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Characterization of a dodecapeptide containing a dominant epitope of Par j 1 and Par o 1, the major allergens of *P. judaica* and *P. officinalis* pollen

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The pollen of *Parietaria*, a weed of the Urticaceae family, is a major cause of respiratory allergy in Europe, where the most common species are *P. judaica* and *P. officinalis*. Previously, we reported that a β -galactosidase fusion protein (6a-BG) expressing a 26-bp cDNA fragment (6a cDNA) contained a dominant IgE-binding epitope (6a epitope) of the major allergens Par o 1 and Par j 1. The present study aimed to define the amino-acid sequence containing the 6a epitope. We analyzed the reactivity of anti-Par o 1 antibodies affinity purified from allergic patient sera with:

- 1) a panel of synthetic peptides deduced from the 6a nucleotide sequence using different reading frames
- 2) glutathione S-transferase (GST) fusion proteins containing selected peptides.

The peptide NSARARADSCRI (p102) specifically bound anti-Par o 1 antibodies affinity purified from allergic patient sera or from rabbit anti-Par o 1 antiserum (ELISA). The related peptide NSARAGTSSCRI (p101) reacted to human but not to rabbit, anti-Par o 1 antibodies. GST fusion proteins containing p101 (GST 3.5) or p102 (GST 3.2) extensively inhibited the binding between Par o 1 and IgE or IgG antibodies from an allergic patient serum pool according to a dose-response curve. Percent inhibition of IgE antibodies binding obtained by absorbing a solution (50 μ l) of affinity-purified antibodies with 5 μ g of GST 3.2 or with 1.2 mg of GST 3.5 was 69% and 66%, respectively. In conclusion, the results of the present study indicate that the amino-acid sequences NSARARADSCRI (p102) and NSARAGTSSCRI (p101) contain the dominant epitope of Par o 1 and Par j 1 for human IgE and IgG antibodies indicated as 6a epitope. Moreover, the study shows that the epitope is conserved in recombinant molecules containing these peptides, irrespective of the fused polypeptide (β -galactosidase or GST). The

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knowledge of the amino-acid sequence of this dominant epitope is important in therapeutic approaches to the development of allergen-derived haptens.

The pollen of *Parietaria*, a weed of the Urticaceae family, is a major cause of respiratory allergy in Europe. Recent reports indicate that the plant, native to the Mediterranean regions, is present also on the Pacific coast of the USA and in Australia, where it is a cause of allergy (1, 2). The prevalence of allergy to *Parietaria* is 60–80% in Italy and Greece and 25–40% in Spain and southern France (4). The prevalence of monosensitization to this pollen is comparatively high and varies from 15% in France to 38% in Italy (3). Allergy to *Parietaria* is frequently characterized by asthma (46–49%) and by a perennial pattern of symptoms (4, 5).

The immune response to the pollen allergens of *P. officinalis* and *P. judaica*, the most common European species, has been investigated. The IgE antibody responses of allergic patients define several minor allergens and the major allergens, Par o 1 and Par j 1, glycoproteins of 10–12 and 14.5 kDa, respectively. Two isoforms of Par o 1 and three isoforms of Par j 1 have been isolated from the pollen extract, and the N-terminal amino-acid sequences have been determined by Edman degradation (6–11). Par o 1 and Par j 1 are highly cross-reactive and show extensive N-terminal amino-acid sequence homology (6, 8, 10, 12). In a consistent proportion of patients, the antibody response appears to be directed mostly or exclusively toward these major allergens (13).

Four *P. judaica* allergens have been obtained as recombinant proteins from clones of a *P. judaica* inflorescence cDNA library: Par j 1.1010 is a polypeptide of 139 amino-acid residues; its isoforms Par j 1.0102 and Par o 1.0201 vary in length and share 89% and 98% homology (14, 15). Par j 2.0101 is a polypeptide of 102 amino-acid residues with 45% sequence homology with Par j 1.0101 (16, 17). Par o 1 has not been cloned.

The definition of the structure of dominant B- and T-cell epitopes of major allergens is a tool to investigate the fine cellular and molecular mechanisms of allergen-specific immune response and is important for the development of new approaches to immunotherapy and diagnosis of allergy. A major obstacle is the fact that B-cell epitopes of major allergens and, in general, of proteins, are conformational. Allergens with conformational dominant epitopes include Amb a 1 (18), Bet v 1 (19), Dac g 1 (20), Lol p 1 (21), Ole e 1 (22), Poa p 1 (20), Sin a 1 (23), Der p 1 (24, 25), Der p 2 (26), Der f 1 (24), Der f 2 (27), and Fel d 1 (28). Synthetic peptides with

minor epitopes have been obtained from Alt a 1 (29), Der p 1 (25), and a wheat allergen (30). A recent study reports that the 1–30 region of Par j 1.0101 contains a conformational epitope (the conservation of a disulfate bridge being essential for IgE-binding activity) (17) shared with Par j 2.0101. Several studies have attempted to identify linear immunodominant epitopes by immunoselection of fusion proteins expressed by cDNA fragment libraries. With the exception of a fragment of Phl p 1 cDNA gene, expressing a 15-amino-acid peptide, which bound IgE antibodies from 30% of grass-allergic patients (31), only products of large cDNA fragments are reported to have IgE-binding activity (32–35).

