

Original article

Monoclonal antibody-based ELISA to quantify the major allergen of *Artemisia vulgaris* pollen, Art v 1

Background: Pollen of *Artemisia vulgaris* (mugwort) is a relevant cause of pollinosis in temperate and humid regions. Recently, the major allergen of this pollen, Art v 1, has been characterized.

Objective: To develop a monoclonal antibody (mAb)-based enzyme-linked immunosorbent assay (ELISA) to quantify Art v 1, and to assess the correlation of Art v 1 content with the biological activity of mugwort pollen extracts.

Methods: Art v 1-specific mAbs were obtained from a BALB/c mouse immunized with high-performance liquid chromatography (HPLC)-purified Art v 1. One of these antibodies (Av 3.7), which recognizes the N-terminal defensin-like domain of Art v 1, was used as the capture antibody in an ELISA method for allergen quantitation. An anti-*A. vulgaris* rabbit serum was used as the second antibody. Art v 1 was purified by immunoaffinity chromatography and used as the standard in the assay.

Results: The purity and identity of the affinity-purified Art v 1 was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), mass spectrometry, amino acid composition, and N-terminal amino acid sequencing. The prevalence of specific IgE against Art v 1, determined by radio-allergosorbent test (RAST) in a population of 44 mugwort-allergic patients, was 79%. The Art v 1-ELISA developed displays a detection limit of 0.1 ng/ml, and a practical working range of 0.2–10 ng/ml. The concentration of Art v 1 was measured in 10 *A. vulgaris* pollen extracts, and a good correlation was observed between the Art v 1 content and the allergenic activity of the extracts.

Conclusions: The results prove the usefulness of the Art v 1-ELISA for the standardization of *A. vulgaris* pollen extracts intended for clinical use.

L. Jimeno, O. Duffort, C. Serrano, D. Barber, F. Polo

Research and Development Department, ALK-Abelló, Madrid, Spain

Key words: Allergen; Art v 1; *Artemisia vulgaris*; immunoassay; monoclonal antibody; mugwort; pollinosis.

Florentino Polo
Departamento de Investigación y Desarrollo
ALK-Abelló
C/Miguel Fleta, 19
E-28037 Madrid
Spain

Accepted for publication 12 November 2003

Mugwort (*Artemisia vulgaris*) is a wind-pollinated perennial weed, which belongs to the Compositae family. Mugwort pollen is considered as the most important cause of pollinosis at the end of summer and beginning of autumn in western Europe.

Mugwort pollen contains approximately 40 extractable proteins of which 10 appear to be allergens (1). The first allergen isolated from this pollen, termed Art v 1 in the article dealing with its purification (2), but not included yet in the list of allergens of the International Union of Immunological Societies (IUIS), is a 60-kDa acidic glycoprotein recognized by the IgE from 73% of mugwort-allergic patients. The allergen named Art v 1 in the IUIS list is a 108-amino acid residues glycoprotein with a high sugar content (30–40%), to which 90% of the patients have specific IgE (3). Art v 1 consists of an N-terminal cysteine-rich domain, homologous to the cysteine-stabilized, $\alpha\beta$ -motif characteristic of the plant defensin family, and a C-terminal domain rich in hydroxyproline residues, some of which are O-glycosylated. The carbohydrate moiety is highly heterogeneous

and plays an important role in the allergenicity (3). The other allergens identified in mugwort pollen to date are the glycoprotein allergen Art v 2 (4), and the panallergens Art v 3, a lipid-transfer protein (5), and the profilin Art v 4 (6), with a rate of IgE-recognition ranging from 30 to 50% of mugwort-allergic patients.

Here, we describe the purification of the major allergen Art v 1 by immunoaffinity chromatography and the development of a monoclonal antibody (mAb)-based enzyme-linked immunosorbent assay (ELISA) to quantify it. The analytical features of this assay and the correlation of the Art v 1 content with the allergenic potency of mugwort extracts are also reported.

Material and methods

Source material and preparation of extracts

Pollen of *A. vulgaris* was purchased from Allergon AB (Aengelsholm, Sweden). The pollen extract was prepared as previously described (2). Other allergenic extracts were obtained from

ALK-Abelló (Madrid, Spain). Protein concentration was determined by the Lowry method (7).

