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Isolation and in vitro Translation of mRNA from Inflorescences of *Parietaria judaica*

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Abstract. The aqueous extract of inflorescences of *Parietaria judaica* contains an allergen homologous to the major pollen allergen *Par o I* (14 kD), as shown by radio-allergosorbent test (RAST) inhibition and immunoblot analysis. Poly(A)⁺ RNA was obtained from inflorescences and was shown to be able to code in vitro for a protein homologous to *Par o I* with respect to sodium dodecylsulphate polyacrylamide gel electrophoretic mobility and to antigenic specificity as defined by the binding, in affinity chromatography, to solid-phase IgG of rabbit anti-*Par o I* antisera, and in RAST inhibition, to IgE antibodies of human reaginic serum pool.

Introduction

The pollen of *Parietaria* (pellitory, wall pellitory) is a major agent of respiratory allergy in Southern Europe and Mediterranean Asia and Africa, where this weed is widely distributed. *Parietaria* is a genus of the family of Urticaceae: the species commonly observed in Italy are *P. officinalis* L. and *P. judaica* L., formerly described as the varieties *P. erecta* [1] and *P. judaica* (L. 1753) of *P. officinalis* L. In Italy approximately 40% of the pollen-allergic adults are sensitive to *Parietaria* [2].

The aqueous pollen extracts (PEs) of *P. judaica* and of *P. officinalis* contain several molecular species. IgE-binding components (allergens) have been separated by electrophoresis, gel filtration, ion exchange chromatography and high-performance liquid chromatography [3-10].

A glycoprotein allergen, of apparently 14 kD, designated as *Par o I* according to Marsh et al. [1], has been purified from the PEs of *P. officinalis* and *P. judaica* [6, 7]. *Par o I* is a major allergen, possessing epitopes for human IgE antibody immunodominant in individual responses and at the population level, shared with IgE-binding (allergenic) molecules of the PE distributed over a wide range of molecular weight [6, 7, 9]. Among the major pollen allergens so far described, *Par o I* has the lowest molecular weight, a

feature that qualifies it as a very convenient tool for studies on the relationship between physicochemical structure and immunological function of antigens and on the genetics of the human immune response.

The presence of a large array of IgE-binding (allergenic) molecules, frequently immunologically correlated, and the existence of structural homologues of purified allergens, have been reported by most authors pursuing the purification of allergens from PEs. This situation may derive from a number of factors, including genetic heterogeneity of the plants, occurrence of enzymatic degradation and/or of aggregation as a result of physiological processes or of reactions taking place in the aqueous PEs.

Recently, some authors have used the methodology of recombinant DNA to obtain allergens synthesized in bacteria; the cDNA copies of the genes for major allergens of the house dust mite *Dermatophagoides pteronyssinus*, a 27 kD protein, and of the venom of the white-face hornet, *Dolichovespula maculata*, a 23 kD protein, have been cloned and expressed in *Escherichia coli* [10-13]. The availability of *Par o I* synthesized in *E. coli* may allow approaches to the analysis of the physicochemical structure of this allergen complementary to those afforded by the traditional methodology of protein biochemistry. The present paper reports the purification of biologically active mRNA from inflorescences of *P. judaica*, the analysis

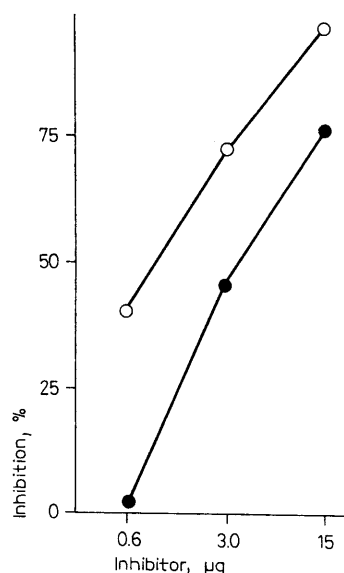


Fig. 1. Analysis of the inhibitory activity of the IE in the PE RAST. Serum: reaginic serum pool. Inhibitors: ● = IE; ○ = PE. The discs were incubated with 20 μ l of serum and 25 μ l of inhibitor and, after washings, with 20 μ l of rabbit antihuman IgE (0.34 μ Ci/ml; PRIST, Pharmacia, Sweden). Abscissa: inhibitor expressed in micrograms per millilitre of protein [14].

of the products of in vitro translation and the comparison of their immunological and biochemical properties with those of *Par o I*, as a preliminary phase of a cDNA-based approach to the molecular cloning of this allergen.

