

Der p 5 allergen from house dust mite: first epitope mapping of rabbit IgG blocking antibodies

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Abstract

Der p 5 is one of the important house dust mite allergens in Algeria; this allergen is frequently recognized by patients with allergic asthma. However, there is no information on its IgG-binding epitopes. In the present study, rabbits were immunized with recombinant Der p 5 allergen, and serum samples were obtained. Recognition of linear IgG epitopes of Der p 5 was determined using synthesized peptides derived from the allergen sequence. The results showed that serum from immunized rabbits recognized three linear epitopes from Der p 5 (²⁸EDKKHDYQNEFDLLMERIHEQIK⁴³), (³⁷IHEQIKKGELALFYLQEQ⁵⁵) and (⁹²LMQRKDLDFEQYNLEMAKKS¹¹²). More interestingly, we observed that the ⁹²L-S¹¹² amino acid sequence is well recognized by both IgE and IgG antibodies. Der p 5 stimulates the synthesis of specific IgG antibodies which recognize common but also novel epitopes compared to IgE antibody binding. Indeed, the potential to induce IgG antibodies can be used to inhibit human IgE binding to allergens which may be part of the mechanism of action of specific immunotherapy.

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Introduction

In Algeria we have demonstrated that 35% of house dust mite (HDM) patients showed IgE binding to Der p 5 allergen. It is interesting to note that, according to our recent studies, the prevalence of this allergen is steadily increasing [1]. In recent years it has become clear that recent advances in the study of mite biology, the molecular characterization of dust mite allergens and the determination of cross-reactivity among these different allergens have greatly facilitated the development of the best approaches for understanding, preventing and

controlling diseases caused by mites, including diagnostic methods and specific immunotherapy [2–4].

The HDM is globally ubiquitous in human habitats. The HDM is a common source of indoor allergens and a major cause of asthma worldwide [5]. Members of the *Dermatophagoides* genus, the HDM is a complex allergen source with more than 20 different reported allergen groups. Although Der p 1, Der p 2 and Der p 23 are reported as the major allergens, others, which are recognized by more than 50% of HDM-allergic patients in some population, are reported as midrange allergens, including Der p 5 and Der p 21 [6]. Group 5 and 21 HDM allergens were identified in *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Blomia tropicalis* and were shown in several studies to be important allergens with high allergenic activity [7–9].

Recently it has been reported that sensitization to Der p 5 is well recognized in children with asthma compared to children with only rhinitis. In addition, children with asthma are more frequently recognized as being allergic to several different HDM

allergens, including Der p 5, compared to children without asthma. Der p 5 is therefore a clinically important allergen.

It has been demonstrated that this allergen is poorly represented in commercialized allergen extracts used for diagnosing allergies [10]. It is therefore difficult to develop HDM allergy vaccines based on natural allergen extracts in order to protect HDM-allergic patients who are sensitized to these allergens. In order to solve this problem of the limitations of natural allergen extracts for HDM-specific immunotherapy, the use of molecular vaccines based on recombinant allergens, allergen derivatives or allergen-derived peptides is a powerful strategy [11–13]. Furthermore, it has been reported that the construction of these vaccines greatly depends on the determination of the antigenic sites of these allergens as well as on a detailed knowledge of IgE epitopes and T cells of the key allergens [14].

The three-dimensional structure of Der p 5 has been revealed and shows a three-helical bundle that can polymerize to create a hydrophobic cavity which could possibly be a ligand-binding site [15]. In addition, chronic exposure to Der p 5 occurs by inhalation and may lead to the production of IgE antibodies in susceptible atopic individuals. It is now well known that IgE antibodies bind to the receptor (termed FcRI) in the absence of antigen; in time, the receptor adopts antigenic specificity. Cross-linking of the receptor through antigen/antibody interactions leads to the initiation of a signal transduction cascade followed by the synthesis and release of many mediators of allergic response.