Human sera

Sera from 44 allergic patients with hypersensitivity to *A. vulgaris* pollen, as demonstrated by clinical history and positive skin reaction to a commercial *in vivo* diagnostic product (ALK-Abelló), were used throughout this work.

Production of antibodies

For mice immunization, high-performance liquid chromatography (HPLC)-purified Art v 1 was used. Purification was carried out by 70% saturation ammonium sulfate salt precipitation of *A. vulgaris* pollen extract followed by sequential size-exclusion and reverse-phase HPLC. Female BALB/c mice were injected intraperitoneally every 15 days with 50 µg of this preparation in Freund's adjuvant (complete for the first injection and incomplete for the rest). Three days before fusion, mice were boosted intravenously with the same dose of antigen in phosphate-buffered saline (PBS). Spleen cells from immunized mice were fused with P3.X63.Ag8.653 myeloma cells according to the method of Galfré and Milstein (8). Ten days after the fusion, cell culture supernatants were screened for anti-Art v 1-specific antibodies by an ELISA with HPLC-purified Art v 1 on the solid-phase. The P3.X63.Ag8 mouse myeloma culture supernatant was used as a negative control. The positive hybrids were cloned and subcloned by limiting dilution.

Polyclonal antibodies against *A. vulgaris* pollen extract were produced in New Zealand rabbits. Each rabbit was injected intramuscularly with 2 mg of extract dissolved in 200 µl of PBS and mixed with the same volume of Freund's adjuvant (complete for the first injection and incomplete for the rest). Injections were repeated every 15 days and sera were collected 10 days after each injection. A rabbit serum pool from four animals was made up of 10 successive bleedings.

Both monoclonal and polyclonal antibody production was carried out in accordance with the Organization of Economic Co-operation and Development (OECD) principles of good laboratory practice (as revised in 1997) and European Council Directive No. 609 (1986).

Purification and characterization of antibodies

Selected mAbs were purified from hybridoma culture supernatants using a Protein G Sepharose affinity column (Amersham Biosciences, Uppsala, Sweden), following the manufacturer's instructions. The isotype of the antibodies was determined by ELISA with antimouse subclass antisera (Nordic, Tilburg, the Netherlands).

The mAb Av 3.7, selected to develop the Art v 1-quantitation ELISA, was further characterized by means of two additional experiments. First, an inhibition ELISA was performed to study the inhibition of the binding of human IgE to Art v 1 by this mAb. Briefly, immunoaffinity-purified Art v 1 (2 µg/ml in PBS) was adsorbed on to polystyrene microtitre wells (EIA/RIA plates, Costar, Cambridge, MA) and, after blocking with 1% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween 20 in PBS (P-B-T), the wells were incubated for 3 h at room temperature with a mixture of 50 µl of a serum pool from mugwort-allergic patients, diluted 1 : 2.5 with P-B-T, and 50 µl of serial dilutions of the mAb Av 3.7 in the same buffer. Detection of bound IgE was performed by sequential incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgE antibodies (Dako, Glostrup, Denmark) and *o*-phenylenediamine (OPD; Dako). After the addition of 2 M HCl,

the absorbance was measured at 490 nm with a reference filter at 650 nm. An inhibition curve constructed with serial dilutions of Art v 1 was included for comparison.

The second characterization experiment consisted in the study of the binding of mAb Av 3.7 to the native or reduced and alkylated (RA) forms of Art v 1. The ELISA plates were coated with native or RA Art v 1 at 2 µg/ml in PBS. After blocking, the wells were incubated for 2 h at room temperature with serial twofold dilutions of mAb Av 3.7 in P-B-T, starting from 0.5 µg/ml. The wells were then sequentially incubated with HRP-conjugated rabbit anti-mouse IgG antibodies (HRP-RAM; Dako) and OPD. A similar experiment was carried out using the anti-*A. vulgaris* rabbit serum instead of the mAb Av 3.7. In this case, detection was accomplished with HRP-conjugated goat anti-rabbit immunoglobulin antibodies (HRP-GAR; Calbiochem, San Diego, CA) and OPD.