Materials and Methods

Plant Material

Inflorescences of *P. judaica* were collected in May 1988, at a stage close to pollen maturation, immediately frozen in liquid nitrogen and stored at -80°C . The pollen of *P. judaica* was purchased from Allergon AB (Sweden).

Human Sera

The reaginic serum pool consisted of equal volumes of sera collected from 20 untreated subjects with a history of clinical allergy to the pollen of *Parietaria*, containing high levels of anti-*Par o I* antibodies as documented by *Par o I* RAST, (ratio: bound cpm/added cpm $\geq 20\%$). The control serum pool was obtained in a similar way from non-allergic subjects negative in PE RAST. The individual sera 33 and 47 were obtained from untreated allergic patients.

Extracts of Pollen and Inflorescence

The PE was obtained as previously described [3]. The inflorescence extract (IE) was prepared according to a similar procedure: briefly, a suspension of 10 g of frozen inflorescences in 200 ml of

0.125 M NH_4HCO_3 (pH 8.7), containing 0.05% NaN_3 and 10^{-4} M phosphomethylsulphonyl fluoride (Sigma, St. Louis, Mo., USA), was stirred at 4°C for 4 h, filtered through N I Whatman paper and centrifuged at 16,000 rpm for 20 min. 10 mg of protein was recovered in the supernatant [14]. The extracts were lyophilized and stored at -20°C .

Antisera

Anti-PE, anti-IE and anti-*Par o I* antisera were raised in rabbits. Subcutaneous injections of 300 μ l of complete Freund's adjuvant emulsified with 300 μ l of saline containing 0.2 mg/ml of *Par o I* or 0.5 mg/ml of PE or IE protein were repeated at 15-day intervals. The antisera were divided into 2-ml aliquots and stored at -20°C .

RAST and RAST Inhibition

Solid-phase allergens for the RAST were obtained essentially according to Ceska [15]. 2,000 CNBr-activated cellulose discs were allowed to react at room temperature for 16 h with 50 ml of a solution containing 0.1 mg/ml of *Par o I* or 0.5 mg/ml of the PE or IE protein.

D. pteronyssinus RAST discs were purchased from Pharmacia Diagnostics (Sweden).

RAST and RAST inhibition assays were carried out as described by Ruffilli et al. [9]. Experimental details are given in the legend to figure 1. Inhibition was expressed as: $[1 - (b - c)/(a - c)] \times 100$, where a = cpm bound in the absence of inhibitor; b = cpm bound in the presence of inhibitor, and c = cpm bound in the absence of a reaginic serum. Assays were performed in duplicate, and the values reported are means.

Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) was performed essentially as described by Laemmli [16]. Samples of the radiolabelled translation mixture and of a solution containing molecular weight standards (range 2.8–78 kD) were subjected to electrophoresis for 4 h at 20 mA in a 10–18% (w/v) polyacrylamide linear gradient gel. The gels were dried, treated with 'Enhance' (NEN, USA) and autoradiographed at -80°C for 1 week.

