

Cross-Reactivity between Major IgE Epitopes of Family 5 Allergens from *Dermatophagoides pteronyssinus* and *Blomia tropicalis*

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Keywords

Allergen · House dust mite · Cross-reactivity · Peptide epitope

Abstract

Background: The aim of this work was to understand the molecular features that trigger the cross-reactivity observed between Der p 5 from *Dermatophagoides pteronyssinus*, Blo t 5 from *Blomia tropicalis*, and Der f 5 from *D. farinae*. **Methods:** We collected serum from 60 house dust mite (HDM)-allergic patients residing in the Dellys area of Boumerdes province in northern Algeria. The presence of specific IgE to Der p 5, Der f 5, and Blo t 5 was analyzed. We performed in silico analysis of the structure of the different allergens in order to identify epitopes that can elicit the cross-reactivity of the sera. Synthetic peptides corresponding to the linear epitope sequence of Der p 5, Der f 5, and Blo t 5 were used to evaluate its implication in the cross-reactivity between the allergens. We also modified the sequence of the conformational epitope of Der p 5 by site-directed mutagenesis to mimic Blo t 5. **Results:** Several sera of patients allergic to HDM contained specific IgE antibodies to Der p 5 and Blo t 5. We demonstrated that the linear epitope of Der p 5 and Blo

t 5 is not involved in the cross-reactivity of the sera. Furthermore, mutations introduced in the sequence of Der p 5 to mimic Blo t 5 could not modulate the cross-reactivity between them. **Conclusions:** The major linear IgE epitopes of Der p 5 and Blo t 5 are involved in species-specific recognition. Our results may be useful for the development of a hypoallergenic vaccine against HDM group 5 allergens.

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Introduction

House dust mite (HDM) species, i.e., *Dermatophagoides pteronyssinus*, *D. farinae*, and *Blomia tropicalis* are dominant sources of allergens worldwide [1–3]. Up to 50% of allergic patients are sensitized to these allergens [4]. HDM allergens are classified into 33 different groups. Among them, Der p 1 and Der p 2 are two major allergens from *D. pteronyssinus* with IgE-binding frequencies >80% [5].

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Group 5 allergens have been isolated from several species of dust mites such as *B. tropicalis* (Blo t 5), *D. pteronyssinus* (Der p 5), *D. farinae* (Der f 5), *Lepidoglyphus destructor* (Lep d 5), and *Suidasia medanensis* (Sui 5 m) [6–10]. Several studies show the importance of group 5 allergens in respiratory diseases [9, 11–14]. Others show that Der p 5 is strongly related to the development of asthma in allergic patients [3, 15, 16] and that significantly higher concentrations of specific IgE (sIgE) to Der p 5 can be observed in asthmatic patients. Furthermore, Der p 5 is secreted in very small quantities, and the number of mite-allergic patients specifically recognizing Der p 5 is disproportionately high considering its low expression level [5]. On the other hand, sensitization to *B. tropicalis* is strongly associated with allergic diseases where Blo t 5 is the major allergen [17–19].

Different studies have investigated the cross-reactivity between allergens from family 5. A low-to-moderate cross-reactivity profile between Der p 5 and Blo t 5 has been shown [17, 20, 21], suggesting that they are species-specific. Moreover, a low cross-reactivity was observed between the different known group 5 and group 21 allergens [22, 23]. Interestingly, Reginald et al. [24] showed significant cross-reactivity between Sui m 5 and Blo t 5 and between Sui m 5 and Der f 5.

Among the allergens from family 5, the epitopes of Blo t 5 and Der f 5 have been experimentally characterized using monoclonal antibodies (mAbs), peptide array, or site-directed mutagenesis [25–28]. The conformational epitope II of Blo t 5 (PBD code 2JMH [27]) is composed of the 4 major residues N62, K65, K70, and R73 (allergome database numbering) located at the end of helix-2 and the beginning of helix-3. The linear epitope I, meanwhile, has 4 key residues (E92, D97, E102, and E107) located in the region surrounding the turn region connecting helix-2 and helix-3 and also on helix-3 [26]. It has been reported that the low cross-reactivity between Blo t 5 and Der p 5 may be explained by the low sequence identity between the epitopes of these 2 allergens.

We investigated the implication of the IgE epitopes in the cross-reactivity between the allergens of family 5. The cross-reactivity of the linear epitope I has been studied by the synthesis of peptides spanning this sequence region in Blo t 5, Der p 5, and Der f 5. As the cross-reactivity of the conformational epitope II cannot be studied by peptide synthesis, we used mutagenesis to produce a mutated Der p 5 form with the same major epitope residues as in Blo t 5.

