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Par *j* 1.0101 is one of the two major allergens of the *Parietaria judaica* (*Pj*) pollen, and its three-dimensional structure was built by three-dimensional structural homology modeling. The resultant model was used to identify putative IgE binding regions. Western blot analysis of gene fragmentation products showed that the 1 to 30 region was capable of binding specific IgE from a pool of sera (*n* = 30) of patients allergic to *Pj* pollen. Using the structural model as a guide, deletion and site-directed mutagenesis of the 1 to 30 region was performed, and the amino acids involved in IgE binding were identified. In addition, a synthetic peptide covering the 1 to 30 region was capable of binding human IgE without triggering histamine release from basophils of *Pj* allergic patients (*n* = 6) and thus represents a haptenic molecule with potential use as an immunotolerant agent. This epitope is also present on the Par *j* 2.0101 major allergen representing a common IgE epitope. It is an immunodominant epitope, since it was capable of inhibiting 30% of all specific IgE against the *Pj* major allergens, and therefore, it might be a candidate for the future development of immunotherapeutics. The *Journal of Immunology*, 1998, 160: 2780–2785.

Allergy is one of the most widespread health problems in humans, affecting approximately 20% of the population in industrialized countries (1). The reaction is a multi-step one. The IgE secreted by B cells binds to the cell surface of IgE receptors present on mast cells and basophils. Upon re-exposure to the specific multivalent allergen, the fixed IgE are cross-linked by the allergen, leading to the release of inflammatory mediators (2). The regions of an allergenic molecule capable of cross-linking IgE are defined as epitopes, and due to their key role in triggering the inflammatory response, they are potential candidates for immunotherapy or diagnosis of the allergic disease. The molecular definition of an epitope presents some difficulty, since most of them are discontinuous and consist of amino acid residues not contiguous in the primary structure but brought together by the folding of the polypeptide chain. Three-dimensional (3D) structural modeling can be used to construct a 3D model of an allergen and to assist the identification of potential epitopes.

The pollen of the weed *Parietaria judaica* (*Pj*) is the main cause of immediate hypersensitivity in the Mediterranean area, and an epidemiologic study conducted on 1878 allergic patients showed that up to 50% reacted to *Pj* pollen extract (3). However, the presence of this weed is not restricted to the Mediterranean, since it has also been described in temperate climates of Central and Eastern Europe, Australia, and California (4, 5). The pollen of *Pj* contains at least nine different allergens with molecular masses ranging from 10,000 to 80,000 Da (6, 7). Two of them, designated Par *j* 1.0101 and Par *j* 2.0101, have already been cloned and sequenced (8, 9). The mature processed allergen Par *j* 1.0101 is a protein of 14,726 Da containing 139 amino acids residues and is capable of inducing histamine release from human basophils of *Pj*-allergic patients (10). It is a major allergen, since it interacts with 95% of the sera from *Pj*-allergic patients and blocks approximately 40% of all the IgEs specific for the *Pj* total crude extract (8, 10). The Par *j* 2.0101 is a protein of 102 amino acids with a molecular mass of 11,344 Da and shows a 45% homology at the amino acids level with the Par *j* 1.0101. Preincubation of sera from *Pj* allergic patients with both rPar *j* 1.0101 and rPar *j* 2.0101 fully abolished IgE recognition of the 10- to 14-kDa native major allergenic area, suggesting that the two allergens contributed to that region. Considering these important immunologic properties we focused our attention on the IgE binding regions of these allergens, and in this manuscript we report the identification of one discontinuous IgE epitope embodied in the first 30 amino acids of the Par *j* 1.0101 allergen. This region contains a common Par *j* 1.0101 and Par *j* 2.0101 dominant epitope and does not induce histamine release, suggesting that this molecule might be used as a specific hapten to block the cytoplilic IgEs bound to the basophil and mast cells.