Immunoblotting

Proteins separated by SDS PAGE were transferred to a nitrocellulose membrane by electrophoresis at 2.8 V/cm overnight [17]. After transfer, the unspecific protein-binding sites were blocked by incubation at 37°C for 1 h in 50 mM Tris (pH 8), containing 150 mM NaCl 0.3% Tween, 3% bovine serum albumin and 0.5% NaN_3 . The nitrocellulose membranes were washed and cut into strips that were incubated at 4°C overnight in 2 ml of undiluted serum each. After repeated washings, the strips were transferred in 2 ml of Tris (pH 8) containing 5% fetal calf serum and ^{125}I -labelled anti-IgE (7×10^4 cpm/ml, 5 ng/ml; PRIST; Pharmacia, Sweden) and incubated at 4°C overnight. After washings the strips were dried and autoradiographed at -80°C for 1 week.

Immunsorbents for Affinity Chromatography

IgG was obtained from rabbit antisera by affinity chromatography on protein A Sepharose CL-4B (Pharmacia, Sweden), according to the manufacturer's instructions. Approximately 6 mg of IgG was obtained from 2 ml of antiserum.

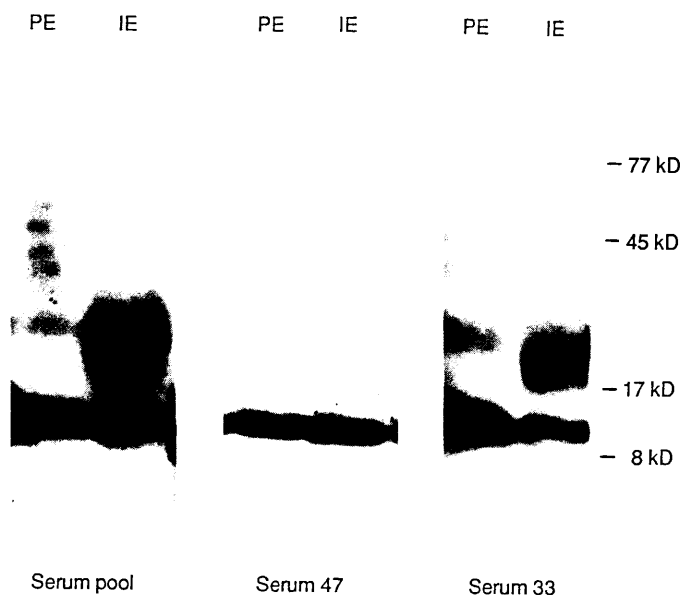


Fig. 2. Immunoblot analysis of IgE-binding bands in the IE and in the PE with the reaginic serum pool and with two individual sera. SDS PAGE was performed on 30 μ l of buffer containing approximately 150 μ g of protein.

Solid-phase IgG and solid-phase IE were obtained by coupling to 1 ml of CNBr-activated Sepharose 4B (Pharmacia, Sweden), approximately 3 mg of protein.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis (CIE) of the IE and of the PE was performed essentially according to Lowenstein [18]. For the analysis of coprecipitation of the translated radioactive proteins with carrier IE, prior to electrophoresis, 0.5 ml of the IE containing 0.05 mg/ml of protein was incubated at room temperature for 30 min with 6 μ l of phosphate-buffered saline containing 0.3 mg/ml *L*-methionine, 1 mg/ml ovalbumin and 10 μ l of the labelled translation mixture [9]. The gels were dried and autoradiographed at -80°C .

Preparation of mRNA

10 g of frozen inflorescences was ground to a fine powder under liquid nitrogen, with a mortar and a pestle; the resulting slurry was added with vigorous shaking to 2 vol of lysis buffer (0.1 M Tris-HCl, pH 8.5) containing 0.001 M EDTA, 1% SDS, 0.05 M β -mercapto-ethanol, 0.05% diethylpyrocarbonate and to 2 vol of hot phenol chloroform (1:1). After 15 min of incubation at 65°C and centrifugation, the same procedure was repeated three times on the aqueous phase that was finally extracted with 2 vol of chloroform/iso-amyl alcohol (24/1). 3 M Na acetate (pH 5.2) was added to a final concentration of 0.3 M, and RNA was precipitated with 2 vol of 96% ethanol. At this stage RNA was contaminated by ethanol-precipitable non-polynucleotide compounds, including polysaccharides and pectins. Polysaccharides were removed by extraction with 2-methoxy-ethanol and by selective precipitation of the nucleic acid with cetyltrimethylammonium bromide [19]. The poly(A)⁺ mRNA was obtained from total RNA by fractionation on oligo deoxythymidine cellulose [20].