Interestingly, it has been shown that FcRI belongs to a family of antibody-binding receptors that also mediate interactions of soluble IgG antibodies with cells of the immune system [16,17]. Indeed, the interaction of the IgE antibody with the high-affinity IgE receptor (FcεRI) is the key step to these immune reactions and provides an attractive target for the inhibition of all IgE-mediated allergic disease [18]. Clinical studies of allergic individuals using anti-IgE monoclonal antibody therapy have shown that this is an effective approach to disease treatment [19]. More interesting, the IgG-Fc receptor is structurally similar to the IgE-Fc and FcεRIα in the complex and potentially could form analogous interactions [20]. In clinical studies it has been proved that fewer children with asthma showed IgG reactivity to HDM allergens than children without asthma [10].

In connection with this, interesting information comes from studies that evaluate early patterns of IgG responses and IgE sensitization (ratio IgG/IgE) to mite and other allergens, which suggest the existence of protection from allergy symptoms in individuals with the highest IgG/IgE ratios [21]. The potential role of IgG antibodies in tolerance acquisition has long been discussed; in particular, the protective or blocking functions of IgG subclass antibodies have been proposed [22,23].

The objectives of this study were to investigate the epitope mapping of IgG antibodies induced in rabbits after immunization with Der p 5 allergen to estimate the capacity of the generated IgG antibodies to block the binding of Der p 5 by human IgE antibodies.

Materials and methods

DNA subcloning, expression and purification of recombinant Der p 5

The complementary DNA insert of Der p 5 was generated by PCR using primers containing *Sna*BI and *A*vrII restriction sites. The DNA insert was ligated into the pJET plasmid (Invitrogen) and transformed into DH5α-competent cells. The complementary DNA of Der p 5 was subcloned into the pPIC9K expression vector.

The expression vectors with Der p 5 insert were subsequently transformed into *Pichia pastoris* SMD 1168 cells for expression of proteins. Cells were grown in BMGY (with 100 µg/mL ampicillin) until A600 reached 6–10. Protein expression was then induced with 0.05% methanol in 1 L of BMMY at 28°C for 48 hours. Protein was further purified using a resin cation exchange SPFF followed by a second separation by an S100HR HiLoad 16/60 Superdex 75 pg (GE Healthcare) gel filtration column and HiTrap QHR on AKTA Fast Protein Liquid Chromatography System (GE Healthcare). Protein concentration was determined by bicinchoninic acid test.

Rabbit immunization

Rabbits were immunized with purified Der p 5 allergen (Eurogentec) with 3 subcutaneous injections of 200 µg of protein per injection. The programme of immunization includes three bleeds and four injections, with day 0 the day of the preimmune bleed with the first injection, then on days 7, 10 and 18 the second, third and fourth injections, respectively. Medium bleed and final bleed were realized at days 21 and 28, respectively. Sera from rabbits immunized with each extract were kept at –20°C. Antibody purification was performed by means of the HiTrap Protein G column (GE Healthcare).

IgE binding by peptides derived from Der p 5

Thirty-four overlapping peptides, each of 15 residues, covering the whole sequence of Der p 5, were synthesized in the format biotin–SGSG–peptide–amide (JPT Peptide Technologies). One microgram of each peptide was coated on a Reacti-Bind streptavidin-coated high-binding-capacity clear 96-well plate (Nunc) at 37°C for 1 hour. Plates were washed with PBS-Tween 20 (PBS-T) three times and were blocked with PBS-T 0.1% bovine serum albumin. Plates were washed again and incubated

separately with purified IgG anti-Der p 5 of rabbit 5 (diluted 1:5000) for 2.5 hours at 37°C, the secondary antibody biotinylated anti-rabbit IgG conjugate and streptavidin–horseradish peroxidase. The reaction made by the tetramethylbenzidine (Sigma-Aldrich). The development of the colour reaction was stopped by adding H₂SO₄ 0.4 M. The optical density reading was made at 450 nm (Novapath–Bio-Rad).