Materials and Methods

Sequence Alignment of Allergens from Group 5

Sequences of Der p 5, Der f 5, Blo t 5, and Sui m 5 were retrieved from the Uniprot database (<http://www.uniprot.org>) [29]. Their accession codes are P14004, A71ZE9, O96870, and B2GM87, respectively. Clustal Omega [30] was used to perform the multiple sequence alignment.

Structures Visualization

PyMOL software (<http://pymol.sourceforge.net/>: [31]) was used to visualize all the 3-dimensional (3D) structures of the studied allergens.

DNA Subcloning and Site-Directed Mutagenesis

DNA inserts of Der p 5 and Blo t 5 were generated by PCR, using an upstream oligonucleotide primer containing an *Sna*BI restriction site and a downstream oligonucleotide primer containing an *Avr* II restriction site. The DNA insert was ligated into a pJET plasmid (Invitrogen) and transformed into DH5α competent cells. PCR screening was performed, and the sequence of the insert was confirmed by DNA sequencing (GIGA, Belgium). The complementary DNA of Der p 5 and Blo t 5 was subcloned into a pPIC9K expression vector and transformed into *E. coli* DH5α cells.

The multiple sequence alignment of group 5 allergens was used to identify residues for site-directed mutagenesis. Mutant constructs were obtained by PCR-overlapping extension using designed oligonucleotide primers (1st BASE) with mismatches. Multiple mutants were generated using the mutated plasmids as a template.

Expression and Purification of Recombinant Proteins

The expression vectors with Der p 5 inserts were subsequently transformed into *Pichia pastoris* SMD 1168 cells for the expression of proteins. Cells were grown in 1,000 mL of BMGY supplemented with 100 µg/mL ampicillin until A600 reached 6. Protein expression was then induced with 0.05% of methanol in 1,000 mL of BMMY at 28 °C for 48 h. Protein was further purified using a resin cation exchange SPFF, followed by a second separation by means of a S100HR HiLoad 16/60 Superdex 75 (GE Healthcare) gel filtration column on an AKTA fast protein liquid chromatography system (GE Healthcare). Protein concentration was determined by BCA.

Circular Dichroism

Circular dichroism (CD) experiments were conducted with 15 µM of protein in phosphate buffer at room temperature. CD spectra were acquired with a Jasco J-810 spectropolarimeter using a Hellma quartz cuvette with a 1-mm path length. The spectra were recorded at a wavelength range of 195–240 nm with 0.2-nm resolution using a scan speed of 50 nm/min and then averaged for 5 scans. Each spectrum was corrected by subtracting the buffer signal.

Patients' Sera and Specific IgE Quantification

Sera from 60 patients with allergic symptoms were collected at the Dellys Hospital (northeast of Algiers). Clinical history was recorded, and a physical examination, skin prick tests, and blood tests for total IgE levels were performed as part of standard clinical care (Table 1). Sera from 10 healthy volunteers were used as con-

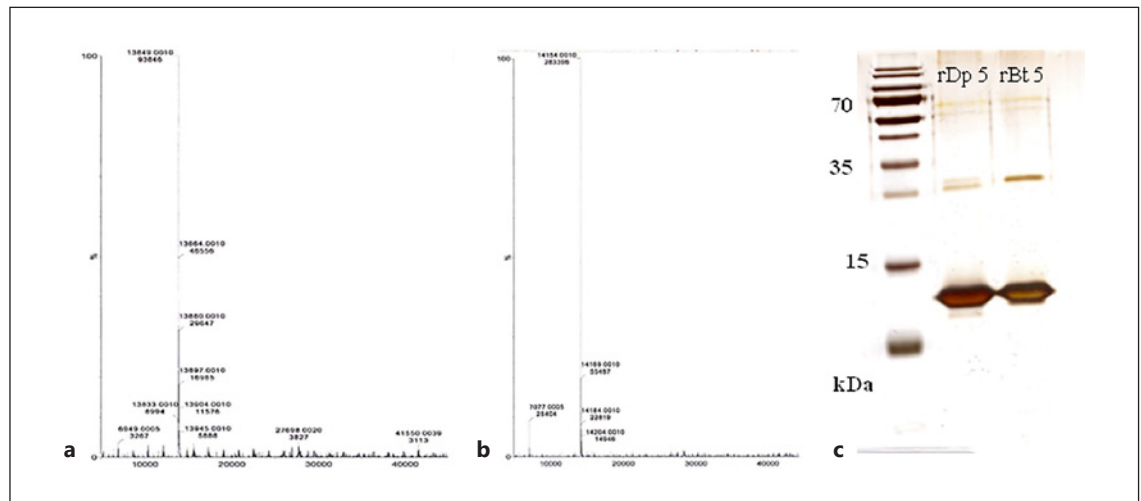


Fig. 1. Characterization of purified recombinant (r)Der p 5 (rDp 5) and rBlo t 5 (rBt 5) produced in *Pichia pastoris*. **a, b** Mass spectroscopic analysis of rDer p 5 and rBlo t 5, respectively, obtained using nano-ESI-Q-ToF MS. **c** Silver-stained SDS-PAGE containing a molecular weight marker (lane 1) and purified allergens (lane 2: rDer p 5; lane 3: rBlo t 5).