Previously, we have characterized a dominant Par o 1/Par j 1 epitope (6a epitope) expressed by a cDNA clone (6a clone) with a 26-bp insert (6a insert) immunoselected from a library obtained by reverse transcription of mRNA from commercial *P. judaica* pollen. The expressed β -galactosidase fusion protein (6a-BG) in immunoassays extensively inhibited the binding of IgG and IgE antibodies from allergic patient sera to purified Par o 1 and Par j 1 and to *P. judaica* and *P. officinalis* pollen extracts and elicited in immunized rabbits an antibody response specific to Par o 1 and Par j 1 (36, 37). The peptide sequence, deduced from the cDNA sequence read in frame with β -galactosidase, could not be located in any of the amino-acid sequences of *P. judaica* allergens deduced from cloned DNA nor in the N-terminal amino-acid sequences of Par o 1 and Par j 1 isoforms obtained by Edman degradation (6–11, 14–17). We considered the possibility that the insert was not expressed in frame with β -galactosidase, and in the present study we report the analysis of the reactivity of synthetic peptides deduced from different reading frames of the cDNA sequence with affinity-purified human and rabbit anti-Par o 1 antibodies. A further aim of the present study was to investigate whether the 6a epitope was conserved in different recombinant constructs.

Material and methods

Human sera and pools

Sera having a high level of anti-Par o 1 IgE antibodies were obtained from *Parietaria*-allergic patients, skin test positive to *P. officinalis* and to *P. judaica* pollen extracts. The pools were obtained from four individual sera. Unless otherwise stated, the serum donors were Italian. Donors were informed volunteers.

Antisera and immunoglobulins

Rabbit antisera to pollen allergens and to fusion proteins were raised as previously described (19). Antipeptide antisera were raised in rabbits immunized with the peptide coupled to a carrier protein (keyhole limpet hemocyanin) with glutaraldehyde.

Goat antiserum to human IgE was purchased from DAKO (Italy). Radiolabeled rabbit antiserum to human IgE (PRIST) was purchased from Pharmacia (Sweden); alternatively, rabbit antiserum to human IgE from Kirkegaard and Perry Labs (USA) was radiolabeled by established protocols (38). The isotype specificity of antisera to human IgE was confirmed by showing that absorption with a human IgE myeloma protein (donated by D.G. Marsh), but not with an IgG myeloma protein, abolished the ability of the antiserum to precipitate allergen-antibody complexes in DARIA.

Alkaline phosphatase-labeled goat antisera to human and rabbit IgG were purchased from Promega (USA), and rabbit antiserum to *E. coli* β -galactosidase was obtained from Boehringer (Germany).

Allergens and peptides

P. judaica and *P. officinalis* aqueous pollen extracts were obtained from the pollens (Allergon AB, Sweden) by the procedure previously described (8). Highly purified Par o 1 was obtained from the pollen extract by the procedure already described (8). Alternatively, Par o 1 was purified from the pollen extract by electroelution (Prep Cell, Biorad) of the 14-kDa band from SDS-PAGE gels.

Peptides p101-p104 and p96 were synthesized with an ABI automated synthesizer (model 438) according to the manufacturer's instructions and purified by HPLC. Other peptides used in the study were purchased from Sigma.

Radiolabeling

An amount of 14–40 μ g of highly purified Par o 1 (11) was labeled with 2 mCi of ^{125} I (3.7 GBq/ml, Amersham, UK) by the chloramine T method (38). Radiolabeled proteins were 75% TCA precipitable. Aliquots of 100 μ l (1 μ g/ml; 10–20 10^6 cpm) in borate-buffered saline (BBS), pH 8.4, containing 5% bovine serum albumin (BSA) were stored at -20°C .

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting were performed with 12% polyacrylamide gel by established protocols (39).