Structure visualization

PyMOL software (<https://pymol.org/2/>) [24] was used to visualize the three-dimensional structures of the Der p 5 allergen.

Result

Production and purification of Der p 5 allergen

We produced a recombinant form of the mite allergen by molecular cloning using the *Pichia pastoris* expression system. The form produced of Der p 5 allergen was purified using chromatography methods. The purification of Der p 5 allergen was carried out in three stages; the containing fractions were analysed by sodium dodecyl sulfate–polyacrylamide gel

electrophoresis (SDS-PAGE). Fig. 1(A) shows the chromatogram corresponding to the last purification stage using the HiTrap QHR column. The purified allergen was analysed by SDS-PAGE. As indicated in Fig. 1(B), the band was present corresponding to the recombinant Der p 5 allergen with 14 kDa. By amino acid (Nt) sequencing and electrospray ionization and quadrupole time-of-flight mass spectrometry, we confirmed that this band corresponds to the mature form of the Der p 5 mite allergen (data not shown).

Induction of specific IgG

Rabbit immunized with purified Der p 5 allergen showed high titres of specific IgG antibodies (Fig. 2). The rabbit serum was diluted five times in 20 mM sodium phosphate buffer pH 7, using the highly specific IgG columns HiTrap Protein G HP Columns. Subsequently, the purity of the antibody was verified by SDS-PAGE gel analysis (Fig. 2(B)).

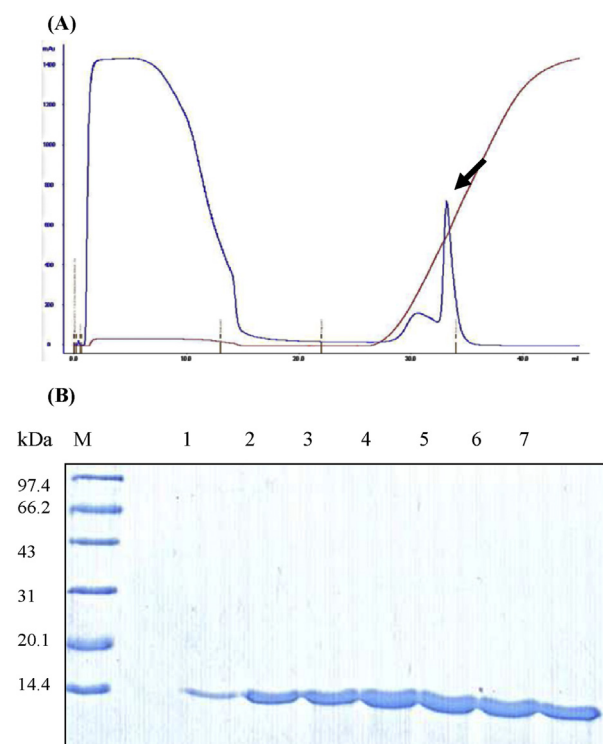


FIG. 1. Purification of recombinant Der p 5 protein. (A) Chromatogram of elution. (B) SDS-PAGE of purified protein using HiTrap QHR chromatography column. Lane M, molecular marker; lanes 1–7, proteins came from elution peak (arrow). SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

