Clinical and Experimental Allergy, 1995, Volume 25, pages 993–999

Quantitation of the major allergen of several *Parietaria* pollens by an anti-Par 1 monoclonal antibody-based ELISA. Analysis of crossreactivity among purified Par j 1, Par o 1 and Par m 1 allergens

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Summary

Background Plants of the genus *Parietaria*, Urticaceae family, represent a major cause of pollinosis in the Mediterranean area. Different *Parietaria* species crossreact to a great extent, but studies on the crossreactivity among the major allergens of these pollens have not been carried out so far.

Objective To develop an immunochemical method to quantify the major *Parietaria judaica* allergen, Par j 1, as well as to verify the presence of Par j 1-like proteins in different Urticaceae pollens. These proteins would be purified in order to study the cross-reactivity among them.

Methods Immunoaffinity chromatography with a monoclonal antibody, solid-phase enzyme-linked immunoassays and SDS-PAGE.

Results A monoclonal antibody-based ELISA for the quantification of Par j 1 has been developed. The assay has a sensitivity of 0·2 ng/mL and shows a high correlation with the allergenic activity of *P. judaica* extracts determined by radioallergosorbent assay (RAST) inhibition.

By means of this assay, proteins homologous to Par j 1 were detected in *P. officinalis* and *P. mauritanica*. These proteins (Par o 1 and Par m 1, respectively) were purified by affinity chromatography using the same monoclomal antibody employed in the ELISA. Crossed-inhibition experiments demonstrated that Par j 1, Par o 1, and Par m 1, competed for the binding of specific IgE from a *P. judaica*-sensitive patients serum pool.

Conclusion The results here described suggest that shared allergenic epitopes are present in the three main allergens investigated, which may simplify the diagnosis and therapy for *Parietaria* allergy.

Keywords: *Parietaria*, Par j 1, Par o 1, Par m 1, allergen, pollinosis, crossreactivity, cross-allergenicity, immunoassay

Clinical and Experimental Allergy, Vol. 25, pp. 993–999. Submitted 1 November 1994; revised 20 January 1995; accepted 18 April 1995.

Introduction

Pollens from plants belonging to the genus *Parietaria*, family Urticaceae, constitute one of the most important causes of pollinosis in Mediterranean countries [1–5]. *Parietaria* grows in coastal Mediterranean areas of European countries such as Spain, France, Italy, the former Yugoslavia, Albania and Greece, as well as in

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Part of this work was presented at the EAACI 1993 Meeting, Rotterdam (Holland), and published in Abstract form in *Allergy* 1993; 48:129.

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Asian and African countries bordering the Mediterranean sea [6]. Its diffusion is not restricted to the Mediterranean area, since *P. judaica* and *P. officinalis* have also been identified in the United States [7] and Australia [8]. However, clinical relevance of *Parietaria* pollen is mainly limited to Italy, the Mediterranean coast of Spain and southern France where it accounts for a high percentage of allergic reactions.

In a previous work, we demonstrated, using human serum, the presence of IgE crossreactive epitopes among pollen extracts from four *Parietaria* species [9], and similar results were found by other authors [10]. However, to our knowledge, studies on the extent of crossreactivity among the major allergens of these pollens have not been reported so far.

Monoclonal antibodies (MoAbs) directed against individual antigenic epitopes on a variety of allergens have also demonstrated the presence of crossreactive epitopes on molecules from different species, and they have proved to be a valuable tool to analyse the molecular bases of crossreactivity among proteins [11,12]. In earlier studies, MoAbs specific to Par j 1, the main allergen of *P. judaica* pollen, were produced and subsequently applied for immunoaffinity isolation of that allergen [13].

In the present study we used one of these specific MoAbs to develop an immmunoassay to quantify Par j 1 allergen, as well as to verify the presence of Par j 1-like proteins in different Urticaceae pollens. In the positive cases, the Par j 1-like proteins were isolated, subsequentially, by affinity-chromatography. Finally, these purified allergens were used in competition immunoassays to study the extent of allergenic crossreactivity among them.

