

ORIGINAL ARTICLE

Immunological characterization of a Blo t 12 isoallergen: identification of immunoglobulin E epitopes

J. Zakzuk^{*,†}, S. Jiménez^{*,†}, N. Cheong[‡], L. Puerta^{*,†}, B. W. Lee[§], K. Y. Chua[§] and L. Caraballo^{*,†}

^{*}Institute for Immunological Research, University of Cartagena, Cartagena, Colombia, [†]Fundemeb, Foundation for the Development of Medical and Biological Sciences, Cartagena, Colombia, [‡]Bioprocessing Technology Institute, #06-01 Centros, Singapore and [§]Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

Clinical & Experimental Allergy

Summary

Background Differences in the IgE response to isoallergens could have clinical implications; therefore, its analysis will contribute to the design of better strategies for the diagnosis and treatment of allergic respiratory diseases. Several isoforms have been described from mites but there is no information about the clinical impact of *Blomia tropicalis* isoallergens.

Objective To evaluate the differences in the IgE response against two Blo t 12 isoallergens.

Methods The IgE-binding properties of Blo t 12 isoallergens were analysed by ELISA, a skin prick test and ELISA cross-inhibition. Epitope mapping was performed using synthetic overlapping peptides. Fold recognition methods were used to model the chitin-binding domain of the two isoallergens.

Results The frequency and strength of the IgE response were greater for Blo t 12.0101 than for Blo t 12.0102. Three IgE-binding areas were identified in Blo t 12.0101; one of them included two residues that are different in Blo t 12.0102. Modelling of the chitin-binding domains of these allergens predicted that they have structural differences that could influence antibody recognition of one of these epitopes.

Conclusion *In silico* structural analysis and immunological characterization of Blo t 12 reveals that allergen polymorphism influences IgE reactivity. Blo t 12.0101 is the most IgE-reactive isoform in Cartagena. The identified IgE epitopes could be mutated to obtain hypoallergenic molecules of potential use for immunotherapy.

Keywords Blo t 12, *Blomia tropicalis*, epitope mapping, IgE, isoallergens

Submitted 27 August 2008; revised 30 October 2008; accepted 2 December 2008

Correspondence:

Luis Caraballo, Institute for Immunological Research, University of Cartagena, Cra. 5 # 7-77. Cartagena, Colombia
E-mail: luiscaraballo@telecom.com.co

Introduction

House dust mite exposure is a major risk for allergic sensitization and asthma development [1]. In tropical and subtropical places, *Blomia tropicalis* is one of the most important sources of clinically relevant aeroallergens [2–4]. Blo t 12 (Blo t 12.0101) was first identified by IgE immunoscreening in a *B. tropicalis* cDNA library constructed from mites collected in Cartagena [5], showing an IgE-binding frequency of 50% when it was tested in 32 individual sera by a plaque assay. No homologous has been described for this allergen in *Dermatophagoides* spp., the other important allergen source in tropical countries, suggesting that it could be included in allergen panels for component-resolved diagnosis and allergen-specific immunotherapy.

Isoallergen is a term used to describe two related allergens with more than 67% similarity in their sequence

[6]. When only a few variations are present, they could also be named as isoforms. Allergen polymorphisms could influence their allergenicity, and their prevalence often varies among different environments [7, 8]. Therefore, identification of allergen variants should be followed by an evaluation of their IgE-binding properties, especially if they have potential use as clinical reagents for allergy diagnosis or immunotherapy. In this study, we evaluated the IgE response to a local isolated Blo t 12 isoallergen (Blo t 12.0101) in asthmatic patients from Cartagena, Colombia, and performed linear epitope mapping. We also isolated, cloned and expressed another Blo t 12 isoform from mites collected in Singapore, Blo t 12.0102, and compared its IgE-binding properties with Blo t 12.0101 to identify whether their amino acid variations influence their allergenicity. To date, only these two Blo t 12 isoforms have been reported.

Materials and methods

The cDNA coding for Blo t 12 isoallergens

Blo t 12.0101. The cDNA coding for Blo t 12.0101 was isolated by IgE immunoscreening from a *B. tropicalis* cDNA library constructed with mites collected in Cartagena, Colombia, as described previously [5].

Blo t 12.0102. 5'-Rapid amplification of cDNA ends (5' RACE and 3' RACE). The 5'-RACE Ready cDNA and 3'-RACE Ready cDNA (mite cDNA library) were generated by following the protocol described in the SMART RACE cDNA Amplification Kit User Manual with slight modifications. Briefly, total RNA was isolated from the live cultured *B. tropicalis* mites [9] (collected in Singapore) with Trizol reagent (Gibco BRL, Life Technologies, Grand Island, NY, USA). One microgram of total RNA was utilized to generate either 5' or 3' RACE Ready cDNA products as described earlier [10, 11] with specific primers and reagents provided by the kit in the presence of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. Subsequently, the 5' RACE was performed using the 5' Universal Primer Mix (Clontech Laboratories, Palo Alto, CA, USA) and a gene-specific primer 1(GSP-1), 5'-GAA GAA GGA CCC ATT CCA CAT-3', which was designed based on the nucleotide sequences of other Blo t 12 mite allergens (corresponding to Glu57-His66 in matured Blo t 12). Similarly, a specific primer (GSP2) was utilized for the generation of 3' RACE fragments. Positive and negative controls were prepared according to the protocol of the manufacturer. GSP 2, 5'-GAG GAA ACT CAT CAT TCC GAT GAC TTG ATC GT-3' (corresponding to residues Glu75-Val 84 in matured Blo t 12 protein), was designed based on the nucleotide sequences of Blo t 12 allergen. The polymerase chain reaction (PCR) was carried at 94 °C for 5 s, 55 °C for 10 s and 72 °C for 3 min for 30 cycles using the PTC-100™ Programmable Thermal Controller (MJ Research Inc, Waltham, MA, USA). Amplicons were analysed by agarose electrophoresis, cloned and sequenced. Clone 1 was isolated from the 5' RACE product with the primer GSP1 whereas clone 2 was isolated from the 3' RACE product with the primer GSP 2 and their DNA sequences were confirmed to be the 5' end and the 3' end of the Blo t 12 gene with overlapping homologous sequences between these two clones.