Antibody purification

Anti-Par o 1 antibodies were purified from individual sera or from serum pools by affinity chromatography on CNBr Sepharose 4B (Pharmacia) coupled to Par o 1 (1.5–3 mg protein/ml of gel). Alternatively, antibodies were eluted from nitrocellulose filters coupled to Par o 1. Each filter (diameter 15 mm) was coupled to 50 mg of Par o 1 in phosphate-buffered saline (PBS), pH 7.2, and exhaustively washed with 10 mM TRIS-HCl buffer (pH 8), 150 mM NaCl, and 0.5% Tween 20 (TBST). To purify anti-Par o 1 antibodies, 5 ml of 1/5 dilution (TBST) of serum was incubated with a filter overnight at 4°C , and, after extensive washing (TBST), antibodies were eluted in 0.1 M glycine-HCl buffer, pH 2.9, 0.5 M NaCl. The eluate was neutralized with 1 M TRIS and dialyzed (PBS), and aliquots (300 ml) were stored at -20°C .

IgG was affinity purified from the allergic patient serum pool by protein A Sepharose (Pharmacia) affinity chromatography, according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed by established protocols (40). Briefly, polystyrene microtiter plates were incubated overnight at 37°C with protein or peptide (1–5 μ g/well) diluted in 0.1 M bicarbonate buffer (pH 9.6) or in PBS, respectively. After washing (PBS containing 0.1% BSA, 0.1% azide, and 0.05% Tween 20), the plates were incubated in PBS containing 1% BSA and 0.1% Na azide, for 1 h at room temperature, to block the remaining binding sites. A volume of 50 μ l/well of serum dilutions (PBS) was added for 1 h at 4°C . After washing (PBS containing 1.5 mM MgCl_2 , 2 mM β -mercaptoethanol, 0.1% Na azide, and 0.1% BSA), bound IgG antibodies were revealed with alkaline-phosphatase-conjugated goat antiserum to human or rabbit IgG (Promega), according to the manufacturer's instructions. Absorbance at 405 nm was revealed with an automated ELISA reader. For the measurement of IgE antibodies, 50 μ l/well of radiolabeled antihuman IgE (10 ng protein/ml, containing approximately 10 000 cpm) was added for 4 h at room temperature, and, after washing, bound cpm was measured by a gamma counter.

Double antibody radioimmunoassay (DARIA)

The DARIA for the measurement of IgE antibodies was carried out essentially as described (41). A volume of 50 μ l of serum was mixed with 50 μ l of the appropriate dilution of an

IgE myeloma protein (IgE carrier). A volume of 100 μ l of the mixture was incubated for 4 h at room temperature with 100 μ l of borate-buffered saline (BBS), pH 8.4, containing 1 ng of radiolabeled Par o 1 (10–20 000 cpm). Antigen–antibody complexes were precipitated by addition of 100 μ l of goat antiserum to human IgE for 16 h at 4°C. Precipitates were collected on Whatman GF/A glass-fiber paper filters and, after washing (10 μ l of BBS), the radioactivity retained was measured by a gamma counter. The antibody level was expressed in arbitrary units, using as a standard the IgG-binding curve obtained with a reference serum.

Inhibition assays

DARIA and ELISA inhibition assays were performed at an antibody dilution which, in direct assays, achieved approximately 50% of the maximal binding to the antigen. Purified antibodies were absorbed with serial amounts of the protein used as inhibitor, for 1 h at room temperature (DARIA) or overnight at 4°C (ELISA). The inhibition was expressed as $100 [1 - (a - b) / (c - b)]\%$, where a and c were cpm bound (DARIA) or OD at 405 nm (ELISA) obtained in essays performed in the presence and in the absence of the inhibitor tested, respectively, and b was the background value.

In all immunoassays, the samples were run in duplicate and the values obtained reported as arithmetic mean. The accepted coefficient of variation from the calculated mean was 12%. The background (defined as the mean value obtained in assays where addition of the first antibody was omitted) was subtracted from the values reported in the figures. The detection limit was defined as $\times 2$ the background.

Fusion proteins

The immunoselection of clone 6a from a *P. judaica* pollen cDNA expression library and the characterization of the expressed 6a β -galactosidase (6a-BG) fusion protein have been described (19). The 6a insert was subcloned in the vectors pGEX-1 λ T, pGEX-3X, and pGEX-2T (Pharmacia), which express the insert in the first, second, and third reading frames respectively, in fusion with glutathione S-transferase (GST). The sequences of the inserts were confirmed according to Sanger (42).

β -Galactosidase fusion proteins were purified by electroelution of the corresponding band from SDS–PAGE gels of bacterial extracts or by affinity chromatography (Protosorb, Promega). GST fusion proteins were purified by affinity

chromatography, according to the manufacturer's instructions (Pharmacia).