Purification of Art v 1 by affinity chromatography

An immunosorbent column was prepared by coupling mAb Av 3.7 to CNBr-activated Sepharose 4B gel (Amersham Biosciences), following the manufacturer's recommendations. Mugwort pollen extract in PBS was passed through the column and, after extensive washing with PBS, Art v 1 was eluted using 100 mM glycine, pH 2.5. Fractions of 1 ml were collected in tubes containing 50 µl of neutralizing buffer (1 M Tris buffer, pH 9.0). Fractions were pooled on the basis of protein content, dialyzed against distilled water, lyophilized and stored at 4°C until use. The purity of Art v 1 was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry. Amino acid analysis was carried out using reagents (AccQ.Tag) and equipment from Waters (Milford, MA).

Reduction and alkylation of Art v 1

Art v 1 (345 µg, corresponding to 256 nmol of cysteine) was dissolved in 345 µl of 0.5 M Tris-acetate buffer, pH 8.2, containing 7 M guanidine and 2 mM ethylenediaminetetraacetic acid (EDTA). Reduction was carried out with a 100-fold molar excess of 2-mercaptoethanol (final concentration 75 mM) for 2 h at 40°C. Iodoacetamide was then added to a final concentration of 150 mM and the reaction mixture was incubated in the dark at room temperature for 2 h. The reaction was quenched by adding 1% (v/v) 2-mercaptoethanol and the excess reagents were removed by dialysis against distilled water.

SDS-PAGE and immunoblotting

SDS-PAGE was performed with tricine 10–20% polyacrylamide gels (Novex, San Diego, CA) (9), under reducing conditions. Electrotransfer of separated polypeptides was carried out by the method of Towbin et al. (10). Immunodetection of IgE-binding polypeptides using a serum pool from mugwort allergic-patients was accomplished with ¹²⁵I-antihuman IgE mAb HE2 (11).

Mass spectrometry analysis

Samples were mixed (1 : 1, v/v) with a matrix solution consisting of aqueous 2,5-dihydroxybenzoic acid (10 mg/ml), and measured on a Biflex III (Bruker-Franzen Analytik, Bremen, Germany) matrix-assisted laser-desorption/ionization time-of-flight mass spectrometer. Mass spectra were recorded in linear positive mode accumulating 100 spectra of single laser shots. The equipment was calibrated using myoglobin.

N-terminal sequence analysis

N-terminal amino acid sequence of RA Art v 1 was determined using an Applied Biosystems Model 477A gas-phase sequenator (Foster City, CA).

Similarity searches were performed in protein and DNA sequence databases (GenBank, EMBL, PIR, NBR, NBR3D and Swiss Prot) using the FASTA3 program.

RAST and RAST inhibition

Determination of specific human IgE in sera was carried out by RAST, with an *A. vulgaris* in-house reference extract or immunoaffinity-purified Art v 1 coupled to cyanogen bromide activated paper discs, essentially as described by De Filippi et al. (12). The allergenic activity of *A. vulgaris* extracts was evaluated by RAST inhibition and expressed in HEP/ml by comparison with an in-house reference, which had previously been calibrated by skin-prick test (13).

Art v 1 ELISA

Microtitre 96-well plates (Costar) were coated overnight at 4°C with 100 µl of anti-Art v 1 mAb Av 3.7 at 5 µg/ml in PBS. After blocking, wells were sequentially incubated with samples and references, anti-*A. vulgaris* rabbit serum (1 : 40000 dilution), and HRP-GAR (Calbiochem). Samples, controls and reagents were conveniently diluted in P-B-T, and all incubations were carried out for 1 h at room temperature with intermediate washes between successive steps. Detection with OPD was accomplished as described above. The Art v 1 concentration of samples was calculated by interpolating from a standard curve constructed with serial twofold dilutions of affinity-purified Art v 1, starting from 25 ng/ml.

Results

Affinity chromatography purification of Art v 1

Nine mAbs of IgG class were obtained from the fusion of spleen cells of a mouse immunized with HPLC-purified Art v 1 and X63.Ag8.653 myeloma cells. One of these mAbs (Av 3.7, isotype IgG2a) was selected to be used for immunoaffinity chromatography and to develop an ELISA to quantitate Art v 1. Affinity chromatography enabled us to efficiently purify Art v 1 from a mugwort pollen extract. Approximately 1.6% of the total protein of the pollen extract was recovered as pure Art v 1 after the chromatographic procedure. The electrophoretic pattern of immunoaffinity-purified Art v 1 (Fig. 1) displayed two broad bands at 26 and 29 kDa. Both bands were strongly immunostained with the sera from *A. vulgaris*-allergic patients in Western blot (Fig. 1C). The identity of the purified protein was established by mass spectrometry, N-terminal amino acid sequencing and amino acid composition analysis. MALDI mass spectrometry analysis showed a large heterogeneity in size of the purified protein, exhibiting two major series of peaks centered around 13 424 and 15 789 kDa (Fig. 2) that correspond to the double-band pattern observed