controls in this study. Total IgE was measured by ImmunoCAP. Written informed consent was obtained before enrollment. Serum was aliquoted into 2 samples. These were stored frozen (-20°C) until use.

sIgE-Binding ELISA Experiment

To identify the sera containing sIgE anti-Der p 5, sera from patients were diluted 1/10 with 1% BSA in PBS. Recombinant (r)Der p 5 was coated overnight at 4°C into Maxisorp ELISA plate (Nunc) at 300 ng protein/well in volumes of 100 μL . Each well was washed 3 times with 200 μL of PBS-T (PBS and 0.1% Tween 80) and blocked with 200 μL of PBS-1% BSA for 1 h at 37°C . Plates were then washed with PBS-T and incubated with 100 μL of the diluted sera solution for 1 h. The plates were washed with PBS-T and then incubated for 1 h at 37°C in the presence of 100 μL of alkaline phosphatase (AP)-conjugated anti-human IgE mAb (Southern Biotechnology) diluted 1:3,000 in PBS-1% BSA.

After washing 3 times with PBS-T, 100 μL of the pNPP substrate (Sigma-Aldrich) was added. After 30 min of incubation of the solution, 40 μL of sodium hydroxy disulfuric acid 3 N was added to stop the reaction. Absorbance measurements were conducted at 405 nm using an ELISA plate reader. All experiments were repeated separately and results were reported as mean values. IgE values were considered positive if equal to or larger than the cut-off calculated as the arithmetic mean of negative controls plus 3 times the standard deviation.

IgE-Binding by Peptides Derived from Der p 5, Der f 5, and Blo t 5

Three peptides corresponding to the linear epitope sequence region 90–108 of Der p 5, Blo t 5, and Der f 5, and 7 corresponding to the mutated region in Der p 5 were synthesized. They are in the “biotin-SGSG-peptideamide” format (JPT Peptide Technologies, Berlin, Germany). One microgram of each peptide was coated on

Table 1. Features of the patients ($n = 60$) included in the cohort of sera used in this study

Variable	<i>n</i> (%)
Gender	
Male	28 (48)
Female	32 (52)
Age	
<20 years	19 (32)
20–30 years	17 (28)
30–40 years	14 (23)
>40 years	10 (17)
Diagnosed with asthma	
Yes	37 (62)
No	23 (38)
Allergic rhinitis	
Yes	53 (88)
No	7 (12)
Skin test	
Positive	55 (92)
Negative	5 (8)
Sera anti-Der p 5 IgE	14 (23)
Sera anti-Blo t 5 IgE	11 (18)

a Reacti-Bind streptavidin-coated, high-binding-capacity, clear 96-well plate at 37°C for 1 h. Plates were washed 3 times with PBS-T and blocked with PBST-0.1% BSA. Plates were washed again and incubated separately with sera (1/5 dilution in PBS) from 10 patients for 150 min at 37°C . Subsequent incubation with Abs and colorimetric development was performed as described above.

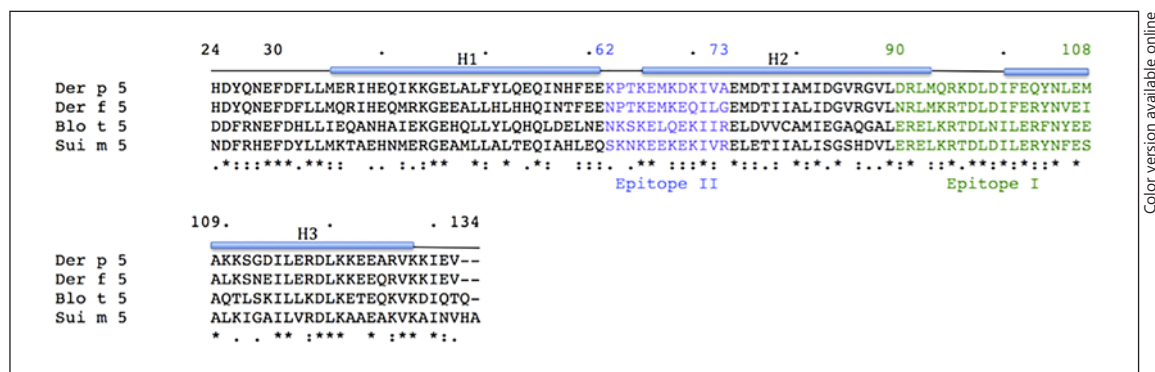


Fig. 2. Multiple sequence alignment of Der p 5, Der f 5, Blo t 5, and Sui m 5. The three α -helices are referenced as H1, H2, and H3. Residues from epitopes I and II of Blo t 5 and their homologs in the 3 other allergens appear in green and blue, respectively.