Long-distance PCR reaction was carried out to generate the full-length Blo t 12 cDNA according to the manufacturer's protocols (Clontech Laboratories). The template DNA was the 5' and 3' RACE products generated from the total mRNA of the mites earlier. The sequences of clone 1 and clone 2 were used to design the forward primer: 5'-AAT ACG ACT CAC TAT AGG GCA AGT AGG TGG-3' and the reverse primer: 5'-TCT AAT ACG ACT CAC TAT AGG GCA AGC AG-3', respectively. Subsequently, the PCR

products were purified with the QIA quick nucleotide removal kit (QIAGEN Inc., Valencia, CA, USA) and ligated into the pCR2.1-TOPO cloning vector (Invitrogen Corporation, Carlsbad, CA, USA). The full-length Blo t 12 cDNA was confirmed using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) with the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Kit.

Expression of Blo t 12 isoallergens in Pichia pastoris

Blo t 12.0101. PCR with specific primers LC-6 (5'-CTGAATT-CGCTGATGAGCAAACC-3') and MR02 (5'-TTGACACCAGACCAACTGGTAATG-3') amplified the sequence coding for mature Blo t 12.0101 protein and add *EcoRI* and *Not I* restriction sites to the 5' and 3' ends, respectively. This nucleotide segment was then cloned into the pPIC9 vector in frame with the α -factor prepro-secretion signal sequence from *Saccharomyces cerevisiae*. pPIC9/Blot12.0101 was linearized with *Sac I* to facilitate homologous recombination with the yeast genome. The expression in *P. pastoris* was performed according to manual recommendations (Invitrogen) as described below for Blo t 12.0102.

Blo t 12.0102. The cDNA encoding for the matured Blo t 12.0102 protein was amplified by PCR using the forward primer: 5'-CG CTC GAG AAA AGA GCT GAT GAG CAA ACC ACT CGC-3' and the reverse primer: 5'-G CGC GGC CGC TTA ATT ATC AAG TAC ACA ATT-3' containing the *XhoI* and *Not I* restriction enzyme sites, respectively. The expression of the PCR product in *P. pastoris* was performed according to the methods described in manual version E (Invitrogen Corporation). Briefly, the gel-purified PCR product was digested with *XhoI* and *Not I*, ligated to the pPIC9 vector and transformed to DH5 α . The plasmid was isolated and linearized with *Bgl II* and then transformed into the yeast strain GS115 by the lithium chloride method for protein expression. Transformants were selected by the slow-growing phenotype (Mut⁻) on the minimum methanol (MM) plates. In order to identify the high expression clones, an individual colony was inoculated in 50 mL of buffered glycerol-complex medium (BMGY, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base with ammonium sulphate, 1% glycerol, 0.4 mg/L biotin and 0.1 M potassium phosphate, pH 6.0) and grown at 30 °C in a rotary-shaking incubator at 250 r.p.m. The cells were harvested by centrifuging at 1500 g for 5 min at room temperature when the optical densities (OD_{600nm}) reached 2.0–6.0. The cell pellet was resuspended in 10 mL of buffered methanol-complex medium (BMMY, replacing the glycerol in BMGY with 0.5% methanol) for induction of the recombinant

expression. The recombinant protein Blo t 12 was harvested after 24 h of induction. It was secreted into the medium.

Subjects

A total of 107 sera from non-immunotherapy-treated asthmatic patients living in Cartagena, Colombia, and allergic to *B. tropicalis* were used in the study. Asthma was defined according to the Global Initiative for Asthma Criteria, GINA [12], using a standardized questionnaire tested previously [13]. All donor patients had a clear history of atopic symptoms related to house-dust mite exposure. Allergy to *B. tropicalis* was diagnosed by specific IgE determination using ELISA. The Bioethical Committee of the University of Cartagena approved the investigation and informed consent was obtained from all patients. The pooled sera from Singapore were from four local children (age between 9 and 15 years old) with allergic asthma, and all these sera showed positive IgE reactivity to recombinant Blo t 12.0102 allergen. Informed consent was obtained from each of these patients and the Research Ethics Committee of the National University of Singapore approved the protocol and the patient recruitment procedures.

Bioinformatics analysis and allergen modelling

PROSITE was consulted for the prediction of functional domains in Blo t 12 isoallergens. BLAST analysis did not identify a homologue sequence with a solved tertiary structure for homology-based modelling. Thus, Metaserver (Bioinfobank) was used to test 10 different folding-based methods and select the best template and secondary structure alignments for protein modelling. Only the predicted chitin-binding domain in both Blo t 12 isoallergens could be modelled, based on spatial restraints from tachycitin, a type II chitin binding protein. Modeller 9v1 software was used for modelling purposes. Energy parameters were calculated with the GROMOS96 implementation of Swiss-Pdb viewer [14]. Prosa-web analysis indicated good overall and local quality for both theoretical models [15]. Imoltalk was used to draw the Ramachandran plots of the models. Procheck was used to assess the geometry of models. HotPatch, an algorithm to detect protein-protein interaction sites, was used for the prediction of IgE-binding regions in Blo t 12 isoallergens [16]. The relative accessible surface area (ASA) for each residue was obtained with Asaview [17]. Pymol version 0.99 (Delano Scientific) was used to visualize structures and generate images.