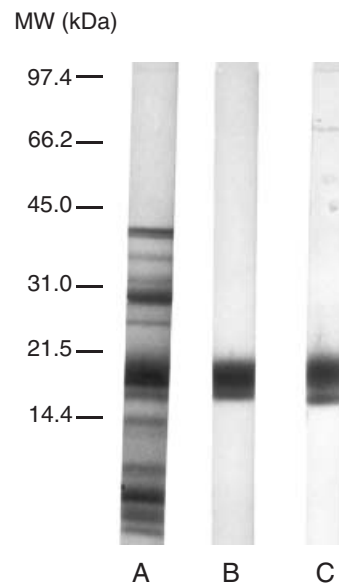


Figure 1. Coomassie Brilliant Blue-stained SDS-PAGE patterns of *Artemisiavulgaris* pollen extract (A) and immunoaffinity-purified Art v 1 (B). (C) Immunoblotting of the same sample as in (B) with a serum pool from mugwort-allergic patients.

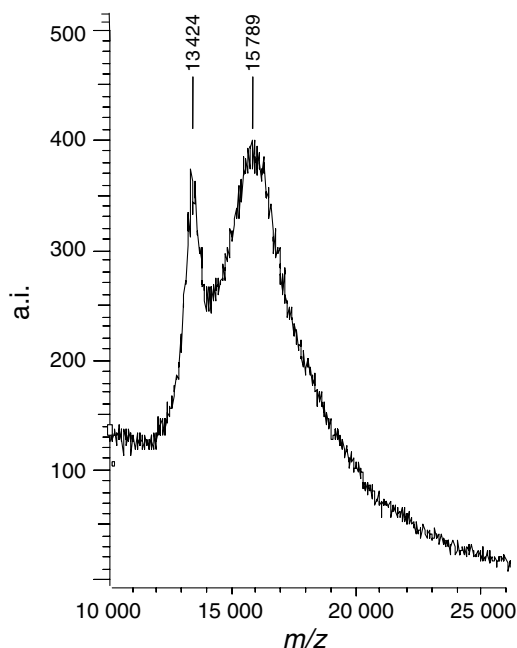


Figure 2. Mass spectra recorded for immunoaffinity-purified Art v 1, exhibiting the single-charged molecular ions. The scale is in arbitrary units.

after SDS-PAGE. These results are in excellent agreement with those described by Himly et al. (3). The discrepancies between the molecular masses and the apparent molecular weight values determined by SDS-PAGE

are attributable to the high sugar content of this allergen (3). The first 20 residues of the N-terminal amino acid sequence (NH₂-AGSKLCEKTSKTYSGKCDNK) matched the sequence translated from the cDNA sequence of mature Art v 1 exactly (3). Moreover, the amino acid composition of immunoaffinity-purified natural Art v 1 completely agreed with that expected from the amino acid sequence deduced from cDNA sequence (3) (data not shown).

Allergenic significance of Art v 1

In order to demonstrate the clinical relevance of Art v 1, the level of specific IgE was determined in serum from 44 selected patients (Table 1). Even after rejecting as negative those sera with RAST class 1, a high proportion of patients (79%) had specific IgE to Art v 1.

Characterization of antibodies

Before proceeding further with the development of a quantitation method using the mAb Av 3.7, additional experiments aimed at the characterization of the epitope defined by this mAb were performed. Thus, a competition assay with human IgE from a serum pool from mugwort-allergic patients showed that mAb Av 3.7 recognizes an epitope that coincides or, at least, overlaps with an important allergenic epitope of Art v 1, as it inhibited the binding of specific IgE to Art v 1 by 30% (Fig. 3). Moreover, the binding of mAb Av 3.7 to Art v 1 was completely abolished upon reduction and alkylation of the allergen (Fig. 4A), which suggests that the mAb does not recognize carbohydrate prosthetic groups, but rather a conformational epitope located on the polypeptide chain of the allergen.