In vitro Translation

Poly(A)⁺ mRNA was translated using a rabbit reticulocyte lysate system (Amersham International, UK) according to the manufacturer's instruction. The reaction mixture contained 20 μ l of lysate, 2 μ l (0.3 μ g) of mRNA and 3 μ l of ^{35}S -methionine (0.045 mCi). Reactions were allowed to proceed at 30°C for 60 min. Aliquots of 1 μ l were incubated on ice for 15 min with 2 ml of 15% w/v trichloro-acetic acid (TCA) and 50 μ l of 5% bovine serum albumin and then filtered on GF/C filters (Whatman, UK). The filters were washed and counted in a liquid scintillation counter.

Autoradiography

Autoradiography was performed at -80°C using a FUJI X-ray film (New Nif, Japan).

Results

Analysis of IgE-Binding Components of *P. judaica* Inflorescence Extract

Before proceeding to the RNA extraction, the aqueous IE was analysed to assess the presence of molecules immunologically and biochemically related to the allergens of the PE and in particular to *Par o I*.

Cross-reactivity between the IE and the PE was assayed by RAST inhibition. Serial dilutions of the IE were used as inhibitors of the PE RAST (fig. 1). The inhibition curve obtained showed that the two extracts were highly cross-reactive; the amounts of the

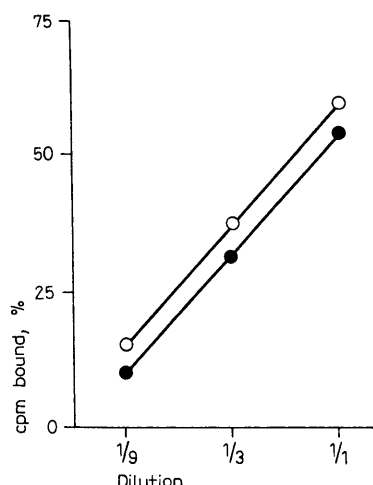


Fig. 3. Analysis of IE-specific human antibodies in the *Paro I* RAST. Sera: ○ = reagenic serum pool; ● = IE-specific retained fraction (400 µg/ml of protein). The discs were incubated with 50 µl of serial dilutions of the reagenic serum pool or of the retained fraction (0.76 mg/ml of protein) [14] and, after washings, with 25 µl of ^{125} I rabbit antihuman IgE (PRIST, Pharmacia, Sweden).

IE and PE protein required for 50% inhibition were 4.2 and 1.1 µg, respectively.

The electrophoretic patterns of the IgE-binding molecules of the IE and of the PE were compared by immunoblot analysis with the reagenic serum pool and with two individual reagenic sera, one of which (serum 33) contained IgE antibodies to minor pollen allergens (fig. 2). In immunoblots of the PE, the serum pool and serum 33 identified multiple bands in the molecular weight range of 30–100 kD and two major bands in the range of 14–17 kD; serum 47 identified only the 14- to 17-kD bands. Immunoblots of the IE, irrespective of the serum used for incubation showed substantially only the two 14- to 17-kD bands. The comparison of the immunoblot patterns suggested that the IE contained the allergens corresponding to the major, lower molecular weight bands of the PE, including *Paro I*, and indicated that the minor allergens identified by serum 33 were absent or present in scarce amounts.

To investigate whether the IE contained an allergen with the immunological properties of *Paro I*, IE-specific antibodies of the reagenic serum pool were separated by affinity chromatography and assayed for their binding activity to *Paro I* in the RAST (fig. 3). Solid-phase IE, obtained as described under

Methods, was used as immunosorbent. 4 ml of the reagenic serum pool was applied to a column (1 ml) of solid-phase IE in 0.2 M Tris (pH 8.5) and 0.5 M NaCl. The retained fraction, eluted with 0.2 M glycine HCl (pH 2.7) and 0.5 M NaCl, contained 0.76 mg of protein; after concentration by lyophilization it was dissolved in 1 ml of RAST buffer and assayed by direct *Paro I* RAST. The binding curve obtained showed that human IgE antibodies specific for *Paro I* had been retained by the solid-phase IE.