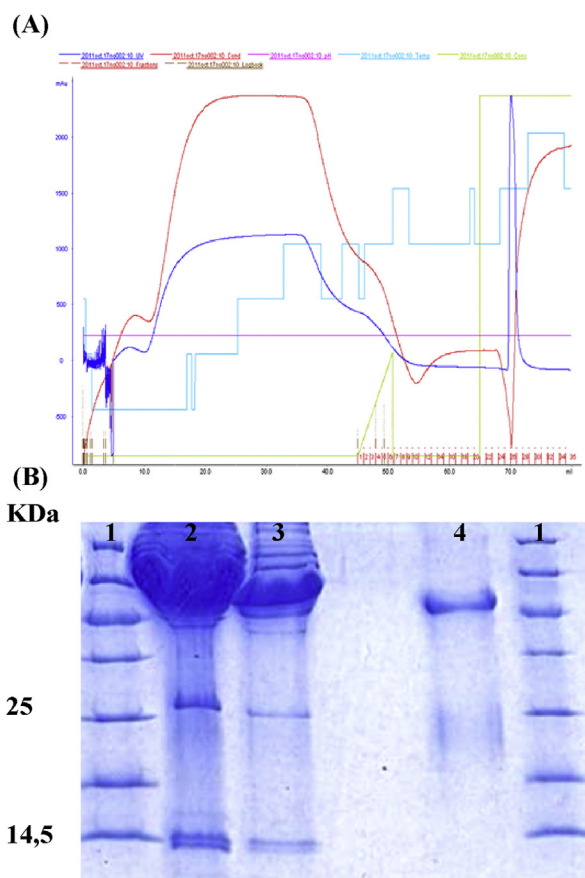


FIG. 2. (A) Purification of IgG-Derp5 antibody by using HiTrap Protein G HP columns. Chromatogram of elution. (B) SDS-PAGE analysis of purified antibodies. Lane 1, molecular marker; lanes 2–4, sera from immunized rabbit before purification, sera from immunized rabbit (dilution 5 ×) and purified IgG antibodies, respectively. SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Recognition of linear epitopes

To assess global epitope binding and to observe epitope-specific IgG production, 34 biotinylated peptides of Der p 5 were synthesized. These peptides were probed with serum samples obtained from rabbits immunized with purified Der p 5 allergen. Fig. 3 shows the results of the IgG binding by the different peptides; the peptides corresponding to the N-terminal region are important in the binding of IgG, predominately peptide 4. A second, less important region comprises peptides 8 and 9, and is also determined as an epitope region with a lower IgG binding intensity. Interestingly, the three peptides P25 (92 LMQRKDLDFEQYNL 106), P26 (95 RKDLDFEQYNLEMA 109) and P27 (98 LDIFEQYNLEMAKKS 112) are also highly recognized by the rabbit-IgG antibody. Thus, peptides P25, P26 and P27 were identified as having significantly greater IgG binding epitope than Der p 5. Furthermore, no reactivity was observed with these other peptides. For negative sera (unimmunized rabbit), no signal was scored for any peptide.

Discussion

Previous studies have documented that the 14 kDa allergen from HDM is an important HDM allergen which is frequently recognized by patients with allergic asthma [8,25]. The structure of recombinant Der p 5 has previously been determined [15]. The conformational epitope of Der p 5 was also determined [26]. In addition, the fine mapping of epitopes of Der p 5 was performed, and the amino acids involved in IgE binding were also determined in our laboratory. Indeed, the amino acid sequence (90 D-M 108) exhibited strong IgE binding; aromatic and hydrophobic residues are recognized as the most critical [1,27].

Many studies performed with recombinant allergens indicate that the induction of blocking IgG antibodies is an important mechanism in allergen-specific immunotherapy with recombinant allergens [28–30]. One of the first indications of successful immunotherapy in humans is the increase in antigen-specific