Materials and Methods

Three-dimensional structural modeling

The technique of protein modeling by homology is used to assign 3D structure to a protein of unknown structure based on structural information from members of the same family (11). The crystal structure of the homologous soybean nonspecific lipid transport (ns-LT) protein (pdb1hyp.ent) was used as a template to generate the Par *j* 1.0101 3D model. The intervening loop structures were obtained by searching the Brookhaven Protein Databank for proteins containing loops of the same
length joining similar secondary structural units, and disulfide bonds were assigned according to the crystal structure of soybean ns-LTP. Calculations were performed using BioSym 95.0 on a Silicon Graphics Indigo 2 workstation (Mountain View, CA). Modeling was performed using the homology module provided by BioSyrm. Energy minimization was performed using BioSyrm’s consistent valence forcefield via a combination of steepest descents and conjugate gradient algorithms to a convergence criterion of <1 Cal/mol-Å, unless otherwise stated. A nonbonded cutoff of 8 Å was used with a switching function applied between 6.5 and 8 Å.

**Eisenberg analysis**

Eisenberg profile analysis (12) was used to verify the predicted structure. Eisenberg et al. suggested that a cutoff of <0.45 × Scale is useful for identifying grossly misfolded structures. Models with scores between 0.45 and 0.60 are considered acceptable, although it is still possible that locally misfolded regions occur. Examination of the profile score in a moving window scan of 10 residues can be used to identify locally misfolded regions.

**Deletion mutants**

Primers for PCR amplification were (lowercase letters indicate the restriction enzyme cloning site, and uppercase letters indicate the coding sequence): oligo 1, 5′-cgggaattcCCGTTTGTGCAGGGGAAAGAG-3′; Cys 30, 5′-ccggaattcCCGTTTGTGCAGGGGAAAGAG-3′; Cys 4, 5′-ccggaattcCCGTTTGTGCAGGGGAAAGAG-3′; Cys 14, 5′-ccggaattcCCGTTTGTGCAGGGGAAAGAG-3′; Cys 29, 5′-ccggaattcCCGTTTGTGCAGGGGAAAGAG-3′; Cys 42, 5′-ccggaattcCCGTTTGTGCAGGGGAAAGAG-3′. Deletion mutants were confirmed.

**Peptide synthesis**

The synthetic peptides were prepared by solid phase synthesis performed in a Milligen 9050 synthesizer (Burlington, MA) as previously described (13). Crude peptides were purified by preparative reverse phase HPLC using a Waters Delta Prep 3000 system (Waters Associates, U.K.). The pPJ1.2 and pPJ1.3 clones were made by annealing the following oligos: pPJ1.2 forward, aattcCAAGAAAACCCTGCGGCTATGTCGAGACCGCTATGC CG; pPJ1.2 reverse, gCGGATCCGAGCTTCATACATCGTCCGCAA GTTTCTTG; pPJ1.3 forward, aattcCAGGCTATGCGGCTATGTCGAGACCGCTATGC CG; and pPJ1.3 reverse, gTTCCTCTCGCAC GAACTCAGACCGAGATCGCG.

**Preparation of the recombinant allergens**

The recombinant clones were grown to 0.5 to 0.6 OD600 in Luria Bertoni broth and induced with 0.3 mM isopropylthiogalactoside for 2 h. Cells were sonicated using a Heat System Ultrasonic W-285, and the cell debris was removed by centrifugation (9000 rpm, 30 min). The supernatant was diluted 1/20 with 10 mM EDTA and concentrated by using a Centriprep concentrator (Amicon, Danvers, MA). The concentration of the recombinant proteins was detec-ted by densitometric analysis of SDS-PAGE gels stained with Coomassie Brilliant Blue.

**Western and dot blot analysis**

Ten micrograms of each recombinant Escherichia coli cell extract and 20 μg of the Pj crude extracts were fractionated on 10 and 16% PAGE-SDS gels, respectively. Gels were stained with Coomassie Brilliant Blue or electrotransferred onto a nitrocellulose membrane. For dot-blot analysis, 10 μg of each peptide was spotted onto a nitrocellulose membrane (Hybond C, Amersham, Aylesbury, UK). Membranes were air-dried, incubated in blocking buffer (PBS supplemented with 3% BSA, 0.5% Tween-20, and 0.02% NaN3), and washed three times in washing buffer (1× PBS/0.1% Tween-20). Subsequently, membranes were incubated overnight with a pool of sera (1/5 dilution; n = 30) from Pj allergic patients with high RAST value.