Results

Reactivity of 6a β -galactosidase fusion protein to allergic patient sera

The β -galactosidase fusion protein expressed by the 6a clone (6a-BG) contains a segment encoded by the oligonucleotide: ggcacgagctcgtgccgactcgtgcc (6a). We have previously documented that 6a-BG contains a dominant epitope of the allergens Par o 1 and Par j 1 and can extensively inhibit immunoassays specific for these allergens (19). Fig. 1 shows the inhibition curves of *P. judaica*- and *P. officinalis*-specific ELISA obtained by absorbing with serial amounts of 6a-BG or of control-BG a pool of sera from *Parietaria*-allergic patients. The serum pool was obtained from Israeli patients exposed to *P. judaica*. At the maximum amount used (2.35 μ g), 6a-BG inhibited 72% and 68% of IgE antibody binding to solid-phase *P. judaica* or *P. officinalis* pollen extract, respectively, whereas the control protein did not cause appreciable inhibition.

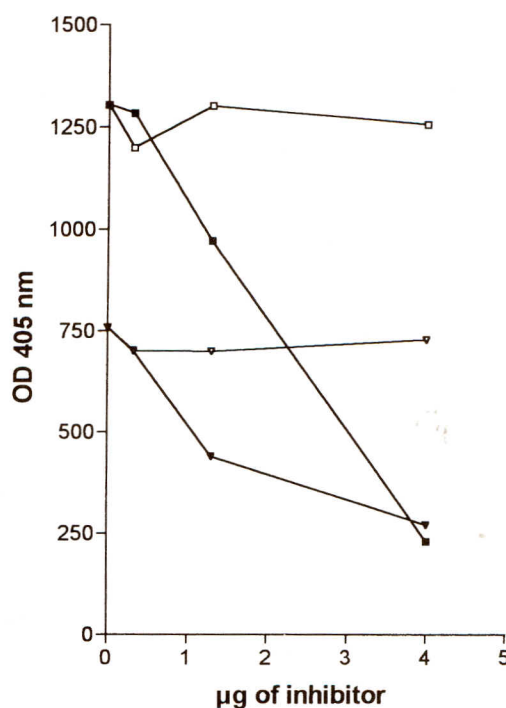


Figure 1. IgE ELISA inhibition. Solid phase: *P. officinalis* (■, □) or *P. judaica* (▲, △) pollen extract (5 μ g/well). One-third dilution (50 μ l) of pool of four sera from Israeli *Parietaria*-allergic patients was absorbed with increasing ($\times 3$) amounts of inhibitor, as indicated in abscissae. Inhibitors: 6a-BG (■, ▲) or control-BG (□, △).

Table 1. Synthetic peptides used in study

p103 (GST 1.8)	E	F	G	T	S	<u>S</u>	<u>C</u>	<u>R</u>	I	V	P	N
p102 (GST 3.2)	N	S	A	R	A	R	A	D	<u>S</u>	<u>C</u>	<u>R</u>	I
p104	I	R	H	E	L	V	P	T	R	A	E	I
p101 (GST 3.5)	N	S	A	R	V	G	T	S	<u>S</u>	<u>C</u>	<u>R</u>	I

In parenthesis, GST fusion proteins containing peptide sequence. Motif SCRI is underlined.

Synthetic peptides and GST fusion proteins

The panel of synthetic peptides used in this study is reported in Table 1. The sequences of peptides p103, p102, and p104 were deduced from the 6a nucleotide sequence, with the first, second, and third reading frames, respectively (including the EcoRI restriction sites).

The 6a oligonucleotide was inserted in pGEX vectors and expressed in different reading frames fused to GST. The fusion proteins GST 1.8, GST 3.2, and GST 3.5 contain the sequences of p103, p102, and p101, respectively. The control proteins were GST obtained from a nonrecombinant clone (pGEX 2.3), and pGEX 2.2, containing an irrelevant insert (encoding the sequence: IRHELVPTRAEF). The SDS-PAGE analysis of the fusion proteins purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia) is shown in Fig. 2.