Total and specific immunoglobulin E

Total serum levels of IgE were quantified in duplicate using a commercial ELISA kit (RIDASCREEN® Total IgE;

R-Biopharm, Darmstadt, Germany). Specific IgE against *B. tropicalis* was detected by ELISA as described previously [18]. For Blo t 12-specific IgE determination, the same protocol was used but with some modifications. Briefly, microtitre plates were incubated with 100 µL of 0.5 µg/mL of rBlo t 12 per well overnight. Then they were washed five times with phosphate-buffered saline (PBS)-0.1% Tween 20 and blocked with 100 µL of blocking buffer (1% bovine serum albumin (BSA), 0.02% sodium azide in PBS Tween 20) for 3 h at room temperature in a wet chamber. After washing again, 100 µL human sera (diluted 1 : 5 in PBS-3% BSA) were added to each well with further overnight incubation at room temperature. Plates were then washed five times and 100 µL of alkaline phosphate-conjugated anti-IgE (diluted 1 : 500 in buffer Tris pH 8.0, 0.05 M, BSA 1%, MgCl₂ 1 mM, sodium azide 0.02%) was added and incubated for 2 h at room temperature. After the final wash (5×), a colorimetric reaction was carried out by adding 100 µL of paranitrophenyl phosphate and then incubating at room temperature for 30 min. Absorbance at 405 nm was determined using a spectrophotometer (Spectra MAX 250, Molecular Device, Sunnyvale, CA, USA). Results were expressed in OD. The cut-off point was defined as the mean of five control-negative sera plus 3 standard deviations.

Skin Prick Test

Skin prick tests (SPT) were carried out in 48 patients for evaluating *in vivo* the allergenicity of recombinant molecules. For skin testing, the appropriate concentration of recombinant Blo t 12 *had been* obtained by titration in an allergic patient. This concentration has been used in several studies involving *B. tropicalis* recombinant allergens [19, 20]. The test was performed in the volar side of the arm using 25 µg/mL of the purified allergens, 50% glycerinated. Histamine dihydrochloride and glycerol were used as positive and negative control, respectively. A result was considered positive when the diameter of the papule was >3 mm. All these patients were also skin tested with *B. tropicalis* and *Dermatophagoides pteronyssinus* extracts (Leti, Madrid).

Enzyme-linked immunosorbent assay cross-inhibition assay

To investigate the presence of shared and unique epitopes in Blo t 12 isoforms, ELISA inhibition assays were performed. Pools 1 and 2 were used with Blo t 12.0101 and Blo t 12.0102 in the solid phase, respectively. Pool 1 was prepared with sera from six Colombian allergic patients with a higher IgE reactivity to Blo t 12.0101 than to Blo t 12.0102. Pool 2 was composed of four sera from Singaporean allergic patients; these sera were previously tested for IgE reactivity to their local isoform. The OD value of

Pool 2 was higher for Blo t 12.0102 than that for Blo t 12.0101. These pools were incubated with each inhibitor (Blo t 12.0101, Blo t 12.0102 and BSA) at different concentrations for 10 h at 4 °C, followed by the respective ELISA.

Peptide synthesis

Ten peptides corresponding to the amino acids 20–39, 30–49, 42–61, 50–69, 60–79, 73–92, 88–108, 101–120, 111–130 and 124–144 of the Blo t 12.0101 protein were prepared by solid-phase synthesis with an automated peptide synthesizer, purified by high-performance liquid chromatography and (HPLC) and analysed by mass spectrometry (SBS Genetech, Beijing, China). Lyophilized peptides were reconstituted to 10 mg/mL in 2.5% dimethyl sulphoxide (DMSO) and then diluted in PBS before their use in ELISA inhibition assays.

Epitope mapping

We used a competitive inhibition enzyme immunoassay to examine the ability of the 10 overlapping peptides to block human IgE binding against Blo t 12.0101 (Fig. 1). Five individual sera, which showed high specific IgE levels to Blo t 12.0101 but low or no reactivity to Blo t 12.0102, were used. End-point inhibition of IgE binding to this allergen was carried out with 1.5 mg/mL of each peptide for 5 h at room temperature. BSA (1.5 mg/mL) and Blo t 12.0101 (0.1 mg/mL) were used as negative and

positive controls, respectively. Peptides showing a significant level of inhibition were then tested at different concentrations to observe the dose–response relationship. The degree of inhibition was expressed as a cross-reactivity index (CRI), using the following formula: (Peptide inhibition %/Homologous inhibition %) × 100. CRI values below 25% were considered as no inhibition, and those between 25% and 50% as a low degree of inhibition.

Statistical analysis

Non-parametric tests were used to compare the specific IgE values obtained by ELISA and SPT results with rBlo t 12 isoforms. Specific IgE values were log-transformed, and correlation was estimated by the Pearson coefficient. Statistical analyses were performed with SPSS 13 for Windows, SPSS Inc., Chicago, IL, USA.

