a1	Immunoblotting rCup a1.02	Immunoblotting nCup a1 deg	ELISA nCup a1	ELISA rCup a1.02	ELISA nCup a1 deg
1	0.45	neg	neg	0.40	0.06	0.06
3	1.39	neg	neg	1.37	0.06	0.06
7	0.75	neg	neg	0.63	0.06	0.07
9	0.50	neg	neg	0.41	0.06	0.06
12	1.03	neg	neg	0.87	0.06	0.05
14	1.22	1.19	1.23	1.50	1.30	1.31
15	0.81	0.90	0.80	0.59	0.50	0.49
16	0.69	0.64	0.77	0.52	0.48	0.48
17	0.42	neg	neg	0.34	0.05	0.05
19	0.53	neg	neg	0.30	0.06	0.06
21	1.29	neg	neg	0.59	0.06	0.06
22	1.17	neg	neg	0.60	0.06	0.06
23	0.43	neg	neg	0.47	0.05	0.06
25	0.47	neg	neg	0.24	0.05	0.06
28	0.78	neg	neg	0.44	0.05	0.06
29	1.33	neg	neg	0.86	0.06	0.05
30	0.63	neg	neg	0.40	0.05	0.06

Negativity cut-off values for direct ELISA have been calculated on the basis of the mean OD₄₉₀ values plus 3 SD, obtained with six sera from non-atopic donors (OD₄₉₀ 0.07, 0.06 and 0.07 for nCup a1, rCup a1.02 and nCup a1 deg, respectively). The negative value for immunoblotting (neg) has been obtained by the densitometric acquisition of a blank lane.

and 16, positive to both the native and the recombinant molecule, were used for inhibition experiments.

As shown in Fig. 3 (a and c, serum 14 and 15, respectively), when the recombinant molecule was used as antigen, both nCup a1 and rCup a1.02 displayed superimposable inhibition patterns on the recombinant molecule up to 100%, suggesting that the two molecules present comparable protein epitopes. Marked differences between the three sera were found when nCup a1 was used as antigen. With serum 14 (Fig. 3b), nCup a1 was able to inhibit the IgE binding up to 100%, whilst the rCup a1.02 reached a maximum value of 65% at the highest concentration used. Serum 15 gave similar inhibition curves with the two inhibitors (Fig. 3d). Serum n. 16 behaved as serum n. 15 using both the recombinant and the native molecule as antigen (data not shown).

Histamine release from basophils

The property of both native and recombinant Cup a1 to trigger basophil degranulation was investigated in a histamine release test, using basophils from a non-atopic donor passively sensitized with IgE from allergic patients n. 3, 12, 14, 15, 21, 28. Sera n. 3, 12, 21 and 28 were chosen as representative of the population displaying anticarbohydrates IgE, whereas sera n. 14 and 15 represented the population recognizing protein epitopes. The dose-related release curves obtained after stimulation with nCup a1 and rCup a1.02 are shown in Fig. 4, panel a and b, respectively. Specific IgE from sera n. 3, 21 and 28 induced a strong histamine release up to 98% at the native antigen concentration of 10 µg/mL, whereas histamine release induced by

serum n. 12 reached 26% at the highest amount of native allergen (Fig. 4a). No histamine release occurred when these sera, whose reactivity was essentially against carbohydrates moieties, were tested with the recombinant molecule in the same range of concentration (Fig. 4b). On the contrary, when sera n. 14 and 15 were used, both nCup a1 and rCup a1.02 were able to induce histamine release in the range of allergen concentration from 10 to 0.05 µg/mL.

Sera from one non-atopic subject and one mite-allergic patient were used as negative controls. They did not induce histamine release when tested with any allergen amount, whereas IgE from the mite-allergic patient were able to elicit histamine release when incubated with a mite extract (data not shown).