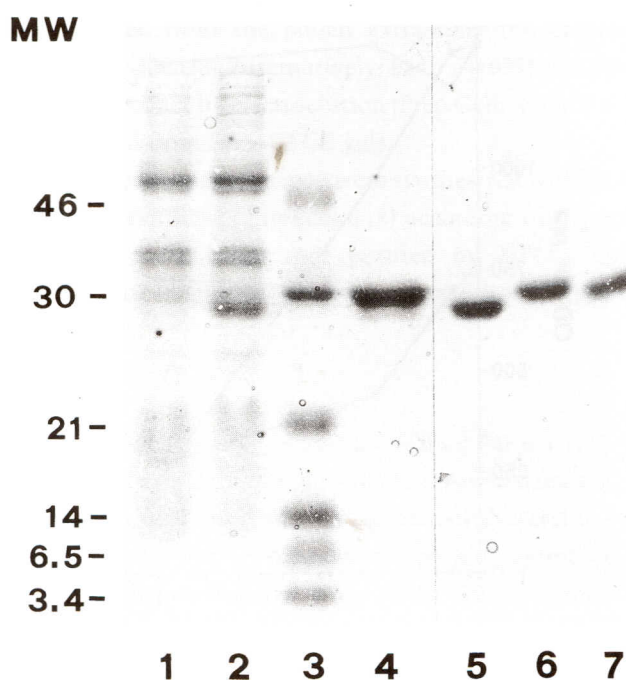


Figure 2. SDS-PAGE analysis. lanes 1 and 2: pGEX 2.3 transformed *E. coli*-non-induced (lane 1) and IPTG-induced (lane 2) extracts; lane 3: MW markers; lanes 4–7: affinity-purified GST fusion proteins GST 1.8 (lane 4), GST 2.3 (lane 5), GST 3.2 (lane 6), and GST 3.5 (lane 7).

Reactivity of rabbit anti-Par o 1 antibodies to synthetic peptides

In order to define the amino-acid sequence containing the 6a epitope, we tested in ELISA the binding between anti-Par o 1 antibodies and the synthetic peptides p101, p102, p103 (Fig. 3), and p104 (not shown). Anti-Par o 1 antibodies were affinity purified from the serum of the rabbit immunized with Par o 1 (the same antiserum used for the immunoselection of the 6a clone from the λ gt 11 library [19]). As a positive control, we used rabbit antibodies specific for the peptide p96 (EFGTSSCRL), which recognize the sequence SCRI. Anti-Par o 1 antibodies bound, in addition to Par o 1, only p102, whereas anti-p96 antibodies bound p101, p102, and p103. The binding of anti-Par o 1 antibodies was very specific, as the p101, differing from p102 for three residues only, was not recognized. The results of these experiments unequivocally indicate that the sequence expressed by the 6a cDNA was that of p102.

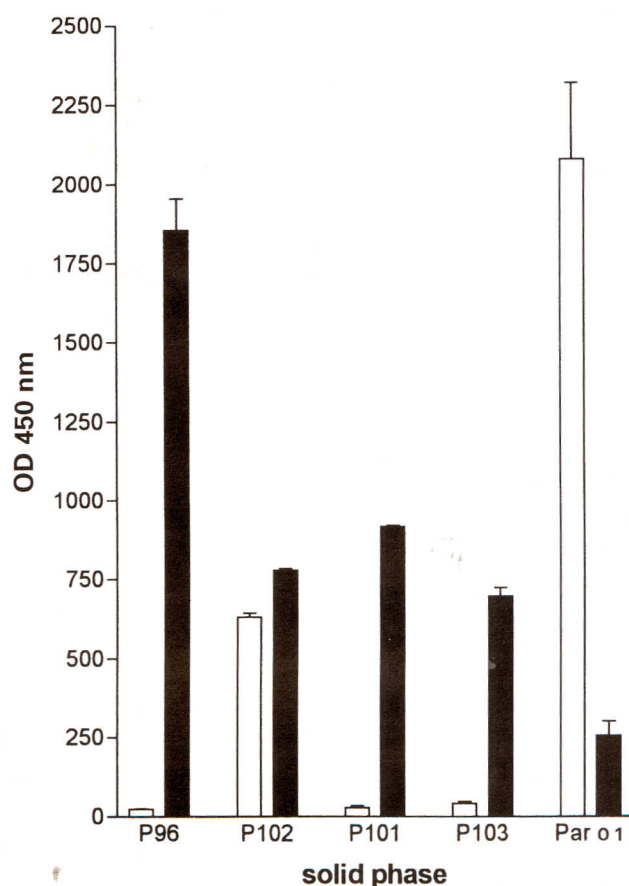


Figure 3. IgG ELISA. Solid-phase synthetic peptides and proteins reported in abscissae (1 μ g/well). Binding of affinity-purified rabbit anti-Par o 1 ($\square$) or anti-p96 ($\blacksquare$) antibodies.