Results

Amino acid variations of Blo t 12 isoallergens could influence their tertiary structure

Figure 2 shows an alignment of the amino acid sequences of Blo t 12 isoallergens. Both proteins contain a predicted type II chitin-binding domain (CBD-II), similar to that found in other invertebrates and human chitinases. Based-folding modelling of Blo t 12 isoallergens could only be carried out for the predicted chitin-binding domain. No

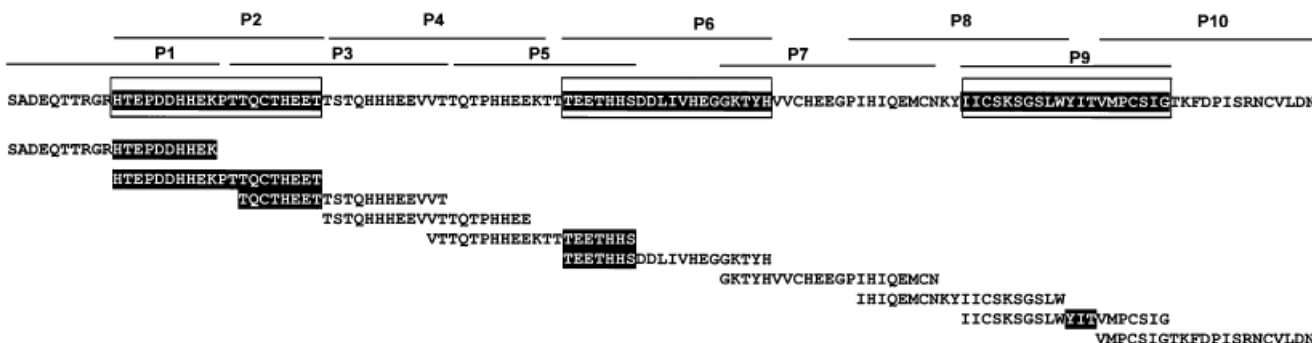


Fig. 1. Amino acid sequences of overlapping peptides covering matured Blo t 12.0101. Antigenic regions identified in epitope mapping experiments are boxed and shaded in the sequence.

Blo t 12.0102	MKSVLIFLVAIALFSANIVSADEQITTRGRHTEPDDHHEKPTTHATHEETTSTQHHHEEVT	60
Blo t 12.0101	MKSVLIFLVAIALFSANIVSADEQITTRGRHTEPDDHHEKPTTQCTHEETTSTQHHHEEVT	
	*****:*****	
Blo t 12.0102	T-QTPHHEEKTTEETHSDDLIVHEGGKTYHVVCHEEGPIHPGNVHKYIICKSGSLW	120
Blo t 12.0101	TTQTPHHEEKTTEETHSDDLIVHEGGKTYHVVCHEEGPIHIQEMCNKYIICKSGSLW	
	* *****:*****	
Blo t 12.0102	YITVMPCSIGTKFDPISRNCVLDN	144
Blo t 12.0101	YITVMPCSIGTKFDPISRNCVLDN	

	Similarity	
	92%	

Fig. 2. Amino acid sequence alignments of Blo t 12.0101 and Blo t 12.0102. Amino acid sequence residues are numbered at the end of each row. The first 20 residues correspond to a signal peptide. The predicted chitin-binding domain type II (CBD-II) is shadowed.

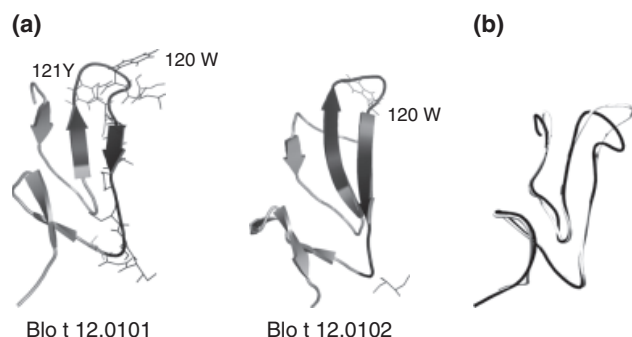


Fig. 3. Identification of E9 epitope in the chitin-binding domain models of Blo t 12 isoallergens. (a) In black, the location of E9 in chitin-binding domain type II (CBD-II) models. Residues identified by Hotpatch as part of this epitope are shown as sticks. Aromatic residues are labelled. (b) Superposition of CBD-II structural models from Blo t 12 isoallergens; Blo t 12.0101 (black), Blo t 12.0102 (grey).

reliable results could be obtained for the complete allergen. Superposition of both tertiary structures indicates that although the CBD-II from Blo t 12 isoallergens share a similar architecture (RMSD: 1.03 Å), amino acid variations clearly influence secondary structure assignments. It is worth noting that loop and β strand lengths are markedly different between these CBD-II domains (Fig. 3a). Although seven consecutive residues in this domain are different between these isoallergens, superposition of the two models showed that these variations did not affect local structural assignment (Fig. 3b); however, it does modify the spatial restraints of the loop region that connect this area with the next β strand.

Asthmatic patients from Cartagena showed a reduced Immunoglobulin E response against a naturally occurring isoallergen of Blo t 12

Specific IgE levels to Blo t 12 isoallergens were analysed in sera from 107 asthmatic subjects. This population had a mean age of 36.23 ± 18 , and 46% were males. Eighty-one percent of them also had a physician diagnosis of allergic rhinitis. In the subgroup that was SPT, 95% were co-sensitized to *D. pteronyssinus*. The cut-off value for ELISA was 0.13 OD. The frequencies of IgE reactivity to Blo t 12.0101 and Blo t 12.0102 were 35.5% and 23%, respectively ($P=0.12$). Twenty-four patients were positive to both isoforms, 14 to Blo t 12.0101 only, one patient was positive to Blo t 12.0102 only and 68 patients were negative for both isoforms. Most patients (10 out of 14) who reacted only to Blo t 12.0101 had lower specific IgE levels than those reacting to both isoforms (mean value, 0.147 vs. 0.535 OD). A high correlation is observed between the log-specific IgE values against these isoallergens (Fig. 4). In contrast, specific IgE to any of the two Blo