Taken together the results of these experiments indicated that the IE contained molecules homologous to the major pollen allergen *Paro I* with respect to the mobility in SDS PAGE and to the allergenic specificity.

Extraction of Poly(A)⁺ RNA from Inflorescences

The extraction of RNA from inflorescences of *P. judaica* and the purification of the poly(A)⁺ RNA fraction were carried out as described under Methods. In vitro translation was carried out in a rabbit reticulocyte lysate system. The translated proteins labelled by ^{35} S-methionine were analysed by SDS PAGE followed by autoradiography; the pattern obtained comprised several radioactive bands in the molecular weight range of 8–77 kD (fig. 4).

Analysis of in vitro Translated Proteins

The presence of proteins with the epitopes of *Paro I* in the translation mixture was investigated by affinity chromatography on solid-phase rabbit antibodies followed by SDS PAGE and RAST inhibition analysis of the retained fractions.

The translation mixture was fractionated by affinity chromatography on solid-phase IgG of an anti-IE rabbit antiserum obtained as described under Methods. 100 µl of the translation mixture was applied to a column (1 ml) of the immunosorbent in 0.2 M Tris (pH 8.5) and 0.5 M NaCl. The fraction unretained was indicated as U1. The retained fraction (R1) was eluted with 0.2 M glycine HCl (pH 2.7) and 0.5 M NaCl. A control sample, containing the same reagents as the translation mixture, with the exception of poly(A)⁺ RNA (–RNA control) was fractionated according to the same procedure, obtaining fractions U2 and R2. Fractions R1, U1 and R2 contained respectively 23,940, 248,000 and 1,250 TCA-precipitable cpm. Fractions R1 and R2 were concentrated by lyophilization and redissolved each in 250 µl of RAST buffer; serial dilutions were assayed for their

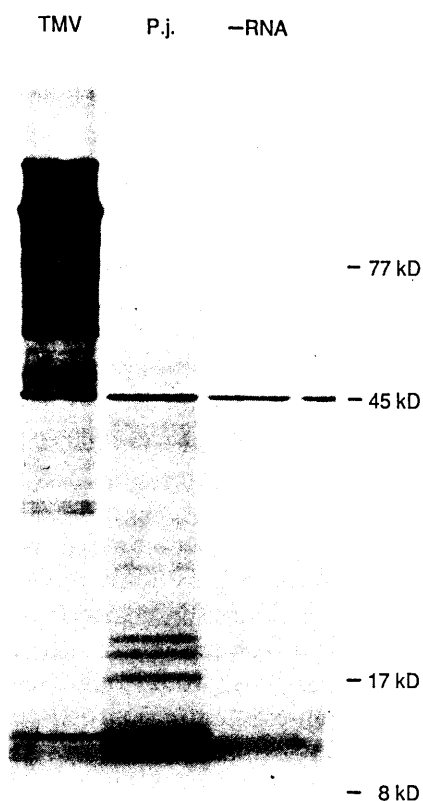


Fig. 4. Autoradiography of SDS PAGE analysis of in vitro translated proteins. Electrophoresis was performed on 3 μ l of translation mixtures containing (left to right): TMV poly(A)⁺ RNA (TMV); IE poly(A)⁺ RNA (P.j.); control (-RNA).