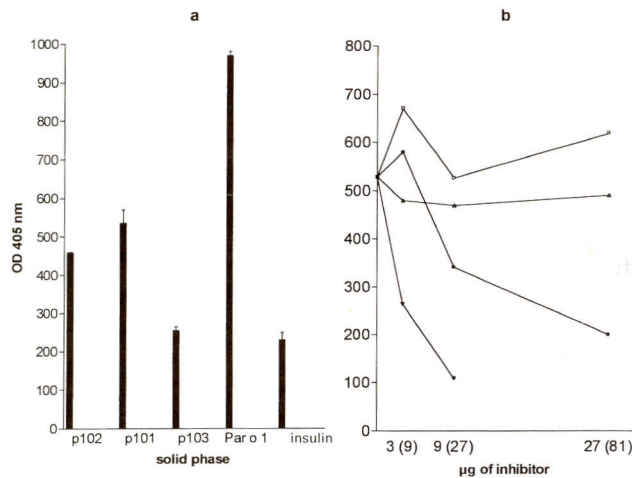


Figure 4. IgG ELISA. a) Solid phase: peptides and proteins listed in abscissae (1 µg/well). Binding of anti-Par o 1 IgG antibodies (affinity-purified from allergic patient serum pool). b) Solid phase: p102 (1 µg/well). Inhibition of binding of IgG affinity purified from allergic patient serum pool. Solution (50 µl) containing 4–8 µg/ml of antibodies was absorbed with increasing ($\times 3$) amounts of inhibitor protein or peptide (l), as indicated in abscissae. Inhibitors: Par o 1 (▲), p102 (■), insulin (□), neurotensin (△).

Reactivity of anti-Par o 1 antibodies from the sera of allergic patients to synthetic peptides

In order to investigate the fine specificity of the antibody response of *Parietaria*-allergic patients, anti-Par o 1 antibodies were affinity purified from a pool of four sera and tested for binding to p101, p102, p103, Par o 1, and insulin in IgG ELISA. Peptide p102 showed significant binding; in contrast to rabbit antibodies, human antibodies bound also p101 (Fig. 4a). The specificity of the binding between anti-Par o 1 antibodies and p102 was confirmed in p102-specific ELISA (Fig. 4b). Absorption of IgG affinity purified from an allergic patient's serum pool with Par o 1 or with p102 extensively inhibited the binding, whereas absorption with a control protein (insulin) or a control peptide (neurotensin: ELYENKPRRPYIL) had no effect.

Reactivity of anti-Par o 1 antibodies from the sera of allergic patients to GST fusion proteins

To confirm the fine specificity pattern obtained in the direct binding assays, the fusion proteins GST 1.8, GST 3.2, and GST 3.5, containing the sequences of p103, p102, and p101, respectively, and the controls GST 2.2 and GST 2.3 were tested for the ability to inhibit the binding between human anti-Par o 1 antibodies and Par o 1 (Figs. 5 and 6).

A volume of 50 µl of a solution containing 5–8 µg/ml of anti-Par o 1 antibodies affinity purified from the serum of a

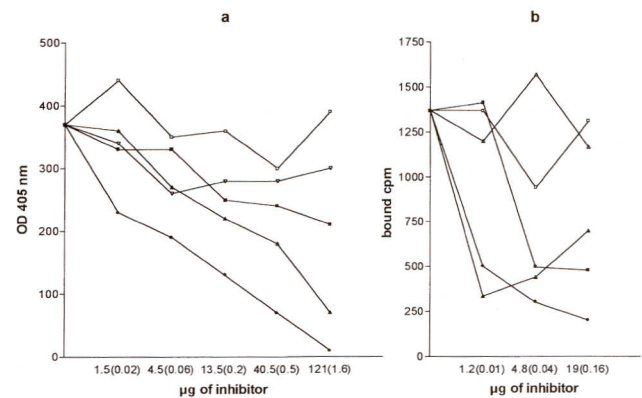


Figure 5. a) IgG ELISA inhibition. Solid phase: Par o 1 (1 µg/well). Solution (50 µl) containing approximately 10 µg/ml of anti-Par o 1 antibodies affinity purified from serum of *Parietaria*-allergic patient was absorbed with increasing ($\times 3$) amounts of fusion protein or of Par o 1 (l), as indicated in abscissae (log scale). b) IgE DARIA inhibition. Radiolabeled Par o 1: 1 ng. Patient serum (50 µl) was absorbed with increasing ($\times 4$) amounts of fusion protein or of Par o 1 (l), as indicated in abscissae (log scale). Inhibitors: GST 1.8 (□), GST 2.2 (△), GST 3.2 (■), GST 3.5 (▲), Par o 1 (●).