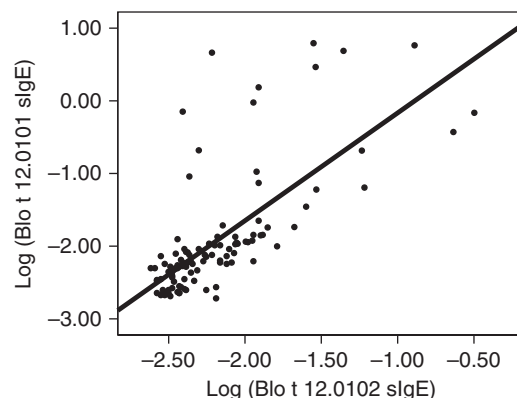


Fig. 4. Correlation of sIgE levels to Blo t 12 isoallergens. Scatter dot plot representing the correlation observed between log-specific IgE levels to Blo t 12.0101 and to Blo t 12.0102. Specific IgE values were measured by ELISA.

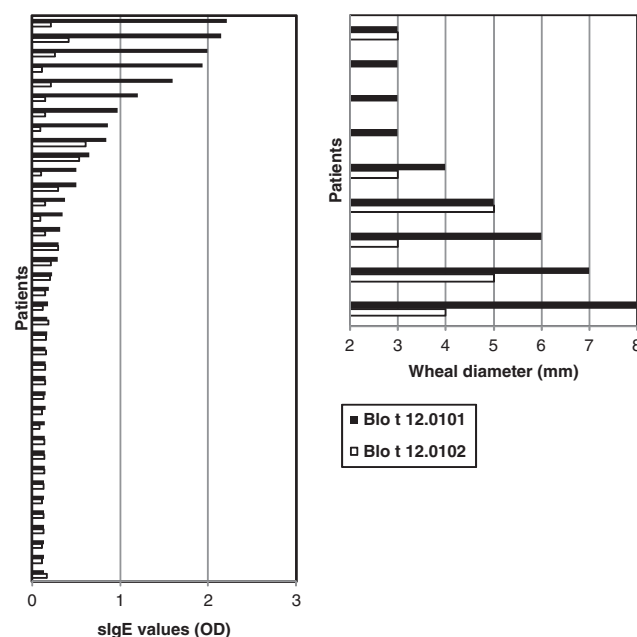


Fig. 5. Specific IgE levels and skin prick test (SPT) results for Blo t 12 isoallergens. (a) Horizontal bars represent specific IgE values against Blo t 12 isoallergens in each Blo t 12.0101 allergic patient; (b) wheal diameter values are induced by these isoallergens in SPT-positive subjects to Blo t 12.0101.

t 12 variants was not correlated with *B. tropicalis*-specific IgE levels, or total IgE.

Specific IgE levels against Blo t 12.0101 were significantly higher than those to Blo t 12.0102 (0.25 ± 0.43 vs. 0.124 ± 0.080 OD, $P=0.001$) (Fig. 5a), especially in those with a high response to Blo t 12.0101, which represents 50% of all positive cases. Similarly, the strength of the IgE response, as determined by the diameter of wheal in SPT-positive subjects to Blo t 12.0101, was greater than that to Blo t 12.0102 (mean values, 4.5 vs. 2.3 mm, $P=0.001$ (Fig. 5b). For both isoforms, a significant correlation was found

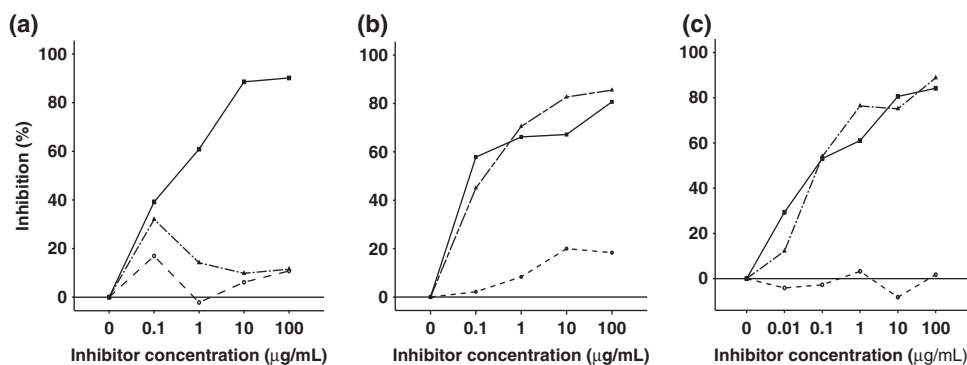


Fig. 6. ELISA inhibition assays for assessment of Blo t 12 isoallergens cross-reactivity. (a) Blo t 12.0101 in the solid phase, using a serum pool from Colombia patients who showed a high reactivity to Blo t 12.0101 but not to Blo t 12.0102. (b) Blo t 12.0102 in the solid phase using a serum pool from Colombian patients or (c) Singaporean allergic subjects. Blo t 12.0101 (■), Blo t 12.0102 (▲) and BSA (○).