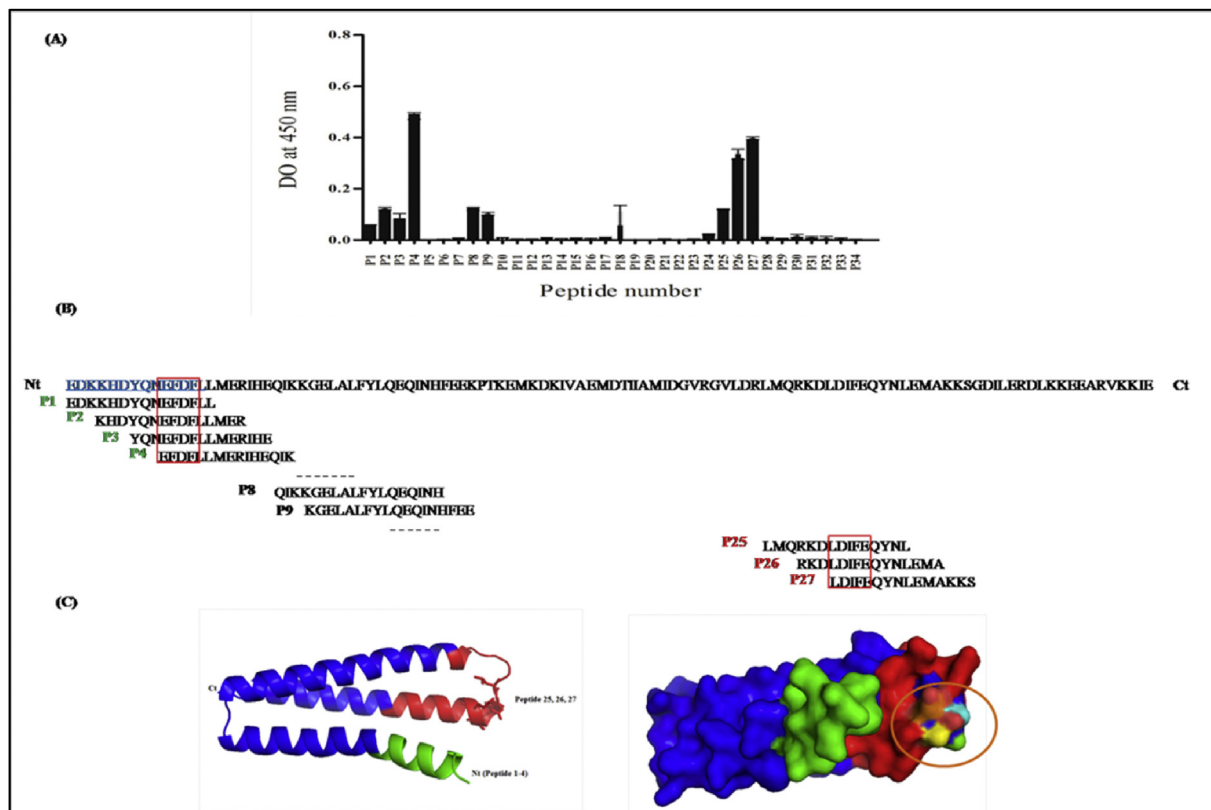


FIG. 3. Epitope mapping of Der p 5 rabbit IgG antibodies. (A) IgG reactivity (y-axis: mean optical density values with 3 standard deviations) of Der p 5-specific IgG and Der p 5 peptides 1 to 34 (x-axis). (B) Overlapping peptides. Letters in blue correspond to amino acids removed in construction of Mueller et al. [15]. Red and green boxes indicate regions at which IgG binding of peptide is of interest. (C) Localization of peptides epitopes in three-dimensional structure of Der p 5. Left images indicate ribbon representation of Der p 5 structure; right images, surface representations of Der p 5 structure, with peptides highlighted in different colours (P1–4, green; P25, P26 and P27, red). Most important sequence in IgG binding corresponds to sequence overlapped between these three sequences is represented in spectrum (orange circle).

IgG levels, which competes and blocks IgE binding to the allergen [31]. To our knowledge, no studies have focused on the IgG binding epitopes of Der p 5 allergen to better understand the molecular basis of this blocking ability against IgE antibodies. Indeed, this study is the first to map rabbit IgG epitopes of this important allergen.

Recombinant peptide libraries and overlapping peptides are two important methods to locate the linear epitopes, but using overlapping peptides seems more promising. In the present study, we synthesized 34 overlapping peptides of Der p 5, and we located three IgG-binding regions corresponding to Der p 5. In fact, we found that IgG antibodies induced by immunization of rabbits with Der p 5 allergen not only recognized the Der p 5 wild-type (WT) allergen but also linear epitopes.