Parietaria-allergic patient was incubated with serial amounts of GST 1.8, GST 3.2, GST 3.5, or the control GST 2.2 before the performance of the immunoassays. The binding to Par o 1 was assessed in ELISA for IgG antibodies and in DARIA for IgE antibodies. IgG antibody binding was

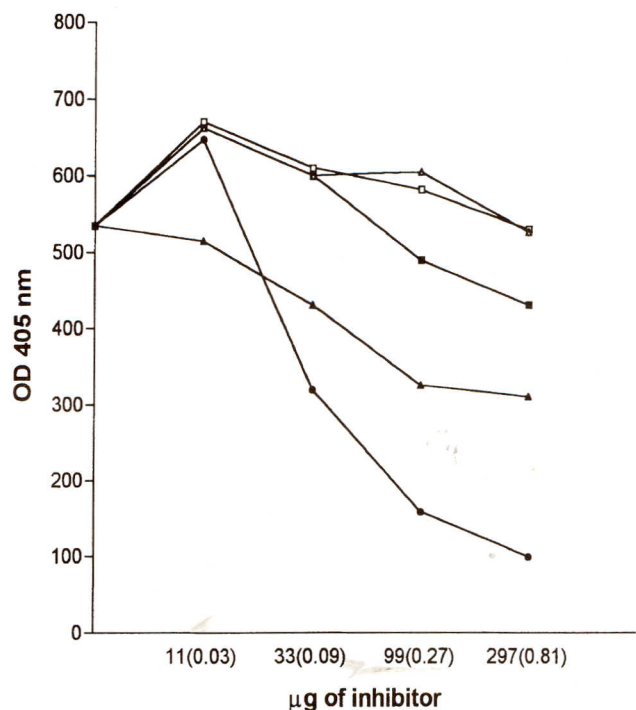


Figure 6. ELISA inhibition. Solid phase Par o 1. Inhibition of binding of IgG anti-Par o 1 antibodies purified from allergic patient serum pool. Solution (50 µl) containing approximately 10 µg/ml of anti-Par o 1 antibodies was absorbed with increasing ($\times 3$) amounts of fusion protein or of Par o 1 (l), as indicated in abscissae (log scale). Inhibitors: GST 1.8 (□), GST 2.3 (△), GST 3.2 (■), GST 3.5 (▲), Par o 1 (●).

inhibited by absorption with GST 3.2 or GST 3.5 according to a dose-response curve, whereas absorption with GST 2.2 or GST 1.8 had no effect (Fig. 5a). IgG antibody binding was inhibited by 54% and 40% by absorption with 121.5 µg of GST 3.2 or of GST 3.5, respectively. A similar pattern was observed in Par o 1-specific DARIA, where IgE antibody binding was inhibited by 69% and 66% by absorption with 5 µg of GST 3.2 or with 1.2 µg of GST 3.5, respectively, whereas GST 1.8 and GST 2.2 had no effect (Fig. 5b).

Inhibition assays performed with antibodies purified from a pool of patient sera yielded a pattern similar to that reported in Fig. 5. Fig. 6 shows that the binding of anti-Par o 1 IgG antibody was inhibited by 42% and 22% by absorption with 300 µg of GST 3.2 or of GST 3.5, respectively, whereas absorption with GST 1.8 and GST 2.3 had no effect. The amounts of GST 3.5 or 6a-BG or Par o 1 required to obtain 50% inhibition of the binding of IgG and IgE antibodies are reported in Table 2.

Taken together, the inhibition experiments confirmed the results of the direct peptide-binding assays, namely, that the p101 and p102 sequences contain a dominant epitope recognized by anti-Par o 1 antibodies from *Parietaria*-allergic patients.

Discussion

We have previously reported that a 26-bp oligonucleotide expressed as a β-galactosidase fusion protein (6a-BG) encodes a dominant epitope of Par o 1 and Par j 1 (6a epitope) (37). The clone containing the insert expressing 6a-BG was immunoselected from a *P. judaica* inflorescence library with an antiserum from a rabbit immunized with Par o 1 and Par j 1 (37). In the present study, we define the amino-acid sequence of the expressed peptide by showing that rabbit anti-Par o 1 antibodies, affinity purified from the same antiserum used in immunoselection, specifically recognize the sequence NSARARADSCRI (p102) corresponding to the second reading frame of the insert (Fig. 3). The observation that an insert is not expressed in frame with β-galactosidase is not unusual, and can be explained by reverse translation of partially degraded mRNA with full or partial loss of codons. The sequence NSARARADSCRI could not be located in any region of *P. judaica* allergen sequences deduced from cloned DNA nor in the N-terminal regions of Par o 1 or Par j 1 isoforms obtained by Edman degradation (14–17).

Par o 1-specific antibodies affinity purified from the allergic patient serum pool specifically bound p102 and p101, a peptide which was not bound by rabbit anti-Par o 1

Table 2. Amount of fusion protein required for 50% inhibition of Par o 1-specific assays

Protein	Assay	
	Ig ELISA	Ig DARIA
GST 3.5	46 µg	0.7 µg
6a-BG	2.9 µg	0.06 µg
Par o 1	0.06 µg	0.007 µg

Amount of GST fusion protein or of Par o 1 required for 50% inhibition of binding between Par o 1 and anti-Par o 1 antibodies purified from allergic patient serum pool.