Materials and methods

Allergen sources

P. officinalis and *U. dioica* pollens were purchased from Allergon AB (Ängelholm, Sweden). Pollen of *P. mauritanica* was kindly provided by Dr J. A. Jiménez, Madrid, Spain. *P. judaica* pollen from several batches were purchased from the above mentioned suppliers. According to the supplier's certificate all pollens contained less than 1% (w/w) foreign particles and less than 0·5% foreign pollens.

Pollens were extracted with a 50 mM NH_4HCO_3 buffer, pH 8·0, at a 1 : 10 (w/v) ratio, for 1 h at room temperature, centrifuged and dialysed against distilled water as previously described for *P. judaica* pollen [14].

Antibodies

Anti-Par j 1 4.1.3 MoAb was produced in Balb/c mice, immunized with HPLC-purified Par j 1 [13], and purified as described elsewhere [15].

Polyspecific antibodies were raised by immunizing rabbits with *P. judaica* pollen extract as described [16].

Goat anti-rabbit IgG labelled with peroxidase was obtained from TAGO, Inc. (Burlingame, California, USA).

Mouse anti-human IgE HE2 MoAb was produced in our laboratory [17] and radioiodinated with chloroglycoluril [18] or labelled with peroxidase.

A human serum pool was made by mixing equal volumes of sera from 40 non-treated *Parietaria* sensitized individuals. Diagnosis was based on clinical history, skin test and detection of *P. judaica*-specific IgE antibodies by radioallergosorbent test (RAST) [19].

Purification of allergens

Affinity chromatography of pollen extracts from *P. judaica*, *P. officinalis* and *P. mauritanica* with an anti-Par j 14.1.3. MoAb-Sepharose column was performed essentially as previously described for the purification of Par j 1 [13]. Briefly, the extracts were passed through the column and, after extensive washing with phosphate-buffered saline (PBS), the column was equilibrated with 10 mM phosphate pH 6·8. Bound proteins were eluted with 100 mM glycine, pH 2·5. The material was collected in 1 mL fractions, neutralized with 1 M phosphate, pH 8·0, and dialysed against 50 mM ammonium bicarbonate, pH 8·0.

Par j 1-ELISA

Polystyrene microtitre 96-well plates (EIA/RIA plate, Costar, Cambridge, MA, USA) were coated with 50 μL of the specific anti-Par j 1 MoAb at 10 μg/mL in PBS, for 1 h at 37°C. The coated wells were blocked with 1% bovine serum albumin (BSA) in PBS, and then incubated for 1 h at room temperature with 50 μL of samples diluted in 0·5% BSA, 0·05% Tween 20 in PBS (dilution buffer). After washing four times with 0·05% Tween 20 in PBS (washing buffer) wells were incubated for 1 h with 50 μL of *P. judaica* rabbit antiserum diluted 1 : 32 000 with dilution buffer. Afterwards, the wells were washed and incubated for 1 h at room temperature with 50 μL of a polyclonal goat anti-rabbit IgG, labeled with peroxidase, diluted 1 : 20 000. After further four washes, 50 μL of orto-phenylendiamine (OPD) in 50 mM phosphate-citrate buffer, pH 6·0, containing H_2O_2 (0·05%) were added. The colour reaction was stopped after 20 min with 50 μL of 2 M HCl and the plates read at 492 nm.