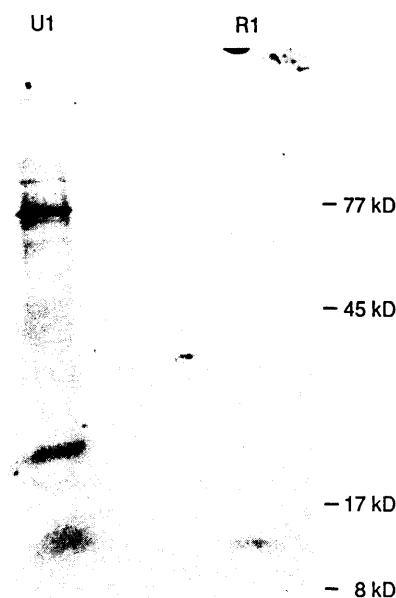


Fig. 6. Autoradiography of SDS PAGE analysis of fractions U1 and R1 of the translation mixture.

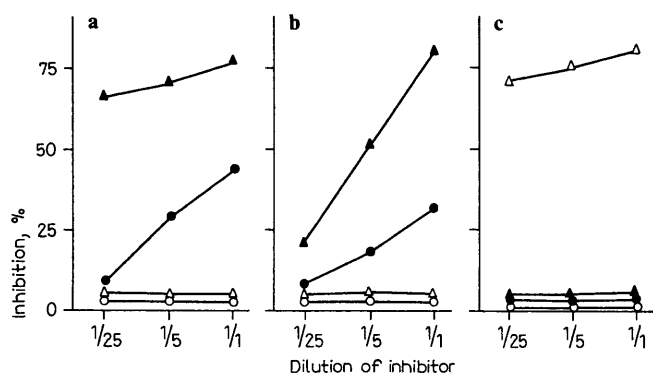


Fig. 5. Analysis of the inhibitory activity of fraction R1 of the translation mixture in the PE (a), *Par o I* (b) and *D. pteronyssinus* (c) RASTs. Serum: reaginic serum pool. Inhibitors: ● = fraction R1; ▲ = *Par o I* (15 μ g/ml of protein); △ = *D. pteronyssinus* extract (100 μ g/ml of protein); ○ = control fraction R2. The assay was performed as described in the legend to figure 1.

ability to inhibit the PE and the *Par o I* RASTs and, as a control for unspecific binding to IgE, the *D. pteronyssinus* RAST (fig. 5a-c). The inhibition values obtained with the highest concentration of fraction R1 were 42 and 30%, respectively, for the PE and for the *Par o I* RAST. From the inhibition curves obtained with *Par o I*, it was extrapolated that 0.03 and 0.022 μ g of this protein were required to obtain 42% inhibition of the PE RAST and 30% inhibition of the *Par o I* RAST, respectively. Fraction R2 did not inhibit either RAST. Neither fraction R1 nor fraction R2 inhibited the *D. pteronyssinus* RAST.

Fractions U1 and R1 were analysed by SDS PAGE and autoradiography (fig. 6). Fraction U1 contained several radioactive bands over a wide range of molecular weights; fraction R1 contained a single band in a position corresponding to 14 kD.

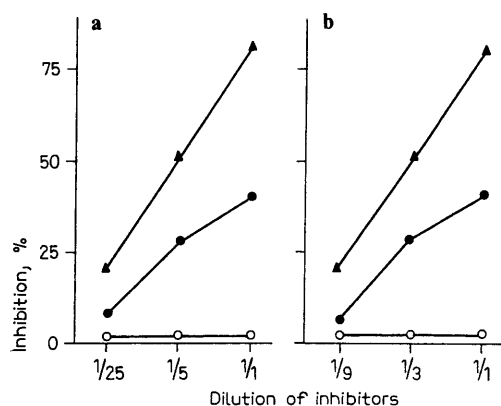


Fig. 7. Analysis of the inhibitory activity of fraction R3 of the translation mixture in the *Par o I* (a) and IE (b) RASTs. Serum: reaginic serum pool. Inhibitors: ● = fraction R3; ▲ = *Par o I* (15 µg/ml); ○ = control fraction R4. The assay was performed as described for figure 1.