Results and Discussion

Production of Recombinant Allergens and IgE-Binding Profiles of Sera from Allergic Patients

The purified rDer p 5 and rBlo t 5 allergens were analyzed by SDS-PAGE and ESI-MS Q-TOF (Fig. 1a–c). We confirmed the expected molecular mass of the mature form of Der p 5 (13,489 Da) and Blo t 5 (14,154 Da). The silver staining of the electrophoresis gel indicated the presence of a minor band at an apparent molecular weight of 30 kDa, suggesting that the proteins can also aggregate into dimers.

The recombinant proteins were used to detect the presence of potential IgE. From the positive sera, the presence of sIgE antibodies against rDer p 5 or rBlo t 5 was found in 14 and 11 samples, respectively. Interestingly, 4 sera (S24, S40, S45, and S58) contained IgE against both tested allergens. Our results showed that *D. pteronyssinus* and *B. tropicalis* are involved in the development of HDM allergy [5]. The prevalence of anti-Der p 5 and anti-Blo t 5 IgE in the cohort of sera from the HDM-allergic patients located in the Dellys region, northern Algeria, was 23 and 18% respectively. We also confirmed that the allergens from family 5 are minor allergens (as described in [5]).

Sequence Alignment of Allergens from Group 5 and Conservation of Epitope Residues

We aimed to determine the molecular features of the family 5 allergens that could favor cross-reactivity. King and Spangfort [32] suggested that sequence identities >70% are required to induce significant cross-reactivity between homologous allergens. This has been confirmed for allergens from group 1 (Der p 1, Der f 1, and Eur m 1)

[33], group 2 (Der p 2, Der f 2, and Eur m 2) [34], and group 10 (Der p 10 and Lep d 10) [35].

In order to analyze the potential cross-reactivities in family 5, we first performed a multiple sequence alignment (see Material and Methods) between allergens from this family (Der p 5, Der f 5, Blo t 5, and Sui m 5) [26, 27]. This alignment revealed that Der p 5 and Der f 5 share a sequence identity of 77%. We also observed that the residues involved in the linear epitope I are better conserved than those of the conformational epitope II (Fig. 2). The major residues of these epitopes are E92, D97, E102, and E107 (linear epitope I) and N62, K63, K70, and R73 (conformational epitope II) (Fig. 2). Three of the four major residues of Blo t 5 epitope I (D97, E102, and E107) are conserved in the sequence of the 4 allergens. On the other hand, the sequence conservation of the major residues of Blo t 5 epitope II is low. Only K70 is strictly conserved in 3 allergens, while N62, K63, and R73 are conserved in 2 allergens. However, until now, no experimental measurement of the cross-reactivity between Der p 5 and Der f 5 had been tested.

Note that the greater cross-reactivity between Blo t 5 and Sui m 5 [24] is in agreement with the good conservation of the major residues of epitopes I and II between these 2 sequences. Low Blo t 5/Der p 5 and Blo t 5/Der f 5 cross-reactivities were measured, which could be explained by conservation of 3 of the 4 major residues of epitope I and almost no conservation of the major residues of epitope II [17, 20, 21].

In the next sections, we analyze the involvement of epitopes I and II in the possible cross-reactivities between Der p 5, Der f 5, and Blo t 5. The sequence alignments were used to design possible mutations that could modify cross-reactivity.

Table 2. Reactivity of the sera against the mature recombinant allergens (rDer p 5 and rBlo t 5) and the peptides corresponding to epitope I of Der p 5 (pDer p 5), Blo t 5 (pBlo t 5) and Der f 5 (pDer f 5), respectively