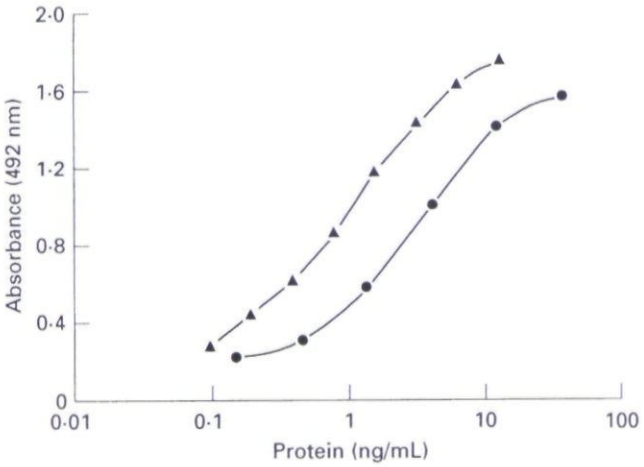

Fig. 1. Dose response curves in the Par j 1 ELISA of (▲) purified Par j 1, and (●) *P. judaica* pollen extract.

The amount of Par j 1 present in the samples is determined from a standard curve using serial dilutions of an affinity-purified Par j 1 preparation, of which the protein concentration had been determined by amino acid composition analysis. The concentration range lay between 0·2 and 25 ng/mL.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Fling and Gregerson [20], using an 8–25% acrylamide gradient in a high molarity Tris buffer system, but excluding urea from the sample buffer and without prior reduction of the samples.

The molecular weight markers used were a Bio-Rad (Richmond, California, USA) low molecular weight range mixture. The proteins were stained with Coomassie Brilliant Blue R-250.

Inhibition ELISA

For competition experiments, polystyrene microtitre 96-well plates (EIA/RIA plate, Costar, Cambridge, MA, USA) were coated with purified allergen Par j 1, Par o 1 or Par m 1, at 10 μg/mL for 1 h at 37°C. The plates were then blocked for 1 h with PBS-BSA 1%. Afterwards, 25 μL of the serum pool (diluted 1 : 3 in PBS) and 25 μL of serial dilutions of a Par j 1 solution were added to the wells and incubated for 2 h at room temperature. Par j 1 concentrations as inhibitor ranged from 2 to 20 000 ng/mL. After washing, wells were incubated with peroxidase-labelled anti-human IgE for 1 h at room temperature. The plates were washed again and developed by incubating with 50 μL/well of a solution of OPD for 30 min. The reaction was stopped by adding 50 μL of 2 M HCl and the absorbance was measured at 492 nm. Assays were performed in duplicate.

RAST inhibition

RAST inhibition tests were carried out according to Yman *et al.* [19] with slight modifications, using paper discs activated with cyanogen bromide and sensitized with a *P. judaica* extract following the procedure described by Ceska *et al.* [21]. Fifty microlitres aliquots of a *P. judaica*-sensitive serum pool (diluted 1 : 3 with PBS) were preincubated with 50 μL of six threefold serial dilutions of every *P. judaica* extract as inhibitors, in protein concentrations from 500 to 2 μg/mL at 37°C for 30 min. Allergen discs were then added to the wells and incubated for 3 h at 37°C. After washing, the discs were incubated overnight at room temperature with radiolabelled anti-human IgE HE2 MoAb [17] (100 000 cpm/50 μL). Finally, the discs were again washed and the bound radioactivity counted in a gamma counter. Samples were assayed in duplicate.

RAST inhibition assays using unrelated allergens such as *D. pteronyssinus* extract or BSA were performed to rule out the possibility of non-specific inhibition by *P. judaica* extracts.

The control representing 0% inhibition was determined by adding PBS instead of allergen extract, while 100% inhibition was determined by adding non-diluted *P. judaica* extract.

Allergenic activity of *P. judaica* extracts was expressed in biological units (BUs) per millilitre by comparison with an in-house reference previously calibrated by skin-prick test [22].