**Histamine release assay**

Human basophils for the histamine release assay were obtained by venipuncture using a heparinized syringe from atopic and nonatopic subjects. The atopics were allergic to Pj pollen and shared a RAST positivity value of 4+. Informed consent and permission was obtained from the subjects. Pj pollen extract (1 μg/ml) and rPar j 1.0101 (1 μg/ml) were used as positive controls. The pep1 synthetic peptide was used at concentrations of 1 and 10 μg/ml. The histamine released in response to the synthetic peptide was determined as previously reported (15). The total histamine contained in the basophils was determined from the supernatant obtained by boiling 200 μl of blood for 10 min. The net histamine released in response to the synthetic peptide was expressed as a percentage of the total cellular histamine after subtraction of the level of histamine spontaneously released in the absence of the stimulus.

**Results**

The soybean ns-LTP three-dimensional structure has been used as a template for 3D structural modeling as described by Kennedy et al. (16). The crystal structure of soybean ns-LTP has an overall α-α-α-β secondary structure with four disulfide bonds (17). The Par j 1.0101 allergen was modeled according to conserved secondary structural regions and disulfide bonds as described in Materials and Methods. The resulting 3D model of the major allergen of Pj was verified by an Eisenberg analysis (12, 18) and gave a profile score of 20.35 (0.45 × Scale = 90.7). This value strongly suggests that the protein may attain a fold consistent with that of the soybean ns-LTP. The 3D structure of the resulting model is shown in Figure 1.

**Epitope mapping**

The Par j 1.0101 cDNA was used as a template in a PCR strategy searching for IgE binding region. In particular, the 1 to 30 region (pPj1 clone) was investigated, since it represents a region of high homology between the Par j 1.0101 and Par j 2.0101 major allergens. The pPj1 fusion protein was capable of binding human IgE by Western blot, suggesting that this region contained at least one IgE binding site. To analyze the region in more detail, additional smaller subclones were made to define the minimum size of the epitope. The 1 to 30 region was split into three overlapping regions from amino acids 1 to 13 (pPj1.2 clone), from amino acids 10 to 21 (pPj1.3 clone), and from amino acids 12 to 30 (pPj1.4 clone) and cloned in the pMALC2 expression vector (Fig. 2A). None of these three clones was capable of binding human IgE, suggesting that the epitope present in this region needs the entire region to attain a functional 3D structure. To investigate the role of the four cysteine residues contained in the region we made deletions of the peptide, as described in Materials and Methods. Examination of the profile score in a moving window scan of 10 residues can be used to identify locally misfolded regions. Examination of the profile score in a moving window scan of 10 residues can be used to identify locally misfolded regions.
whereas the pPJ1.6 clone had no IgE binding activity, suggesting
that the C30 did not play any role in IgE Ab recognition. The
pPJ1.7 clone was still capable of binding IgE, whereas the pPJ1.8
clone showed a very weak IgE binding activity (Fig. 2A). To con-
firm the importance of the disulfide bonds between cysteines 14
and 29, we performed a dot-blot assay with the Par j 1.01 allergen
and the pep1 peptide under reducing conditions. This ex-
periment showed that the pep1 peptide structure is destroyed by
2β-ME treatment, confirming that the cysteines contained in that
region are essential for IgE binding, as previously shown by site-
directed mutagenesis (Fig. 2B).

In addition, the modeled structure of the Par j 1.01 allergen was
used to identify potential amino acids residues directly involved in
IgE binding. The region encompassing residues 1 to 30 was examined
to identify any exposed side chains that would be capable of in-
teracting with IgE. Residues Q19, K21, E22, K23, E24, and K27
were identified as potential candidates, and site-directed mutagen-
esis was used to mutate these residues to alanine. The IgE binding
activity of the resultant mutants was tested by Western blot anal-
ysis (Fig. 3B). From this analysis we showed that mutation of the
K21, K23, E24, and K27 amino acids caused loss of binding.