Sera	A ⁴⁰⁵				
	Recombinant allergens		Peptides “epitope I”		
	rDer p 5	rBlo t 5	pDer p 5	pBlo t 5	pDer f 5
S5	0.08±0.001	0.18±0.001	–	0.117±0.009	–
S6	0.07±0.001	3.10±0.100	–	0.168±0.001	–
S10	0.08±0.001	0.86±0.001	–	0.344±0.001	–
S12	0.08±0.001	0.34±0.004	–	0.08±0.003	–
S21	0.07±0.001	0.22±0.001	–	–	–
S31	0.07±0.001	0.11±0.001	–	–	–
S60	0.07±0.001	0.12±0.001	–	0.104±0.001	–
S15	0.22±0.001	0.08±0.001	0.115±0.002	–	0.085±0.002
S17	0.23±0.002	0.08±0.001	0.182±0.001	–	0.083±0.001
S19	0.59±0.003	0.08±0.001	0.322±0.001	–	0.082±0.001
S25	0.13±0.001	0.07±0.001	0.078±0.003	–	0.077±0.001
S32	0.10±0.001	0.08±0.002	–	–	–
S35	0.13±0.001	0.08±0.001	0.107±0.001	–	0.076±0.001
S41	0.25±0.001	0.08±0.001	–	–	–
S54	0.15±0.001	0.08±0.001	–	–	–
S57	0.18±0.002	0.08±0.001	0.117±0.004	–	0.082±0.002
S59	0.11±0.001	0.08±0.001	–	–	–
S24	0.18±0.001	0.42±0.001	0.13±0.002	0.079±0.002	0.087±0.003
S40	0.42±0.004	0.87±0.008	0.082±0.001	0.082±0.004	0.076±0.002
S45	0.35±0.004	0.42±0.001	0.203±0.004	0.113±0.001	0.078±0.001
S58	1.78±0.005	3±0.01	1.259±0.001	0.362±0.002	1.719±0.005

The absorbance at 405 nm (A⁴⁰⁵) was measured in triplicate for each serum (– = not determined). A positive signal (written in bold) is reached if A⁴⁰⁵ > 0.1.

Importance of the Linear Epitope I of the Group 5 Allergens

We synthesized peptides that cover the sequence region of the linear epitope I (90–108) of Der p 5, Blo t 5, and Der f 5, and searched for IgEs that recognize them. As shown in Table 2, 80 and 67% of the peptides corresponding to epitope I of Der p 5 (P20) and Blo t 5 (P9), respectively, were recognized by sera displaying anti-Der p 5 IgE and anti-Blo t 5 IgE. Most of the IgEs to Der p 5 or Blo t 5 that are present in the sera bind the peptide that correspond to epitope I. More interestingly, seras 45 and 58 displayed IgE that recognized both pDer p 5 (P20) and pBlo t 5 (P9).

We then decided to check the potential interaction between the peptide of epitope I of Der f 5 (pDer f 5) and our sera. Recently, Cui et al. [28] determined, by means of peptide array, that the region 90–108 corresponds to a major epitope region of Der f 5. A single serum (S58) recognized the peptide from Der f 5, meaning that there is no cross-reactivity between Der p 5 or Blo t 5 and Der f 5

through this sequence region (Table 2), although Der p 5 and Der f 5 share a high sequence identity. This suggests that *D. farinae* is also maybe not endemic to this region of Algeria. On the other hand, this sequence region could be a promising candidate for immunotherapy of HDM-allergic patients against family 5 allergens.

The data obtained by Miyaji et al. [36] show that antibodies can bind to the original epitope but also to sequences that may have several different amino acids. We therefore synthesized mutated pDer p 5, with the aim of increasing the cross-reactivity between Der p 5 and Blo t 5. We introduced some charged residues of the Blo t 5 epitope I sequence into pDer p 5 (Table 3). Our results demonstrated that these substitutions are insufficient to increase the cross-reactivity between these allergens. The sIgE anti-Blo t 5 did not recognize the tested peptide, except for P9 that corresponds to pBlo t 5 (Fig. 3a). These data suggest that the key amino acid for the interaction between IgE and Blo t 5 is not present in these peptides.

Fig. 3. IgE reactivity of wild-type and mutated peptide sequence 90–108 from Der p 5 and Blo t 5. P9 and P20 are the wild-type sequence of Blo t 5 and Der p 5, respectively. See Table 3 for the sequence of the peptides. All peptides were tested individually with 4 sera with sIgE to Blo t 5 (S5, S6, S10, and S60) (**a**) and 4 sera with sIgE to Der p 5 (S17, S19, S24, and S57) (**b**). The peptides P9 and P20 (derived from Blo t 5 and Der p 5 allergens, respectively) were used as a positive control with 100% IgE-binding in tests (**a**) and (**b**), respectively. Experiments were performed in triplicate, and data are expressed as mean values $\pm$ SD from triplicate determinations.