In contrast, the polyclonal antibodies raised in rabbit against mugwort pollen reacted strongly to the reduced and alkylated allergen, although 2.5-fold more concentrated solutions of the serum were needed to reach absorbance values similar to those of the native allergen (Fig. 4B).

ELISA for Art v 1 quantitation

The method we have developed to quantify Art v 1 consists in a sandwich ELISA with the mAb Av 3.7

Table 1. Specific IgE levels to *Artemisia vulgaris* pollen extract and purified Art v 1 in mugwort-allergic patients

Immunosorbent	RAST class					Positivity (%)
	4	3	2	1	0	
<i>Artemisia vulgaris</i>	12	22	9	1	0	97
Art v 1	5	21	9	6	3	79

Data show the number of patients with the indicated RAST class. Only those sera with RAST class 2 or higher are considered positive.

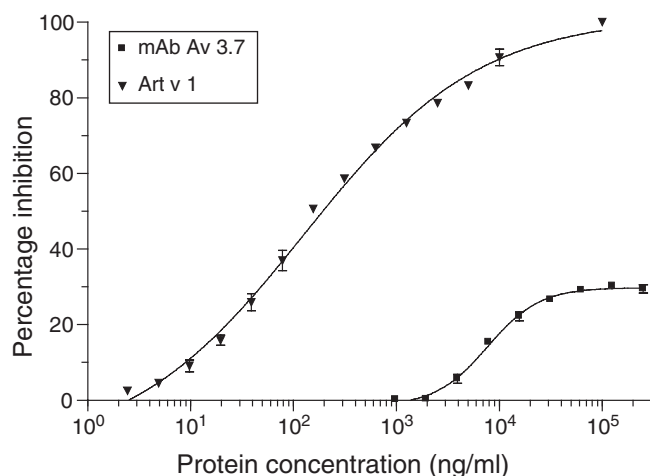


Figure 3. Inhibition of IgE-binding to Art v 1 by monoclonal antibody (mAb) Av 3.7. Native Art v 1 was adsorbed to the solid-phase and binding of specific IgE from a serum pool of mugwort-allergic patients was inhibited by increasing concentrations of mAb Av 3.7. An inhibition curve with Art v 1 was included for comparison. Detection was accomplished with HRP-conjugated rabbit anti-human IgE antibodies and OPD, as described in 'Materials and Methods'. Determinations were carried out in triplicate. Bars indicate standard error of the mean.

on the solid-phase and an anti-*A. vulgaris* pollen rabbit serum as the second antibody. Detection of the rabbit antibodies bound is accomplished with goat anti-rabbit immunoglobulin antibodies conjugated with HRP.

The specificity of the method was evaluated against a large battery of extracts from pollens of grasses, trees, and weeds, including *Ambrosia trifida*, *Helianthus annuus*, *Taraxacum officinale*, and *Chrysanthemum maximum* belonging to the Compositae family. Only the latter, which belongs to the same Anthemidae tribe as mugwort, gave a significant response in the ELISA method, but the rest were negative in the assay, even when they were tested at a protein concentration as high as 100 µg/ml (data not shown).

The dose-response curves obtained in the ELISA with affinity-purified Art v 1 and *A. vulgaris* pollen extract were parallel (Fig. 5), suggesting that the allergen had not been altered during the purification process and that the assay was measuring the same molecule in the different preparations. Therefore, the concentration of affinity-purified Art v 1 was accurately measured by quantitative amino acid analysis, and this preparation was used as the primary standard in the ELISA method.

The assay developed in this work is very sensitive, with a detection limit of 0.1 ng/ml and a practical working range of 0.2–10 ng/ml. The lower limit of this range (0.2 ng/ml) can be considered the limit of quantitation of the method. Moreover, the assay is highly reproducible. The precision was determined by analyzing six replicates of the same