IgG antibodies have been proposed to act as blocking antibodies. Our data support this concept by showing that IgG antibody binding patterns were globally similar to that of IgE (Fig. 3), with P1–4: E28–K43 (²⁸EDKKHDYQNEFDLLMER-IHEQIK⁴³), P8–9: I37–Q55 (³⁷IHEQIKKGELALFYLQE⁵⁵) and P25–27: L92–S112 (⁹²LMQRKDLDFEQYNLEMAKKS¹¹²).

These observations support the understanding of the competitive blocking potential of IgG-Der p 5 observed in previous studies. Indeed, it has been reported that specific polyclonal rabbit IgG antibodies induced by rabbit immunization with Der p 5 allergen are able to block the binding of allergic patients' IgE to Der p 5. In the majority of tested patients, anti-Der p 5 IgG strongly inhibited IgE binding to Der p 5 [8]. In a study that aimed to investigate the IgG mapping of Der p 5 as a potential producer of blocking antibodies, specific IgG antibodies were raised in rabbit by immunization with WT Der p 5, and inhibition of human IgE binding to WT Der p 5 was assessed by enzyme-linked immunosorbent assay [26]. Rabbit IgG antibodies against WT Der p 5 showed almost complete inhibition of human IgE binding to Der p 5 at more than 90% inhibition.

Interestingly, the Nt-IgG epitope determined in this study (²⁸EDKKHDYQNEFDLLMERIHEQIK⁴³) is a potential rabbit IgG epitope (Fig. 3), but it showed little IgE binding to simple sera (unpublished data). Coincidentally, Mueller et al. [15], who designed a construct of Der p 5 encoding residues Leu³⁴-Val¹³² for Der p 5, sought to reduce the number of disordered residues for crystallization. The prevalence of IgE antibody binding to the shorter Der p 5 construct used by Mueller et al. is the same as reported for the full-length allergen, indicating that this construct has the same ability to binding IgE antibodies compared to complete protein. This allows us to suggest that IgE antibodies are not directed against the flexible N-terminal residues of Der p 5, which are absent in the shorter construct.

In our study, two important regions for antibody binding were determined the Nt of the protein and the 90 to 108 aa

sequence. The results of Mueller et al. [15] implied that the Nt sequence is not important for IgE binding; removal of 34 residues from this part of the protein does not seem to change the allergenicity of the allergen. It is then suggested that the ⁹⁰D-M¹⁰⁸ sequence is the most robust epitope region.

It may therefore be expected that immunotherapy of HDM-allergic patients with Der p 5 will also induce a robust Der p 5-specific and protective (blocking) IgG response. Indeed, the Der p 5 molecule described may be a useful tool for the diagnosis and specific immunotherapy of HDM allergy.

We also demonstrated that the protein sequence ⁹⁰D-M¹⁰⁸ (corresponding to the sequence of P25, P26 and P27) has an overall conservation and forms the IgE epitope region for group 5 and 21 structurally similar allergens, which exhibit low or no IgE cross-reactivity (unpublished data). It will be important to design a mosaic protein which carries the epitope sequences of amino acid ⁹⁰D-M¹⁰⁸ allergens of groups 5 and 21 in order to generate a vaccination protein because these allergens do not have any cross-reactivity between them. Furthermore, our results indicate the presence of sequences that are not recognized by antibodies; these sequences are also important for the generation of these vaccines. In addition, recent studies have clinically demonstrated the efficacy of this type of vaccine [14,32–34].

In summary, we assume that the protective effect of the IgG response induced by Der p 5 is due to its polyclonal nature, thus recognizing more epitopes; more interestingly, the most important epitope is also recognized by IgE antibodies. Our work will therefore be important for the rational engineering of recombinant epitope-based vaccines for group 5 and 21 allergens based on 90 to 108 aa sequence.

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Conflict of interest

None declared.

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