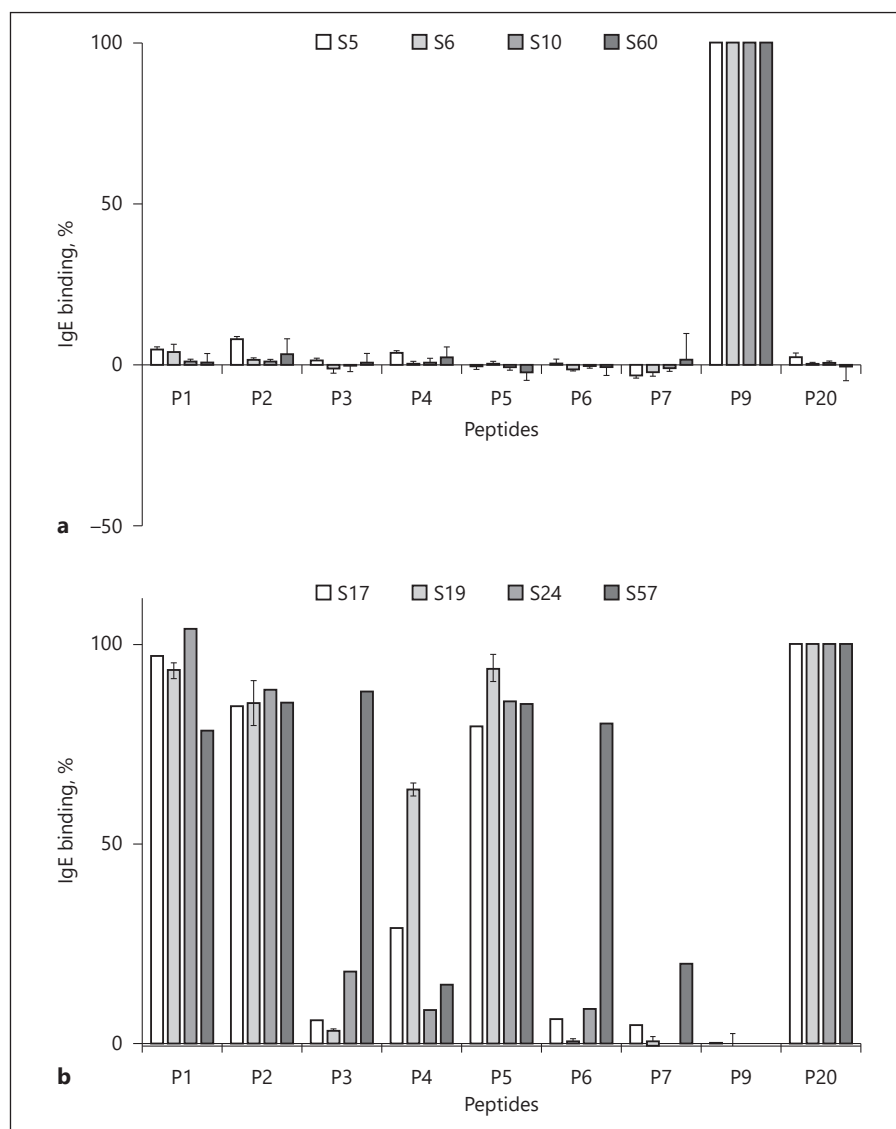
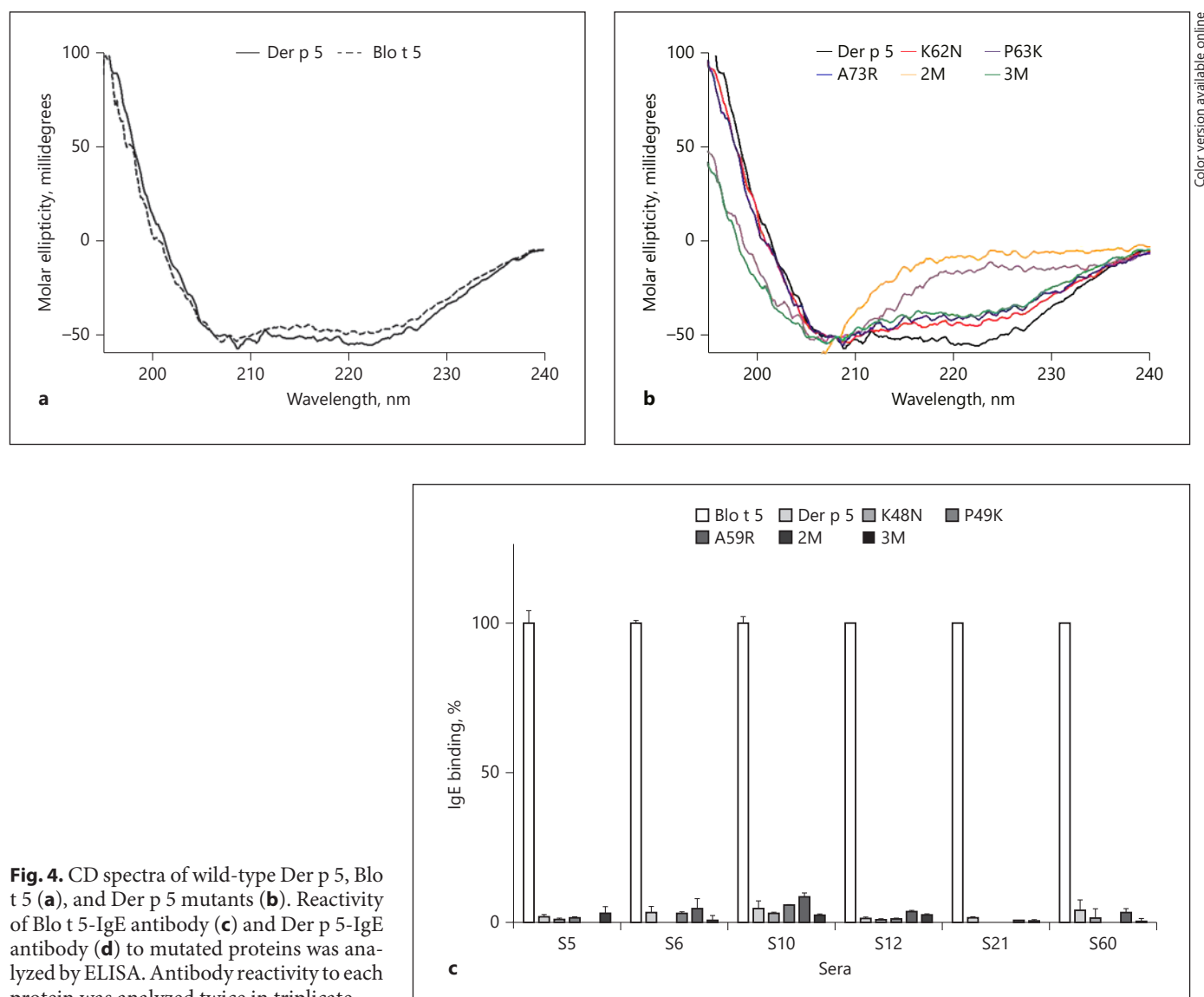