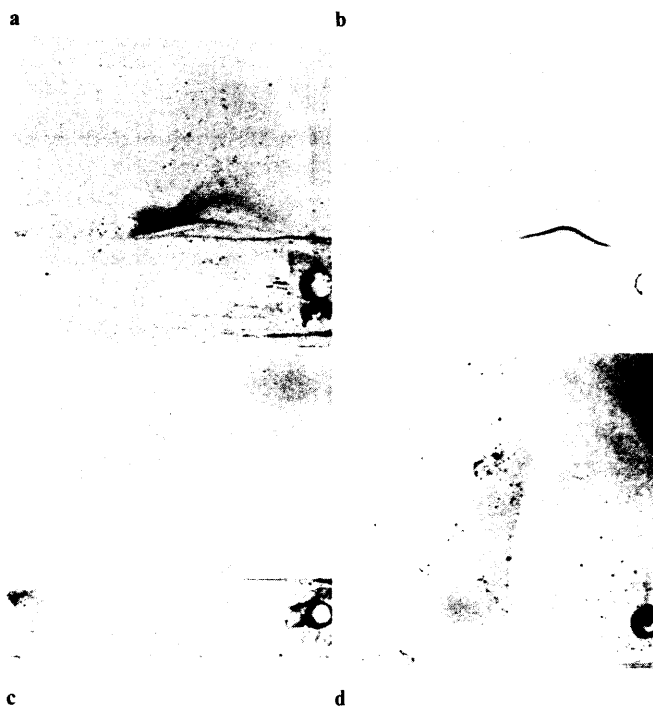


Fig. 8. CIE analysis of the translation mixture performed with an anti-IE rabbit antiserum a, b Translation mixture coprecipitated with the IE as a carrier, as described under Methods. a Autoradiography. b Staining with Coomassie brilliant blue. c, d Autoradiography of controls consisting, respectively, of a CIE of the -RNA translation mixture and of a CIE of the translation mixture where the rabbit antiserum was substituted by serum of non-immunized rabbits.

The translation mixture was fractionated by affinity chromatography on solid-phase IgG of anti-*Par o I* rabbit antiserum. 100-µl samples of the translation mixture and of the -RNA control mixture were applied to the immunosorbent and eluted as previously described, obtaining the retained fractions R3 and R4 (containing respectively 24,000 and 1,000 TCA-precipitable cpm). After concentration by lyophilization, serial dilutions of fractions R3 and R4 were assayed for their ability to inhibit the *Par o I* and the IE RASTs (fig. 7a, b). The values of inhibition corresponding to the highest concentration of fraction R3 were 42 and 40%, respectively, for the *Par o I* and the IE RAST. From the inhibition curve obtained with *Par o I* it was extrapolated that 0.032 µg of this protein was required to obtain 40% inhibition of the IE RAST. Fraction R4 had no inhibitory activity.

The results of these experiments indicated that the poly(A)⁺ mRNA extracted from inflorescences were specified in vitro for allergenic proteins and that a major component was represented by a protein or a group of proteins homologous to *Par o I* for electrophoretic mobility in SDS PAGE and for antigenic specificity as defined by rabbit IgG and human IgE antibodies.

To find further evidence of the presence of a protein homologous to *Par o I*, the translation mixture was analysed by CIE. Prior to electrophoresis, 10 µg of the radiolabelled translation mixture, containing 100,000 TCA-precipitable cpm, was incubated with carrier IE as described under Methods. CIE analyses of the IE and of *Par o I* were performed under the same conditions. Autoradiography of the gels revealed an arc of radioactive precipitate corresponding for the position and shape to that obtained with *Par o I* (fig. 8).

Discussion

Recently, groups working on allergens have been attracted by the potential that the technology of recombinant DNA offers for the study of the physico-chemical structure and immunological properties of protein antigens. The cDNA copies of the genes for the major allergens of the house dust mite *D. pteronyssinus* and of the venom of the vespid *V. maculata* have been cloned and expressed in *E. coli* [10-13]. As a preliminary phase of a cDNA-based cloning strategy, a recent report describes the isolation of biologically

active mRNA from inflorescences of *Betula verrucosa*. The translation mixture was analysed by immunoblotting: a serum pool revealed several IgE-binding bands; analysis with pools of selected individual sera showed that these did not include the major allergen Bet v I in spite of the presence of a 17-kD band [21].