Synthetic peptide analysis
To confirm that the IgE binding activity of the 1 to 30 clone was
due exclusively to the Pj sequence, we used synthetic peptides
covering the 1 to 30 and 1 to 28 regions (pep1 and pep2, respec-
tively) and tested them in a dot-blot assay. They showed a pattern
of reaction identical with that of the pPJ1 and pPJ1.6 clones (Fig. 2).

Inhibition assay
The recombinant Par j 1.01, Par j 2.01, and pPJ1 proteins
were fractionated on PAGE-SDS gel, transferred to a nitrocellu-
lose membrane, and incubated either with a pool of sera from
patients allergic to the Pj pollen (Fig. 4A) or with the same pool of
sera treated with 50 μg/ml of rPar j 1.01 (Fig. 4B) or rPar j
2.01 (Fig. 4C). These data showed that the rPar j 2.01 allergen
is capable of blocking the specific IgE vs the 1 to 30 region of the
Par j 1.01 molecule, suggesting the presence of a common
epitope between the two allergens.

The Pj pollen crude extracts were fractionated on a 16% PAGE-
SDS gel, transferred to a nitrocellulose membrane, and incubated
either with a pool of sera (1/5 dilution; n = 30) from patients
allergic to the Pj pollen or with the same pool of sera preincubated
with increasing amounts of the pPJ1 clone (see Fig. 5A for details).
Results are displayed as a mean of triplicate determinations. The
Western blot analysis in Figure 5B shows the different level of IgE
complexes, and a densitometric analysis performed for the two
lanes showed a reduction of approximately 30% of all the Ig complexes
in lane 2 around the 10,000 to 14,000 Da native
allergenic area.
Histamine release

Pep1 was used to test its capacity to induce histamine release from basophils of Pj allergic patients. Basophils from six patients with high concentrations of Pj-specific IgEs did not show any release after pretreatment with different concentrations of the 1 to 30 synthetic peptide. Total Pj crude extract and rPar j 1.0101 protein were used as positive controls (Table I). This result indicates that the 1 to 30 region contains only a single IgE epitope, since the release of histamine detected during an allergic reaction requires cross-linking between at least two epitopes on the allergenic molecule complexed to the IgEs bound to the basophil surface.

Discussion

In the last few years a large number of cDNA coding for allergenic molecules have been isolated (20–23), but very little information is available on the IgE binding regions. The isolation and characterization of an IgE epitope are of paramount importance, since the inhibition of IgE cross-linking on the membrane of mast cells or basophils may represent a safe immunotherapy with reduced anaphylactic reaction.

The Pj pollen contains at least nine allergens, of which the two major allergens have been isolated and characterized (8, 9). Sequence homology analysis using a Blast database search from the European Molecular Biology Laboratory Service of the current databases has shown a high degree of amino acid identity between the two Pj major allergens and a family of proteins from plants whose function is to facilitate in vitro ns-LTP between membranes (24–26). They contain a conserved secondary structure forming a α-α-α-β fold with a subset of specific amino acids required to maintain a tertiary structure, including eight cysteine residues forming four disulfide bonds that compact the four helices. The overall effect of the secondary structures is to form a right hand winding of the α helixes with a hydrophobic pocket for lipid interactions; meanwhile, the C-terminal, which is not clearly defined, may form a cap to the pocket. We used the soybean ns-LTP crystal structure as a template for the Par j 1.0101 allergen 3D structural modeling, since it provides satisfactory resolution at 1.8

![FIGURE 2](image_url)

**FIGURE 2.** IgE binding activity of the 1 to 30 region and its mutants. A. Amino acids are indicated in one-letter code. Numbers indicate the positions of the amino acids on the wild-type protein. Underlined amino acids indicate substitutions. Gaps indicate deletions of the wild-type sequence. + and − indicate the IgE binding activity of the recombinant protein. B. Dot blot indicates the IgE binding activity of pep1 in the presence or the absence of a reducing agent.