Table 3. Native (P20) and mutated sequences of Der p 5 epitope I

Peptide	Epitope I	Sequence
P20	WT (pDer p 5)	D ₉₀ RLMQRKDLDIFEQYNLEM ₁₀₈
P1	L92E	D ₉₀ REMQRKDLDIFEQYNLEM ₁₀₈
P2	Q94K	D ₉₀ RLM K RKDLDIFEQYNLEM ₁₀₈
P3	Q103R	D ₉₀ RLMQRKDLDIFERYNLEM ₁₀₈
P4	M108E	D ₉₀ RLMQRKDLDIFEQYNLE E ₁₀₈
P5	L92K-Q94K	D ₉₀ REM K RKDLDIFEQYNLEM ₁₀₈
P6	L92E-Q94K-Q103R	D ₉₀ REM K RKDLDIFERYNLEM ₁₀₈
P7	L92E-Q94K-Q103R-M108E	D ₉₀ REM K RKDLDIFERYNLE E ₁₀₈
P9	pBlo t 5	E ₉₀ RELKRTDLNILERFN Y EE ₁₀₈
P8	pDer f 5	N ₉₀ RLMKRTDLDIFERYN V EI ₁₀₈

The sequences of peptides P9 and P8 correspond to epitope I of Blo t 5 and Der f 5, respectively. The positions of the amino acid substitutions of pDer p 5 are indicated in bold and underlined.