The present paper describes the isolation from inflorescences of *P. judaica* of a poly(A)⁺ RNA that was biologically active and coded in vitro for protein(s) homologous to the major pollen allergen *Par o I*, a 14-kD glycoprotein. It also reports the presence of allergens homologous to *Par o I* in the aqueous IE.

The IE contained IgE-binding molecules with the electrophoretic mobility of the pollen allergens in SDS PAGE and was highly cross-reactive with the PE in RAST inhibition; immunoblot analysis with individual sera suggested that it did not contain minor, high molecular weight allergenic components of the PE (fig. 1, 2). The presence of *Par o I* was demonstrated by the high inhibitory activity of the PE RAST shown by the IE and by the high binding activity in the *Par o I* RAST shown by IE-specific human antibodies, separated by affinity chromatography; these data were in agreement with the presence of a diffuse IgE-binding band with the electrophoretic mobility of approximately 14 kD in the immunoblot pattern of the IE (fig. 1-3).

Poly(A)⁺ RNA purified from inflorescences of *P. judaica* was biologically active and coded for several proteins (fig. 4). Evidence for the presence in the translation mixture of a protein homologous to *Par o I* was obtained by experiments combining affinity chromatography, RAST inhibition and SDS PAGE. A fraction of the translation mixture retained by solid-phase IgG of anti-IE rabbit antiserum was shown to consist of a single radioactive band with an apparent molecular weight of 14 kD in SDS PAGE and to possess a high inhibitory activity of the PE and of the *Par o I* RASTs; conversely, a fraction of the translation mixture retained by solid-phase anti-*Par o I* rabbit antibodies possessed a high inhibitory activity of the IE RAST (fig. 5-7). Taken together the results of these experiments indicate that the poly(A)⁺ RNA isolated from inflorescences of *P. judaica* contained a mRNA specific for protein(s) homologous to *Par o I*, with respect to SDS PAGE electrophoretic mobility and antigenic specificity as defined by IgE antibodies of human allergic subjects and IgG antibodies of rabbit antisera.

The observation that *Par o I* was expressed during pollen maturation raises interesting questions concerning its role in the physiology of the plant: the absence of minor pollen allergens in the IE may be attributed to a number of factors; as the pollen and inflorescences had a different origin, these may include differences in the geographical localization of the areas where the plants were collected; alternatively, the aqueous extraction may be inadequate to obtain higher molecular weight molecules from the inflorescence tissues; it is also possible that our observations reflect a developmentally or tissue-regulated expression of these molecules. It is possible that an understanding of the relationship between the structure and the physiological (in the plant) function of these allergens will afford a clue for the study of properties relevant for their immunological activity, such as cross-reactivity. Considering that in vitro synthesized *Par o I* presumably did not possess the carbohydrate moiety of the homologous molecule purified from the PE our data indicate that the epitopes of *Par o I* for human IgE and rabbit IgG antibodies, defined by the RAST and by immunoblotting, reside on the protein moiety of the molecule.

The presence of mRNA coding for *Par o I* in the poly(A)⁺ RNA of inflorescences is encouraging in the perspective of cloning *Par o I* cDNA genes in *E. coli*. The human immune response to *Par o I* could be a convenient model for studies of allergy at the molecular levels, considering the relatively low molecular size of this allergen and the high prevalence of sensitive subjects in the Parietaria-allergic population. The availability of *Par o I* homogeneous at a molecular level is essential to identify T and B cell epitopes, to define them with respect to dominance in the human response and to cross-reactivity with genetic variants and with other allergenic molecular species, and to investigate genetic determinants of the fine specificity of T and B cell responses. The elaboration of reagents and protocols for the diagnosis, the prevention and the therapy of allergy may benefit from these studies.

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