Discussion

The molecular biology applied to the production of recombinant allergens has brought great advantages in the field of allergy diagnosis [9, 10, 25–27]. The availability of a complete panel of cloned allergens would be of great help in understanding the potential clinical importance of allergen cross-reactivities. Moreover, the individual patient's sensitization profile could be determined by using purified recombinant allergens, thus allowing *in vivo* and *in vitro* component-resolved diagnosis [9] to be performed.

On the other hand, CCDs have been demonstrated in several cases to act in binding assays as important epitopes recognized

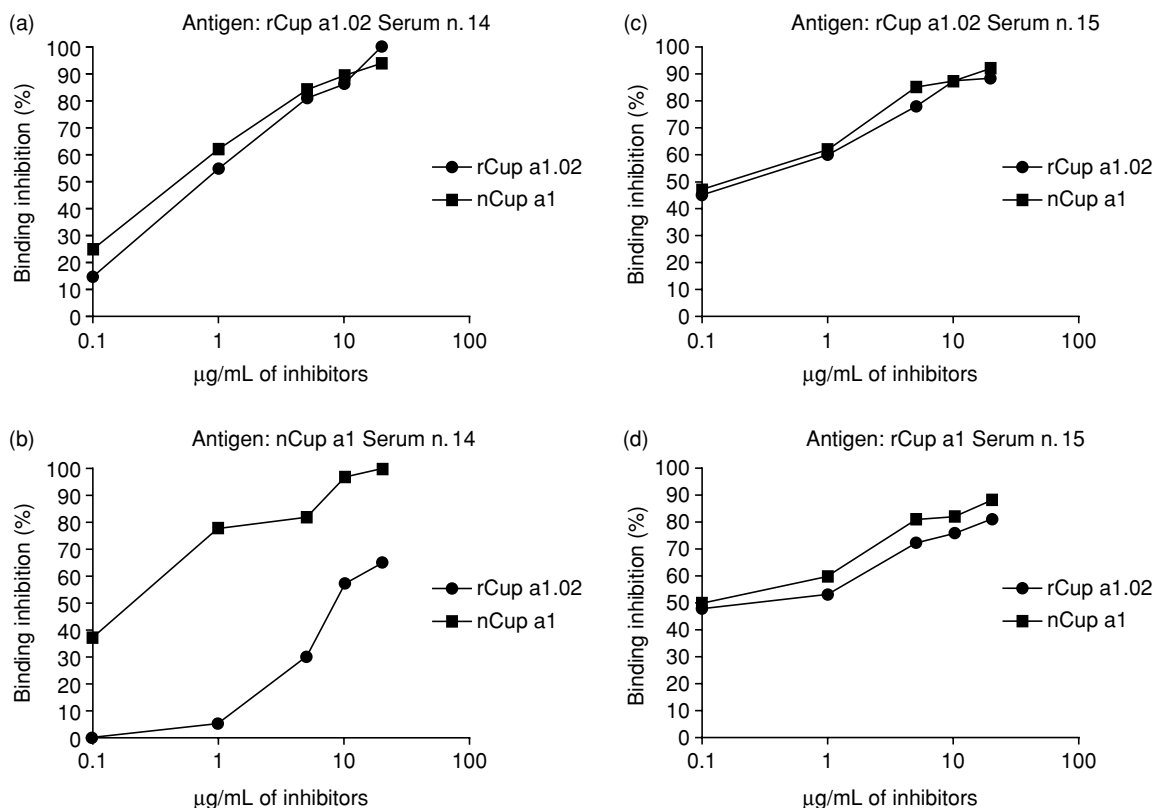


Fig. 3. (a) and (c): IgE binding inhibition to rCup a1.02 as antigen by rCup a1.02 (circles) and nCup a1 (squares). (b) and (d): IgE binding inhibition to nCup a1 as antigen by rCup a1.02 (circles) and nCup a1 (squares). Inhibitors were used at the concentration of 20, 10, 5, 1 and 0.1 µg/mL.