Results

Par j 1 ELISA

In this study, we developed a solid-phase ELISA for the determination of Par j 1 in *P. judaica* extracts, in which the anti Par j 1 4.1.3. MoAb was used as the capture antibody, and a polyclonal rabbit anti-*P. judaica* serum as the second antibody. Figure 1 shows the binding curve of purified Par j 1 preparation in this ELISA. Using this preparation as a standard, it is possible to quantify the Par j 1 content in the range from 0·2 to 25 ng/mL. The same figure shows the binding curve for a *P. judaica* extract. As can be seen, this curve is parallel to that obtained with pure Par j 1, suggesting that the allergen had not been altered during the purification process and

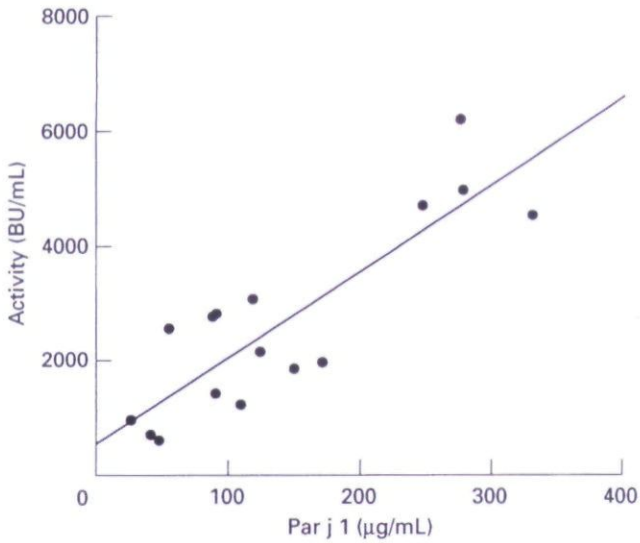

Fig. 2. Correlation between Par j 1 content in µg/mL (measured by MoAb-based ELISA) and activity values in BU/mL (measured by RAST inhibition) in 16 *Parietaria judaica* pollen extracts.

that the assay is measuring the same molecule in the different preparations.

As Par j 1 is by far the most important *P. judaica* allergen, accounting for more than 80% of the IgE-binding capacity of the whole extract [12,21,22], a correspondence between Par j 1 content and total allergenic activity of the pollen extract might be expected. To assess this relationship we analysed, by the Par j 1 ELISA and RAST inhibition, 16 *P. judaica* pollen extracts that had been prepared in our lab, between 1987 and 1992, from different batches of pollen supplied by Allergon and Dr J. A. Jiménez and that had been kept under different conditions. Figure 2 shows the comparison of the results obtained by both techniques. A good correlation was obtained between these two assays ($r = 0{\cdot}8544$; $P < 3{\cdot}10^{-5}$). Besides, an average Par j 1 content of 5·7 µg/mL in a whole *P. judaica* extract with an activity of 100 BU/mL could be estimated from the equation of the line of best fit.

Par j 1-like proteins in other Urticaceae

The Par j 1 ELISA also allowed the detection of Par j 1-like proteins in pollen extracts from different Urticaceae plants. Results obtained are listed in Table 1. In three different *Parietaria* species, significant amounts of this common allergenic component was found. In contrast, Par j 1-like proteins do not seem to be present in *U. dioica* pollen, since only such a minute amount of Par j 1 was detected that even a contamination of *U. dioica* pollen with less than 0·5% *P. judaica* pollen could account for it.

Table 1. Nanograms of *Par j* I-like protein per µg of total protein of extract determined by a MoAb-based ELISA as described in Materials and methods

Pollen extract	ng allergen/µg of total protein
P. judaica	64·96
P. officinalis	112·15
P. mauritanica	11·25
U. dioica	0·02
*D. pteronyssinus**	0·00

* *D. pteronyssinus* was included as a negative control.

The proteins homologous to Par j 1 detected in *P. officinalis* and *P. mauritanica* were isolated from the pollen extracts using an anti-Par j 1 4.1.3 MoAb-Sepharose affinity chromatography column. The affinity-purified proteins were analysed by SDS-PAGE, along with a sample of purified Par j 1 for comparison (Fig. 3). All of them exhibited two bands with slightly different apparent molecular weights close to 12 kDa.