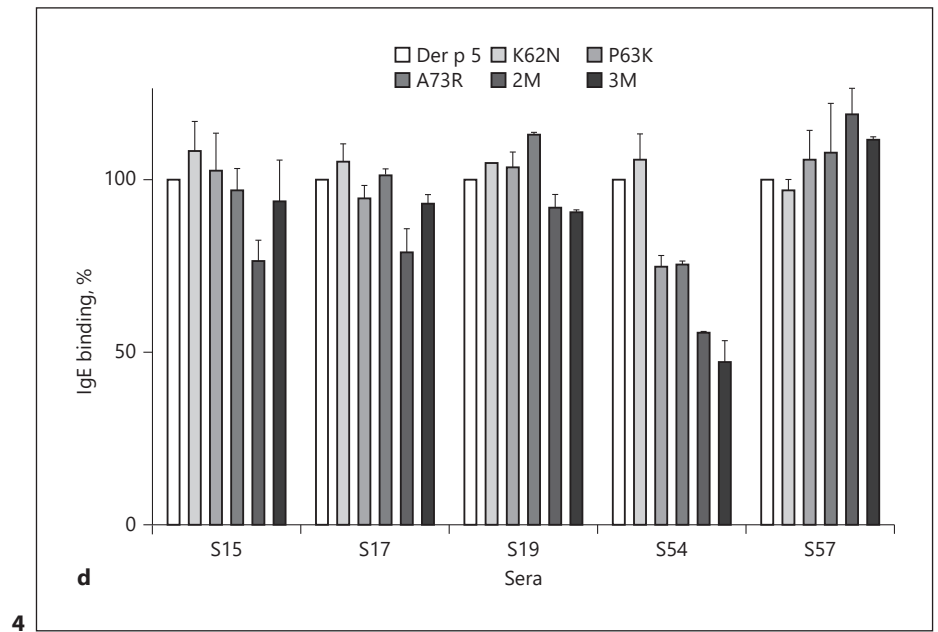
(Figure continued on next page.)

The impact of the mutations for the binding of IgE to Der p 5 was also studied (Fig. 3b). We observed that the single mutations of L92E (P1) and Q94K (P2) did not change the IgE-binding capacity when compared to pDer p 5 (P20). The same result was obtained for the double mutant L92E-Q94K (P5). In the case of the substitution Q103R (P3) and M108E (P4), fewer interactions with the anti-Der p 5 IgE were detected. Both residues seem to play a major role in epitope I and the allergenicity of Der p 5 (Fig. 3b).

According to these results, the amino acid sequence 90–108 is a linear IgE epitope in all 3 allergens, but the key residues for the IgE-binding differed in each allergen.

Site-Directed Mutagenesis of the Der p 5 Conformational Epitope II

The comparison between the sequence of Der p 5 and Blo t 5 revealed that epitope II contains 4 conserved amino acids (K65, E66, K70, and I71; Fig. 2). We produced, by site-directed mutagenesis, 5 Der p 5 variants, namely K62N, P63K, A73R, K62NP63K, and K62NP63KA73R. The proteins were produced and purified to homogeneity as described for the wild-type protein. The influence of the mutations on the secondary-structure content of Der p 5 was analyzed by far UV CD (Fig. 4a, b). As expected, the CD spectra of the native Der p 5 and Blo t 5 are in agreement with α -helical secondary structures; they



show 2 minima, at 208 and 222 nm. The comparison of this spectrum with those recorded for the Der p 5 mutants indicated that the P63K and K62NP63K mutations resulted in the denaturation of the protein. For the other mutants, the increase of the molar ellipticity values at 222 nm can be related to a loss of the α -helical structure. These mutations appear to be structurally destabilizing. This could suggest the importance of these residues for the stability and correct folding of this allergen.

We measured IgE-binding by the different mutants using sera with sIgE to Blo t 5 only, with no sIgE to Der p 5 (Fig. 4c). The tested mutants did not react with the sera. The proposed mutations were unable to increase the cross-reactivity between these allergens. On the other hand, as shown in Figure 4d, Der p 5 mutants showed an important capacity for IgE-binding.

Conclusion

We showed that Der p 5 and Blo t 5 are important allergens in Dellys in northeastern Algeria. We determined that Der f 5 possesses a linear epitope (epitope I), located in the same region as in Der p 5 and Blo t 5. It is a species-specific epitope, since we were not able to increase the allergen's cross-reactivity using site-directed mutagenesis of epitope I. The different amino acid substitution within epitope II modified the native structure of the allergen; as

a consequence, the importance of this epitope was not evaluated. These results highlight the essential structural function of the first β -loop and the N-terminal sequence of the second α -helix of Der p 5, and, more generally, of all the allergens belonging to family 5.

Finally, we can conclude that (i) the modification of the epitope I sequence is a promising strategy to generate the specific hypoallergens of family 5, and (ii) allergen cross-reactivity is not mediated by the known epitopes. Although we cannot exclude the presence of not-yet-identified minor epitopes in type 5 HDM allergens, the study performed by El Fekih et al. [37] showed that the prevalence of sensitization to *B. tropicalis* in Algeria concerns 25% of the tested patients. This could lead to cosensitization as well as the cross-reactivity detected in our study.

Disclosure Statement

The authors have no conflict of interest to declare.

Author Contributions

L.S., D.G., and M.-E.D. designed the experiments. L.S., B.I., D.G., and M.G. analyzed the data and drafted the manuscript. M.-E.D. helped L.S. conduct the experiments. D.G. and M.G. assisted in data analysis and editing of the manuscript. All authors read and approved the final manuscript.

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