DARIA and ELISA were performed as described in Material and methods.

antibodies (Figs. 4a and b). Differences in the epitope specificity between human and rabbit anti-Par o 1 antibodies may be explained by different conditions at immunization/sensitization and/or by different composition of the immunizing/sensitizing allergen mixture. Since the ELISA binding pattern of human anti-Par o 1 antibodies was less clear-cut than that of rabbit antibodies, as reported also in studies on other antigens (43), and we wished to characterize further their peptide specificity, we developed GST fusion proteins containing the peptide sequences and tested their ability to inhibit the binding between Par o 1 and anti-Par o 1 IgG and IgE antibodies (Table 1). The results of ELISA and DARIA inhibition experiments showed that the fusion proteins containing p101 (GST 3.5) or p102 (GST 3.2) specifically inhibited Par o 1 binding of IgG or IgE anti-Par o 1 antibodies affinity purified from human sera, confirming the results of the direct peptide-binding assays (Figs. 5 and 6). Taken together, these experiments also show that the fused polypeptide (GST or β-galactosidase) did not influence peptide reactivity with the tested antisera, and that the 6a epitope was conserved in different recombinant constructs.

In a previous study, we reported that the 6a epitope was epidemiologically dominant, since antibodies specific for this epitope (expressed in fusion with β-galactosidase) were revealed in 28/28 sera from *Parietaria*-allergic patients from Italy and Israel. In addition, based on the results of inhibition experiments, we hypothesized that the individual anti-Par o 1 antibody response was restricted and focused on the 6a epitope (37). This hypothesis is supported by the results of the inhibition experiments discussed in the present study (Figs. 4b, 5, and 6). However, there are several caveats to the interpretation of these experiments in terms of restriction of the antibody fine specificity, as it has to be

demonstrated that the experimental conditions of the immunoassay allow monitoring of the complete fine specificity repertoire of anti-Par o 1 antibodies, a condition that can only be approximated in immunoassays performed with antibodies elicited by natural immunization (20, 44). The repertoire of Par o 1 epitopes monitored in immunoassays performed with serum pools is limited to epidemiologically dominant epitopes, as the proportion of antibodies specific to these epitopes is increased in pools. In addition, the amplitude of the repertoire monitored depends on the matching between species variants or isoforms of allergen contained in the commercial pollen and those responsible for natural sensitization of the serum donors and, in conditions without such matching, the epitope repertoire monitored is restricted to cross-reacting epitopes. If this was the case, however, we would expect that, in essays performed with sera from donors prevalently exposed to *P. judaica*, the 6a epitope would inhibit to a greater extent the binding to *P. officinalis* than to *P. judaica* allergens. However, in the experiments illustrated in Fig. 1, in which we used a serum pool from allergic patients from Israel (where the prevalent species is *P. judaica* and *P. officinalis* is not reported to grow), the species of the solid-phase allergens did not appear to influence the dose-response inhibition curve obtained by absorption of the serum pools with the 6a fusion protein.

It is possible that the conditions of sensitization (repeated exposure to allergens deriving from plants which are genetically heterogeneous and/or belong to related species, exposure to cross-reacting allergens/antigens), or the mechanisms of immune response in allergy favor antibody responses of restricted specificity. A restriction of the fine specificity repertoire of the IgE antibody response in allergic

patients has been hypothesized for some allergens, but not for other. Studies on the allergens Lol p 1 (44), Poa p 1 (45), Sin a 1 (46), and Cry j 1 (47) have reported that monoclonal antibodies defining conformational epitopes extensively compete with human antibodies for binding to the allergen. Other allergens are reported to possess multiple epitopes for human IgE antibodies (48–50). Dominance of a few epitopes in the individual antibody response has been reported also in studies on other antigens, including autoantigens (51) and helminth parasite antigens (52). To explain repertoire restriction and dominance of selected epitopes, various cellular and molecular mechanisms have been postulated, including B-cell tolerance, antigenic competition, imprinting by cross-reacting antigens, and action of B-cell superantigens (51–53).

The knowledge of the linear sequence containing the dominant epitope of Par o 1 is important in allergen-specific approaches to therapy. Short synthetic peptides or related constructs behaving as haptens, i.e., unable to cross-link receptor-bound IgE, could be used to saturate mast-cell-bound IgE, in order to reduce anaphylactic side-effects of allergen exposure (30, 31). Such therapies could be particularly appropriate for subjects monosensitized, who, in the area of highest exposure, represent a consistent proportion of *Parietaria*-allergic patients (3).

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