The binding of specific IgE from a *P. judaica*-allergic patients serum pool to the purified proteins was measured by means of an ELISA (Hamlet-IgE, Alergia e Inmunología Abelló S.A.), and absorbance values greater than the highest value of the standard curve (equivalent to RAST class 4) were obtained with the three purified allergens (data not shown). The extent of allergenic crossreactivity among them was evaluated by an inhibition ELISA, using the same serum pool (Fig. 4). Par j 1 inhibited the IgE binding to the other *Parietaria* allergens in a dose-dependent manner, reaching a complete inhibition in both cases, and the slopes of the linear range of the inhibition curves were statistically parallel, suggesting the existence of common or related epitopes in the three allergens. Nevertheless, it should be noted that the IgE-binding to Par m 1 was inhibited by a concentration of Par j 1 lower than those needed for the inhibition of IgE-binding to Par o 1 and Par j 1, which were very similar.

Discussion

In previous works, Par j 1 was purified and it was demonstrated to be the most important allergen from *P. judaica* pollen, to which almost all the *P. judaica*-sensitive patients had specific IgE [14,23–25]. Moreover, Par j 1 was able to inhibit in a great extent (approximately

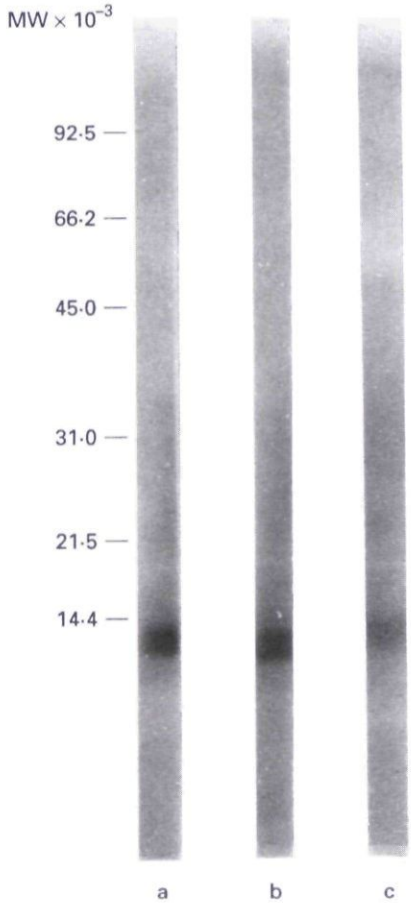

Fig. 3. SDS-PAGE analysis of proteins isolated by affinity chromatography with an anti-Par j 1 4.1.3 MoAb-Sepharose column from (a) *P. judaica* extract, (b) *P. officinalis* extract, (c) *P. mauritanica* extract. Positions of molecular weight markers are shown on the left-hand side. Proteins were stained with Coomassie Brilliant Blue.

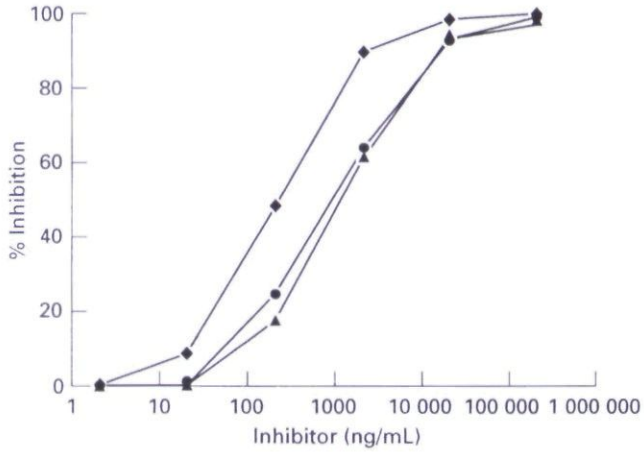

Fig. 4. Competition ELISA curves. The binding of specific IgE to plastic wells coated with the affinity-purified allergens from (▲) *P. judaica*, (●) *P. officinalis*, and (◆) *P. mauritanica*, was inhibited by adding increasing concentrations of Par j 1, as described in Materials and methods.