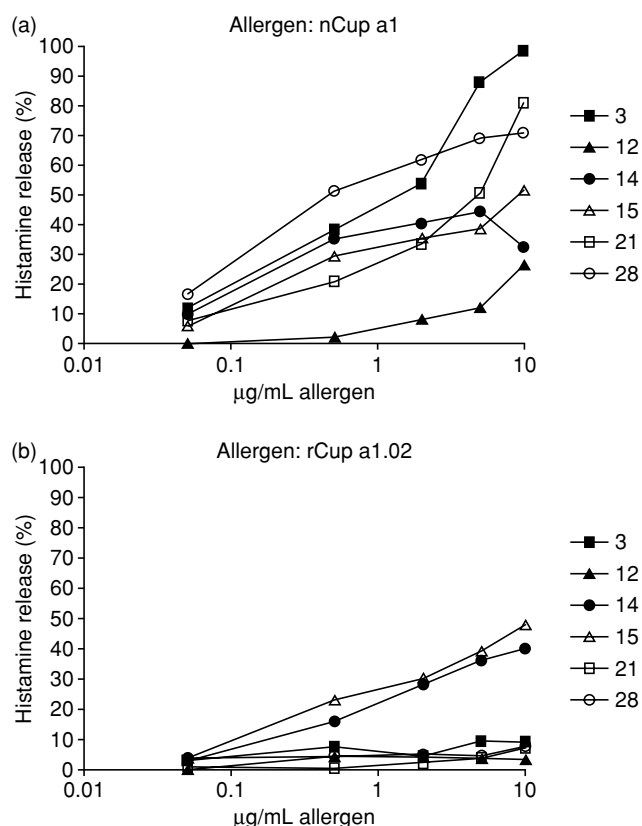


Fig. 4. Basophil histamine release test performed with IgE from sera n. 3 (filled squares), 12 (filled triangles), 14 (filled circles), 15 (open triangles), 21 (open squares) and 28 (open circles). Panels (a) and (b) show the dose-related release curves obtained after stimulation with nCup a1 and with rCup a1.02, respectively. Allergens were used at the concentration of 10, 5, 2, 0.5 and 0.05 $\mu\text{g/mL}$.

by IgE from allergic patients [28–34]. Very little information about the histamine-releasing activity of such CCDs is available.

We have previously described in the field of Cupressaceae allergy the important role of carbohydrate moieties in the IgE binding on allergenic components of *C. arizonica* pollen extract [11]. To better characterize the role of sugar moieties present on *C. arizonica* major allergen, the native Cup a1 has been purified and its N-glycans have been analysed [13]. The carbohydrate structures identified (xylose, a core α 1,3-fucose and Le^a epitope) are known to be responsible for IgE binding as well as for IgE cross-reactivity between foods and pollens [35].

In the present study, we cloned and expressed a cDNA of Cup a1 in a bacterial system; we used the recombinant allergen to further study the nature of IgE epitope in cypress allergy and to evaluate the IgE binding on the native glycosylated molecule as compared with a non-glycosylated recombinant molecule. The deduced amino acid sequence alignment of our molecule to the molecule cloned and expressed by Aceituno et al. [14] revealed 11 amino acid substitutions, thus suggesting that Cup a1 isoforms might exist. For this reason, our clone was named Cup a1.02. As the primers were designed on the basis of Jun a1 cDNA and the 3' primer region was different between Jun a1 and Cup a1 by Aceituno et al. at the level of two nucleotides, the amino acid exchange at the C-terminus in our molecule (see Fig. 1) could be an artefact resulting from the primer design and

it should be better investigated. However, the replacement of Ala by Thr is a conservative one and should not affect the folding of the molecule.

Three subjects (out of the 17 reactive with nCup a1) were able to recognize the recombinant non-glycosylated molecule; these data were confirmed by chemical deglycosylation of the nCup a1. On the other hand, IgE from the other 14 sera that bound the native molecule only, lost their reactivity after such treatment. These results confirmed our previous data on the low frequency of recognition of protein epitopes by specific IgE in cypress-allergic patients [11].