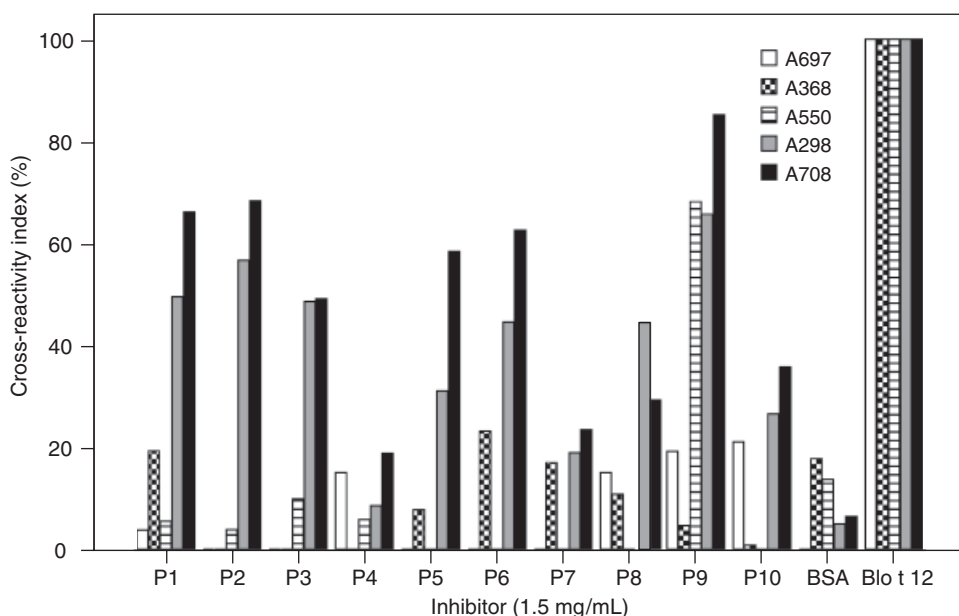


Fig. 7. Cross-reactivity index between 10 synthetic peptides and complete Blo t 12.0101. Diluted sera were preabsorbed with each of the 10 overlapping peptides, BSA or Blo t 12.0101 and reacted with the plate-bound Blo t 12.0101. When the optical density (OD) was reduced by more than 50%, it was considered a significant inhibition. Bars represent the results obtained for each of the five tested sera.

between log-specific IgE levels and weal diameter (Blo t 12.0101: $r=0.32$, $P=0.027$; Blo t 12.0102: $r=0.51$, $P<0.001$). Blo t 12.0101 and Blo t 12.0102 inhibited 95% and 20% of IgE binding, respectively, when the former was in the solid phase. Inversely, Blo t 12.0102 in the solid phase was inhibited by Blo t 12.0101 as much as its homologous inhibition under both conditions, using a serum pool from Colombian patients and another from Singapore asthmatic individuals (Fig. 6).

Linear epitope mapping reveals three immunoglobulin E-binding regions in Blo t 12.0101

Inhibition ELISA experiments with overlapping synthetic peptides identified three IgE-binding areas in Blo t

12.0101 (Figs 1 and 7). Peptide 9 inhibited IgE binding in three of five Blo t 12.0101 allergic sera, reducing OD values in $>60\%$ and identifying the E9 epitope. Peptides 8 and 10, which share 10 and seven residues with peptide 9, respectively, showed null or low inhibition of IgE binding to the full-length isoallergen, indicating that the three amino acids (Y121, I122 and T123) found only in E9 are critical for antibody recognition. Overlapping peptides 1–3 also identified a region in Blo t 12.0101 that binds IgE (E2). In one patient, peptides 5 and 6 inhibited $>50\%$ of IgE binding from the complete allergen. These two peptides have an overlapping sequence of six amino acids and identify the E6 epitope. Two amino acid variations between these isoallergens are included within this IgE-binding epitope. Furthermore, a dose-response

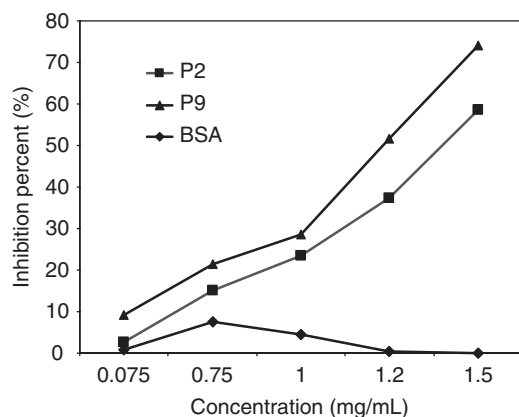


Fig. 8. Dose-response curves for peptides 2 and 9 inhibiting IgE binding to the Blo t 12.0101 isoallergen. Peptides 2 and 9 showed 58% and 74% of maximum inhibition, respectively. Bovine serum albumin (BSA) was used as a negative control.

relationship was observed when different concentrations of peptides 2 and 9 were used for inhibition experiments (Fig. 8). In two patients, none of these peptides inhibited IgE binding to the full-length allergen. BSA, which was used as a negative control, did not inhibit IgE binding to this allergen. Patients A550 and A368, who were also SPT, showed a lower response to Blo t 12.0102 than to Blo t 12.0101; however, linear IgE epitopes were only recognized in one of these two patients (A550).

In silico structural analysis concurred with experimental data in identification of E9 as an epitope

The location of E9 in the tertiary structure of Blo t 12.0101 CBD-II showed that this region is a solvent-accessible area composed by two β strands connected by a loop. In Blo t 12.0101, the most extensive stretch of accessible residues is included in the epitope identified by peptide 9 (Fig. 3a). Using Hotpatch, an algorithm that predicts sites in proteins that participate in protein binding (in this case, IgE antibodies), we identified two solvent-accessible patches in the CBD-II of Blo t 12.0101 that correspond to most of the P9 sequence. In contrast, the same analysis, when applied to Blo t 12.0102, identified only two common residues.

Discussion

In this study, we found that 35.5% of 107 asthmatic subjects living in Cartagena, Colombia, were sensitized to Blo t 12.0101, the locally isolated isoallergen. In this population, higher IgE sensitization rates to *B. tropicalis* allergens have been observed only for the recombinant BtM, a partial clone of Blo t 5 [21]. According to this, Blo t 12.0101 is an important *B. tropicalis* allergen in this tropical city where this mite, with *D. pteronyssinus*, is a

cause of allergic sensitization and a risk factor for emergency visits due to asthma exacerbations [22]. Because no homologue cross-reactive allergen has been reported in *Dermatophagoides* spp., it could be included as a component for allergy diagnosis and immunotherapy in this population.