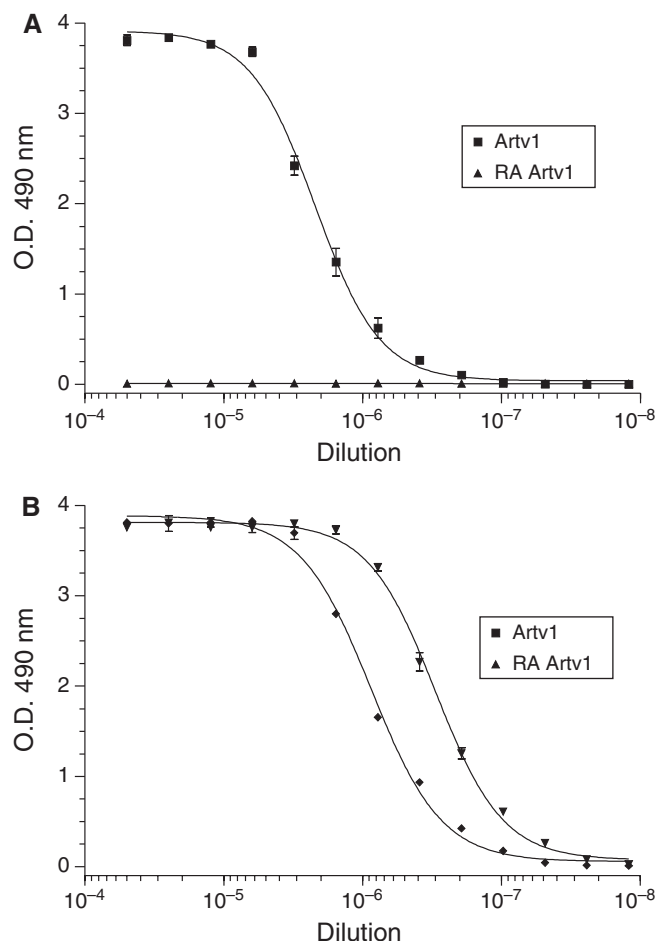


Figure 4. Binding of antibodies to native and to reduced and alkylated (RA) Art v 1. (A) Native or RA Art v 1 was adsorbed to the solid-phase and incubated with serial twofold dilutions of monoclonal antibody (mAb) Av 3.7, starting from a 1 : 20 000 dilution of a stock solution at 10 mg/ml. Detection was accomplished with HRP-conjugated rabbit anti-mouse and OPD. (B) The same experiment as above using a rabbit serum raised against *Artemisia vulgaris* pollen extract; detection was performed with HRP-conjugated goat anti-rabbit and OPD. Determinations were carried out in triplicate. Bars indicate standard error of the mean.

sample in eight independent assays. The mean intra-assay coefficient of variation in these assays was 5.5%, and the interassay coefficient of variation was 14.2%.

Quantitation of Art v 1 in pollen extracts and correlation with allergenic activity

Ten *A. vulgaris* pollen extracts were analyzed by Art v 1-ELISA and RAST inhibition. A comparison of the results obtained by both techniques is shown in Fig. 6. A very good correlation was observed, suggesting that the Art v 1 content is highly representative of the total allergenic potency of the whole pollen extract. Moreover,

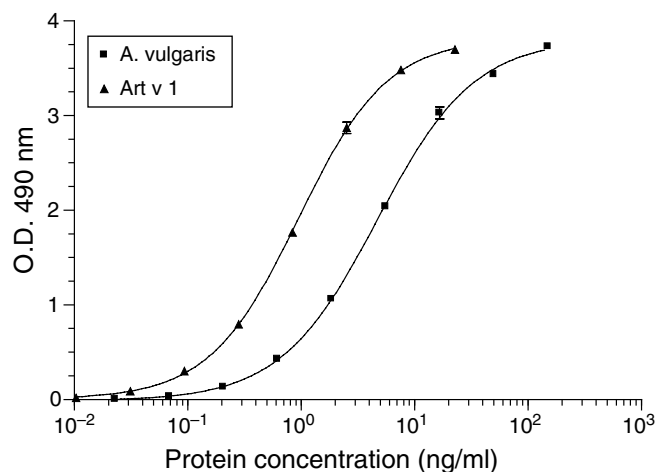


Figure 5. Dose-response curves obtained in the mAb-based Art v 1 ELISA for an *Artemisia vulgaris* pollen extract and affinity-purified Art v 1 allergen. Determinations were carried out in triplicate. Bars indicate standard error of the mean.