On the basis of the reactivity of individual sera with recombinant and native Cup a1, we were able to group nCup a1 positive subjects into two populations, the largest one displaying IgE reactivity essentially restricted to CCDs (82%) and the other one (18%) recognizing protein epitopes.

To rule out the possibility that the observed differences could be ascribed to different protein epitopes represented on the native and the recombinant molecule, ELISA inhibition experiments were carried out using the three sera recognizing protein epitopes. When the recombinant allergen was used as antigen, both the molecules were able to inhibit the IgE binding with similar curves, suggesting that the protein epitopes recognized by these sera are represented in a comparable amount on the two molecules. The results obtained with the sera recognizing protein epitopes allow us to claim similarity between rCup a1.02 and nCup a1 at the level of protein epitopes. This finding is further confirmed by the reactivity of monoclonal antibodies specific for two different protein epitopes on the Cup a1 molecule. On the other hand, the native glycoprotein was a better inhibitor in the test performed using the homologous antigen and serum n. 14, thus suggesting that carbohydrates on the native Cup a1 can bind IgE present in this group of sera. As an alternative explanation, the higher inhibiting capacity of nCup a1 vs. the binding of serum n. 14 could be due to differences in the folding of the recombinant molecule. These differences would lead to the loss of some protein epitopes specifically recognized by serum 14 only.

The two molecules have been then evaluated for their capability of triggering histamine release from basophils passively sensitized with sera from cypress-allergic patients. To our knowledge, two groups only have until now carried out histamine release tests to demonstrate that carbohydrates can trigger basophil degranulation. Batanero et al. [7] showed that the free carbohydrates from the major olive tree allergen, Ole e1, induced histamine release from sensitized human basophils. Fötisch et al. [2] demonstrated a strong *in vitro* histamine release by IgE from a celery-allergic patient but in this case the stimulation was carried out with a synthetic conjugate consisting of BSA and some glycopeptides purified from bromelain. In the present paper, a different approach was followed, using the recombinant and the native Cup a1 first to characterize a panel of sera and then to assess the properties of some of them, chosen as representative, to trigger histamine release upon challenge by the two molecules. These experiments were performed with basophils from a non-atopic donor sensitized with IgE from cypress-allergic subjects: four sera (n. 3, 12, 21, 28) recognizing sugars moieties only and two sera (n. 14 and 15) recognizing proteins. The results obtained demonstrated that nCup a1 only was able to induce a strong histamine release from basophils sensitized with sera containing IgE specific for CCDs (up to

98%). On the other hand, IgE from sera n. 14 and 15 were able to induce basophil degranulation after stimulation with both the molecules, thus confirming that rCup a1.02 can trigger histamine release when specific antiprotein IgE are present in the serum.

These findings could have important practical implications for the study of different pollinosis. In fact, our data would suggest that recombinant allergens expressed in procaryotic systems would not be suitable to correctly diagnose cypress allergy, due to the lack of carbohydrates that represent important allergenic epitopes able to induce histamine release. The expression of Cup a1 in different eucaryotic systems such as plants could be of great interest to evaluate its reactivity in the histamine release test.

In conclusion, data reported in our study show that IgE binding on *C. arizonica* major allergen involves protein as well as carbohydrate epitopes and that the latter are not only recognized by a large majority of the subjects, but also capable of inducing *in vitro* histamine release from basophils sensitized with IgE specific for the carbohydrates on nCup a1. These results support the hypothesis that these IgE could be biologically active in cypress allergy, and suggest that the expression of the recombinant allergen in glycosylating systems is needed for their use as an alternative to native Cup a1 in the diagnosis of cypress allergy.

The recombinant molecule expressed in *E. coli* could be regarded as a useful tool to discriminate the different characteristics of IgE recognition by heterogeneous populations of Cupressaceae-sensitized subjects.

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