![FIGURE 3](image_url)

**FIGURE 3.** Mapping of the 1 to 30 IgE epitope. A. Amino acids are indicated in one-letter code. Numbers indicate the positions of the amino acids on the wild-type protein. Arrows indicate the mutated residues. + and − indicate the IgE binding activities of the recombinant mutants. B. Western blot analysis of the Par j 1.0101 allergen, rpPJ1, and its mutants. Lane UN indicates an uninduced pPJ1 E. coli crude extract.

![FIGURE 4](image_url)

**FIGURE 4.** Inhibition assay of the pPJ1 with the rPar j 1.0101 and rPar j 2.0101 proteins. A. Lanes 1, 2, and 3 show the presence of IgE complexes toward the recombinant pPJ1, rPar j 1.0101, and rPar j 2.0101 proteins using a pool of sera of Pj allergic patients. B. The recombinant extracts incubated with the same pool of sera pretreated with 50 μg/ml of rPar j 1.0101. C. The same recombinant extracts incubated with the same pool of sera pretreated with 50 μg/ml of rPar j 2.0101. The amino acid alignments indicate the maximum homology between the Par j 1.0101 and Par j 2.0101 allergens in the 1 to 30 region.
FIGURE 5. Western blot analysis of the Pj pollen crude extract. A, Dose-response curve showing the capacity of the recombinant pPJ1 protein to inhibit binding to natural Par j 1.0101 and Par j 2.0101. B, Numbers on the left indicate the molecular mass markers. Lane 1 shows the presence of specific IgE complexes from a pool of sera from Pj allergic patients. Lane 2 shows the depletion of the 1 to 30 region-specific IgE complexes after preincubation of the pool of sera with the recombinant pPJ1 protein (30 µg/ml).

and has the same topographical fold as the other members of this family. By this techniques, the Par j 1.0101 major allergen has been shown to adopt the same structural fold as members of the plant ns-LTP family although no allergenic activity has been shown for the other members of this family to date.

Mapping an epitope by gene fragmentation and/or point mutations is often expensive and time consuming. Analysis of a 3D model can be used to detect the amino acids residues that are exposed to the solvent surface and thus represent potential epitope residues. In particular, the recombinant pPJ1 fusion protein was investigated by Western blot, since it contains a cluster of residues within the region presenting side chains exposed to the solvent surface and containing the properties (bulky and/or charged side chains) to form an epitope. In addition, the 1 to 30 region represents a region of high homology between the Par j 1.0101 and Par j 2.0101 major allergens (65% amino acids identity; see the alignment of highest homology at the bottom of Fig. 4), and preliminary results by cross-inhibition experiments suggested the presence of a common epitope(s) between the two molecules. In this paper we describe the identification of an IgE binding region in the first 30 amino acids of the major Pj pollen allergens and its characterization as a haptenic molecule. In particular, either deletion or serine substitutions of the cysteine residues at positions 14 and 29 suggested that these amino acids were essential for IgE binding. More interestingly, comparison sequence analysis and cross-inhibition experiments showed that this region contains a common epitope between the major Par j 1.0101 and Par j 2.0101 allergens (Fig. 4) and that the preincubation of a pool of sera from Pj allergic patients with the recombinant pPJ1 protein caused a decrease in binding of about 30% of all the IgE toward the 10 to 14 kDa major allergenic area (Fig. 5). It has also been shown that the 1 to 30 region contains only a single epitope capable of binding IgE; therefore, it is unable to trigger any release of histamine. A consequence of this property is that the 1 to 30 region is an IgE hapten and may be a useful therapeutic candidate, since it can specifically bind the IgE present on the basophil and mast cell membranes, thus preventing the binding of native allergenic molecules.

In conclusion, definition of the IgE epitopes of an allergenic molecule is a key step in the understanding of the allergic reaction, since they represent the effectors of the immune reaction. In particular, the Pj 1–30 IgE hapten can be used to reduce at least 30% of the allergenic response toward the Pj major allergens, supporting the idea that it might represent an important tool for the development of a therapy against the Pj-induced allergy.

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