Genetic variation of isoallergens could influence their IgE binding, T cell response and clinical reactivity [23]. Variations in IgE reactivity to isoforms can be due to differences in exposition, because the distributions of isoallergens vary among environments. In the case of group 2 allergens from *D. pteronyssinus*, it has been shown that there are distinct prevalent isoforms in European and Asiatic countries, the IgE response against them being directly related to their presence in those regions [23, 24]. It is also possible that some amino acid changes affect the allergenic potential of the molecule. For example, Bet v 1 isoforms isolated from the same birch extract showed a markedly different serological and clinical IgE response [7]. In this study, we compared the IgE response against two isoallergens isolated from different continents (America and Asia) because this could help to understand the importance of considering isoallergens when designing allergy diagnosis and treatment. We have detected that polymorphisms of Blo t 12 influence its IgE binding and reactivity in asthmatic patients, suggesting clinical relevance. Because Blo t 12.0101 inhibited Blo t 12.0102 as much as its homologous inhibition when using the serum pool from Singapore, we hypothesize that all the Blo t 12.0102 epitopes are present in Blo t 12.0101, but unique epitopes exist in Blo t 12.0101, indicating that in spite of the immunological importance of Blo t 12.0102 in our population, Blo t 12.0101 is representative of both isoforms regarding IgE epitopes. Thus, the locally isolated isoform is the most appropriate one for diagnosing IgE sensitization against Blo t 12 in Cartagena.

Blo t 12.0101 could also be clinically relevant in other populations, especially in America. Fifty percent of IgE reactivity against Blo t 12.0101 was observed in 36 allergic subjects from Tampa, USA [7]. Preliminary results from Havana, Cuba, showed an IgE sensitization frequency of 37.2% in the 42 tested patients (L. Puerta et al. unpublished). Although it is possible that the 0101 isoform is more relevant in this continent, IgE reactivity against Blo t 12.0102 has to be assessed to confirm it. Identification and quantification of these and other natural isoallergens by proteomic approaches must be performed using allergen extracts to evaluate whether there are differences in their geographical distribution. [25].

We further characterized at the epitope level the IgE response to Blo t 12.0101, the most important isoallergen in Cartagena. Three IgE-binding regions were detected by means of ELISA inhibition: E2, E6 and E9. Two residues of E2 are not conserved in these isoallergens. Thus, E2 could be absent in Blo t 12.0102, explaining why we found a

lower antibody reactivity to this isoform. E9 was the most immunoreactive epitope detected in Blo t 12.0101. Using overlapping peptides, we could identify that Y121, I122 and T123 are critical residues for IgE binding to E9. Its amino acid composition is in agreement with a recent analysis of common residues in antigen-antibody complexes that found, as we observed in Blo t 12.0101, that tyrosines and tryptophans are more frequent in antigenic sites than in non-immunogenic surfaces [26]. Interestingly, mapped IgE epitopes from *Hev b 6*, another CBP, are also enriched with aromatic residues [27].

Conventional methods for B cell epitope prediction do not consider that aromatic amino acids have a high probability to participate in antigenic sites because of their hydrophobic character. In contrast, some structural-based algorithms assign high scores to those patches including hydrophobic solvent-accessible residues to predict protein-protein interaction interfaces. Hotpatch, one of them, predicted most residues from E9 as part of a potential protein-binding site, but only for Blo t 12.0101. Relative ASA value analysis also supported our experimental finding: the most extensive stretch of solvent-exposed residues found in Blo t 12.0101 (>8 amino acids) is included in the IgE-binding region detected by peptide 9; however, in Blo t 12.0102, this region is less accessible, and some residues are buried in the molecular structure. Thus, it is possible that although the E9 sequence is common in the two isoforms, the accessibility differences influence antibody recognition. IgE specific to the E9 epitope found in Blo t 12.0101 could bind with a lower affinity to the corresponding region in Blo t 12.0102.

Linear epitopes were not recognized by IgE antibodies from the sera of two patients, suggesting that conformational epitopes are also present in this allergen. Identification of these epitopes with a more refined technology is required to identify all clinically relevant IgE-binding areas of Blo t 12. Noteworthy, a high molar excess of peptides with respect to the complete allergen was needed to achieve a significant IgE-binding inhibition, indicating that Blo t 12-specific IgE antibodies have a lower affinity to these reactive peptides than that to their respective epitopes in the complete allergen. A low affinity between complementary free peptides and antibodies could be explained by a loss of entropy in the binding reaction due to a high flexibility and mobility of the former [28]. However, it is also possible that the identified linear epitopes are only a segment of larger conformational epitopes.

In conclusion, immunological characterization of two Blo t 12 isoforms helped us to identify Blo t 12.0101 as the most important isoallergen in Cartagena. Epitope mapping experiments identified three IgE-binding regions in this molecule that could be mutated to obtain hypoallergenic variants. Because the IgE response to Blo t 12.0102 is lower than that to our local isoform, Blo t 12.0101

should be selected for diagnosis. Identification of the most important isoallergen in other populations should also be addressed locally.

Acknowledgements

This work was partially supported by a grant from the University of Cartagena and Fundemeb.