80%) the binding of specific IgE to the whole pollen extract in inhibition immunoassay experiments [14,23,24]. These data suggested that the content of Par j 1 might be correlated with the allergenic potency of *P. judaica* extracts, such as it has been reported for other major allergens [26–29]. Therefore, we exploited the availability of a specific anti-Par j 1 MoAb [11] to develop a solid-phase ELISA for the quantitation of the allergen in mass units, that could be very useful for the evaluation of the allergenic activity of *P. judaica* extracts. The good correlation obtained between the content of allergen, as determined by Par j 1-ELISA, and the allergenic activity, determined by RAST inhibition, for 16 *P. judaica* pollen extracts showed that the content of Par j 1 is representative for the overall allergenic potency in *P. judaica* extract, and hence Par j 1 ELISA might be an alternative to RAST inhibition for quality control of extracts, avoiding the main drawbacks with RAST inhibition assays, such as the use of human serum as well as *P. judaica* extract-sensitized paper disks that are difficult to standardize.

In a previous work [11], we demonstrated that Par j 1 consists of two isoforms that were purified and characterized, exhibiting some slight differences in their physico-chemical properties as well as in the N-terminal amino acid sequence. In this study we detected the presence of allergens homologous to Par j 1 in *P. officinalis* and *P. mauritanica*, Par o 1 and Par m 1, respectively, by means of the Par j 1 ELISA, and we purified them by immuno-affinity chromatography using the same MoAb as in the ELISA. SDS-PAGE analysis of purified Par o 1 and Par m 1 revealed that they also contained a doublet of protein bands with apparent molecular weight in the range 10–13 kDa. As far as Par o 1 is concerned, this result is in agreement with those reported by Oreste *et al.* [30], who also found a doublet of distinct bands for this allergen, although they described slightly higher molecular weight values, which might be due to the different electrophoretic system used. Regarding Par m 1, to our knowledge this is the first time that the purification and preliminary data on its physico-chemical and immunochemical features have been reported.

In the same way, allergenic crossreactivity among different *Parietaria* species has been previously reported [9,10,31], but all the studies were carried out with the whole pollen extract. In this study we have analysed the crossallergenicity among the affinity-purified allergens by means of an inhibition ELISA. Par j 1 completely inhibited the IgE-binding to Par o 1 and Par m 1. Then,

Par 1 allergen might represent a major shared allergen in all the *Parietaria* species studied and, probably, in others such as *P. lusitanica* that has also been described to be crossreactive with *P. judaica* [7]. In addition, inhibition curves of IgE-binding to Par o 1 and Par j 1 were almost equivalent, demonstrating the high allergenic homology between them. However, Par m 1 showed a slightly different behaviour in the same experiment, the IgE-binding to it being inhibited by a low amount of Par j 1. This suggests that the relatedness of Par j 1 to Par m 1 is not so close as to Par o 1. Consequently, further studies will be needed to characterize the allergenic properties of Par m 1 and its degree of crossreactivity to Par j 1.

On the other hand, *Urtica dioica*, a non-allergenic plant, which also belongs to the Urticaceae family, does not crossreact with *P. judaica* [7,8]. The fact that Par j 1 was not detected in *U. dioica* seems to suggest that Par 1 is a genus-specific protein whose presence determine the allergenicity of some Urticaceae species.

From the results presented in this article, we can conclude that crossreactive determinants with Par j 1 are located on homologous components present in *P. officinalis* and *P. mauritanica* pollens. The immunoassay developed for the quantification of Par j 1 can give a measure of the Par j 1 equivalent in *P. officinalis* and *P. mauritanica*, and can be a useful tool for the standardization of *Parietaria* extracts.

Acknowledgements

This work was supported by Alergia e Immunología Abelló S.A. We gratefully thank I. Courinha for her expert secretarial assistance in the preparation of the manuscript.

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