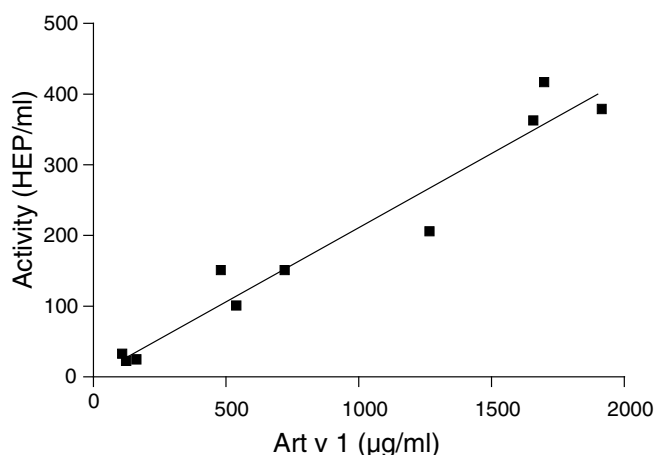


Figure 6. Correlation between Art v 1 content in µg/ml (measured by mAb-based ELISA) and allergenic activity values in HEP/ml (determined by RAST inhibition) in 10 *Artemisia vulgaris* pollen extracts.

an average Art v 1 content of 450 µg/ml in *A. vulgaris* pollen extracts with an activity of 100 HEP/ml was estimated from the equation of the line of best fit.

Discussion

Monoclonal antibodies are very useful tools in many fields of biology and medical research as well as for diagnostic and therapeutic applications. Particularly in allergy, the quality of allergenic extracts intended for clinical use has notably improved in the last years with the use of mAb-based methods for the measurement of

the concentration of allergens. In this work, we have produced mAbs against a major allergen from the pollen of a clinically relevant species, *A. vulgaris*, and have used one of them to purify this allergen and to develop an immunoassay to measure its content in allergenic extracts. This allergen was purified in an immunoreactive state using an efficient one-step procedure based on the specificity of the mAb Av 3.7. The results obtained using different techniques to characterize the purified allergen allowed the unambiguous identification of the protein as Art v 1. Furthermore, results from immunoblotting and RAST with individual sera demonstrated that IgE-binding epitopes are preserved in immunoaffinity-purified Art v 1. The prevalence of recognition of Art v 1 by the specific IgE from the study population was very high (79%). Moreover, it should be noted that this value was estimated after excluding the borderline results as positive (six patients with RAST class 1, Table 1). Thus, if we had considered these results as positive, the prevalence would have increased up to 93%, a value very close to the 90% reported by Himly et al. (3), who regarded results of ≥ 0.3 IU/ml as positive in a RAST screening performed in a population of 55 mugwort pollen-allergic patients.

Our results demonstrate that the mAb Av 3.7 defines an epitope which is identical or very close to a relevant allergenic epitope, the structure of which depends on the integrity of the disulfide bridges. Taking into account the 'head' and 'tail' structure of Art v 1 (3), with a cysteine-stabilized, compact globular N-terminal domain and an extended left-helical structure, with extensive carbohydrate substitutions, at the C-terminal end, this epitope must be located on the defensin-like N-terminal

domain, which is therefore involved in the allergenic activity of Art v 1.

It has been demonstrated that Art v 1 exists in nature as a mixture of O-glycosylated isoforms bearing a previously unknown type of O-arabinogalactosyl chain in plants (3). These carbohydrates constitute relevant allergenic epitopes, since up to 71% of sera from mugwort pollen-allergic patients that recognize natural Art v 1 do not recognize the recombinant Art v 1 expressed in *Escherichia coli* (3). This feature anticipates the difficulties that might be encountered in producing an antigenically equivalent recombinant Art v 1. Therefore, the availability of a mAb-based ELISA for allergen quantitation is particularly important in the case of Art v 1, because it seems that it will be very difficult to replace the natural allergen for the diagnosis of mugwort sensitization. Furthermore, we have shown in this work that the concentration of Art v 1 is a good indicator of the total allergenic potency of mugwort pollen extracts, as a very good correlation between both parameters was observed.

Finally, the ELISA method to quantitate Art v 1 will be also a very useful tool for studies on the stability of the allergen in preparations intended for clinical use, as well as for monitoring the potential contamination of other pollen raw materials with mugwort pollen.

Acknowledgments

This work was supported by ALK-Abelló. Authors thank Linda Puebla for language revision of the manuscript, and Carmen Barrio for expert secretarial assistance.

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