References

- 1 Sporik R, Chapman MD, Platts-Mills TA. House dust mite exposure as a cause of asthma. *Clin Exp Allergy* 1992; 22:897-906.
- 2 Caraballo L, Puerta L, Fernandez-Caldas E, Lockey RF, Martinez B. Sensitization to mite allergens and acute asthma in a tropical environment. *J Invest Allergol Clin Immunol* 1998; 8:281-4.
- 3 Fernandez-Caldas E, Fox RW, Bucholtz GA, Trudeau WL, Ledford DK, Lockey RF. House dust mite allergy in Florida. Mite survey in households of mite-sensitive individuals in Tampa, Florida. *Allergy Proc* 1990; 11:263-7.
- 4 Sanchez Palacios A, Schamann Medina F, Garcia Marrero JA, Sanchez Palacios MA, Perez Griera J, Lamas-Figueroa A. Prevalence of sensitization to *Blomia* in Gran Canaria. *Allergol Immunopathol (Madr)* 1995; 23:105-10.
- 5 Puerta L, Caraballo L, Fernandez-Caldas E *et al.* Nucleotide sequence analysis of a complementary DNA coding for a *Blomia tropicalis* allergen. *J Allergy Clin Immunol* 1996; 98:932-7.
- 6 Chapman MD. Allergen nomenclature. *Clin Allergy Immunol* 2008; 21:47-58.
- 7 Ferreira F, Hirtenlehner K, Jilek A *et al.* Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J Exp Med* 1996; 183:599-609.
- 8 Eriksson TL, Gafvelin G, Elfman LH, Johansson C, Van Hage-Hamsten M, Olsson S. T cell responses to recombinant isoforms, synthetic peptides and a mutant variant of Lep d 2, a major allergen from the dust mite *Lepidoglyphus destructor*. *Clin Exp Allergy* 2001; 31:1881-90.
- 9 Yi FC, Chew FT, Jimenez S, Chua KY, Lee BW. Culture of *Blomia tropicalis* and IgE immunoblot characterization of its allergenicity. *Asian Pac J Allergy Immunol* 1999; 17:189-94.
- 10 Ramos JD, Cheong N, Lee BW, Chua KY. cDNA cloning and expression of Blo t 11, the *Blomia tropicalis* allergen homologous to paramyosin. *Int Arch Allergy Immunol* 2001; 126:286-93.
- 11 Cheong N, Yang L, Lee BW, Chua KY. Cloning of a group 3 allergen from *Blomia tropicalis* mites. *Allergy* 2003; 58:352-6.
- 12 Global Strategy for Asthma Management and Prevention: Global Initiative for Asthma.: NHLBI/WHO, 2002.
- 13 Dennis R, Caraballo L, Garcia E *et al.* Asthma and other allergic conditions in Colombia: a study in 6 cities. *Ann Allergy Asthma Immunol* 2004; 93:568-74.
- 14 Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997; 18:2714-23.

- 15 Wiederstein M, Sippl MJ. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res* 2007; **35**:W407–10.
- 16 Pettit FK, Bare E, Tsai A, Bowie JU. HotPatch: a statistical approach to finding biologically relevant features on protein surfaces. *J Mol Biol* 2007; **369**:863–79.
- 17 Ahmad S, Gromiha M, Fawareh H, Sarai A. ASAView: database and tool for solvent accessibility representation in proteins. *BMC Bioinformatics* 2004; **5**:51.
- 18 Martinez B, Barrios K, Vergara C *et al.* A NOS1 gene polymorphism associated with asthma and specific immunoglobulin E response to mite allergens in a Colombian population. *Int Arch Allergy Immunol* 2007; **144**:105–13.
- 19 Yi FC, Cheong N, Shek PC, Wang DY, Chua KY, Lee BW. Identification of shared and unique immunoglobulin E epitopes of the highly conserved tropomyosins in *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 2002; **32**:1203–10.
- 20 Kuo IC, Cheong N, Trakultivakorn M, Lee BW, Chua KY. An extensive study of human IgE cross-reactivity of Blo t 5 and Der p 5. *J Allergy Clin Immunol* 2003; **111**:603–9.
- 21 Jimenez S, Puerta L, Mendoza D, Chua KY, Mercado D, Caraballo L. IgE antibody responses to recombinant allergens of *Blomia tropicalis* and *Dermatophagoides pteronyssinus*. *Allergy Clin Immunol Int* 2007; **9**:33–8.
- 22 Fernandez-Caldas E, Baena-Cagnani CE, Lopez M *et al.* Cutaneous sensitivity to six mite species in asthmatic patients from five Latin American countries. *J Investig Allergol Clin Immunol* 1993; **3**:245–9.
- 23 Park JW, Kim KS, Jin HS *et al.* Der p 2 isoallergens have different allergenicity, and quantification with 2-site ELISA using monoclonal antibodies is influenced by the isoallergens. *Clin Exp Allergy* 2002; **32**:1042–7.
- 24 Piboonpocanun S, Malainual N, Jirapongsananuruk O, Vichyanond P, Thomas WR. Genetic polymorphisms of major house dust mite allergens. *Clin Exp Allergy* 2006; **36**:510–6.
- 25 van Ree R. Isoallergens: a clinically relevant phenomenon or just a product of cloning? *Clin Exp Allergy* 2002; **32**:975–8.
- 26 Rubinstein ND, Mayrose I, Halperin D, Yekutieli D, Gershoni JM, Pupko T. Computational characterization of B-cell epitopes. *Mol Immunol* 2008; **45**:3477–89.
- 27 Beezhold DH, Kostyal DA, Sussman GL. IgE epitope analysis of the hevein preprotein; a major latex allergen. *Clin Exp Immunol* 1997; **108**:114–21.
- 28 Aalberse RC. Structural biology of allergens. *J Allergy Clin Immunol* 2000; **106**:228–38.