Nuclear Magnetic Resonance Structure and IgE Epitopes of Blo t 5, a Major Dust Mite Allergen[1]

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A high incidence of sensitization to *Blomia tropicalis*, the predominant house dust mite species in tropical regions, is strongly associated with allergic diseases in Singapore, Malaysia, and Brazil. IgE binding to the group 5 allergen, Blo t 5, is found to be the most prevalent among all *B. tropicalis* allergens. The NMR structure of Blo t 5 determined represents a novel helical bundle structure consisting of three antiparallel α-helices. Based on the structure and sequence alignment with other known group 5 dust mite allergens, surface-exposed charged residues have been identified for site-directed mutagenesis and IgE binding assays. Four charged residues, Glu76, Asp81, Glu86, and Glu91 at around the turn region connecting helices α2 and α3 have been identified to be involved in the IgE binding. Using overlapping peptides, we have confirmed that these charged residues are located on a major putative linear IgE epitope of Blo t 5 from residues 76–91 comprising the sequence ELKRTDLNILERFNYE. Triple and quadruple mutants have been generated and found to exhibit significantly lower IgE binding and reduced responses in skin prick tests. The mutants induced similar PBMC proliferation as the wild-type protein but with reduced Th2:Th1 cytokines ratio. Mass screening on a quadruple mutant showed a 40% reduction in IgE binding in 35 of 42 sera of atopic individuals. Findings in this study further stressed the importance of surface-charged residues on IgE binding and have implications in the cross-reactivity and use of Blo t 5 mutants as a hypoallergen for immunotherapy. *The Journal of Immunology,* **2008, 181: 2586–2596.**

Dust mites are one of the major causes of allergy in the world. As high as 10% of the general population and 90% of people with allergic asthma are sensitive to dust mites (ACAAI online). *Dermatophagoides farinae, Dermatophagoides pteronyssinus,* and *Blomia tropicalis* are the main species of dust mites implicated in allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis. Allergenic proteins such as those from dust mites, pollens and cockroaches bind and cross-link IgE molecules bound to the surface of mast cells, thereby triggering release of various pro-inflammatory mediators, and, consequently, leading to atopic diseases.

Allergens identified from dust mites are highly diverse, with more than 20 groups of them having been identified so far. Structures of several of these allergens, such as Der p 1, Der p 2, and Der f 13, have been determined by using x-ray crystallography and NMR spectrometry (1–3). These structures have enabled us to locate the site of IgE binding, while facilitating the generation of hypoallergens for allergen-specific immunotherapy. One of the major goals in preparing hypoallergens is to generate a protein derivative with reduced IgE binding, while retaining its ability to generate blocking IgG Abs to inhibit the binding of IgE Abs to the wild-type allergen. To achieve this, one can utilize the protein structure and sequence information, and selectively mutate unique surface residues to remove IgE epitopes. The advantage of this approach has been demonstrated successfully in generating potential hypoallergens against Bet v 1 and Der f 13 (3, 4). Hence, the structural information of allergenic proteins proves to be useful in identifying and locating putative IgE binding residues.

Group 5 allergens have been isolated from several species of dust mites such as *B. tropicalis* (Blo t 5) and *D. pteronyssinus* (Der p 5) (5–7). In *D. pteronyssinus*, Der p 1 and 2 bound 50–65% of the IgE Abs, and only around 10% of the IgE bound to each of Der p 4, 5, and 7 (8). In contrast, among the seven allergens evaluated in *B. tropicalis*, Blo t 2a, 3, 5, 10, 11, 12, and 13, Blo t 5 is by far the most important allergenic component and Blo t 5-specific IgE is found in 40–60% of *B. tropicalis*-sensitive individuals (9). Blo t 5 contains a 17-residues signal peptide and a 117-residues mature protein, while Der p 5 is made up of 19-residues signal peptide and a 113-residues mature protein (10). Der p 5 and Blo t 5 share 43% identity in their amino acid sequences, and cross-reactivity analysis has shown that both these allergens only share a low degree of cross-reactivity and are species specific (11–13). However, it is also reported that 79% of 48 sera of atopic individuals were radioallergosorbent test positive to both recombinant Der p 5 and BtM, a C-terminal 72-residues fragment of Blo t 5 (14).

Previous studies have shown that Der p 5 contains heptad repeats and could potentially form a coiled-coil structure at low pH conditions and arranging into a filament-like structure (10). The preliminary x-ray diffraction of Der p 5 and NMR resonance assignments of Blo t 5 have been reported (15, 16). In this present study, we report the NMR solution structure as well as mapping of the IgE binding epitope of Blo t 5. In contrast to the predicted coiled-coil structure, Blo t 5 adopts a novel helical bundle structure comprising three helices arranged in an antiparallel fashion. Using

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site-directed mutagenesis and overlapping peptides, a major putative linear IgE binding epitope of Blo t 5 has been mapped to around the turn region, connecting helices α2 and α3 containing four charged residues, Glu76, Asp81, Glu86, and Glu91. The implication in cross-reactivity with other group 5 allergens and the potential of Blo t 5 mutants as hypoallergen for immunotherapy are discussed.

Materials and Methods

Subcloning

DNA insert of Blo t 5 was generated by PCR, using upstream oligonucleotide primer containing *Bam*H I restriction site, and downstream oligonucleotide primer containing stop codon and *Xho*I restriction site. In our Blo t 5 construct, the first 22 residues in the N-terminal region of Blo t 5 were removed during subcloning to eliminate the signal peptide region as well as residue Pro21. This construct was used in all experiments and regarded as wild-type Blo t 5. For nuclear magnetic resonance (NMR)[4] experiments, Blo t 5 recombinant protein was expressed as a His-tag fusion protein. The DNA insert was ligated into a modified pET-32a plasmid (Novagen) and transformed into DH5-α competent cells. PCR screening was performed and the sequence of the insert was confirmed by DNA sequencing (Big Dye v3.1, Applied Biosystems).

Expression and purification of recombinant Blo t 5

Plasmid containing cDNA of Blo t 5 was transformed into *Escherichia coli* strain BL21 (DE3) for protein expression. Bacterial cells were grown in 1 liter of Luria Broth supplemented with 100 μg/ml ampicillin and protein expression was induced with 0.3 mM of isopropyl-β-D-1-thiogalactopyranoside at 20°C. For triple resonance NMR experiments, ^{15}N and/or ^{13}C labeled samples were obtained from cultures grown in M9 media supplemented with ^{15}N-labeled ammonium chloride and/or ^{13}C-labeled glucose as sole nitrogen and carbon sources, respectively. The protein was expressed as a His-tagged soluble protein and purified using the nickel-nitrilotriacetic acid resin (Qiagen). The His-tag was cleaved by thrombin (0.5 unit/mg protein, Sigma-Aldrich) and the protein was further purified using a HiLoad 16/60 Superdex 75 pg (GE Healthcare) gel filtration column on AKTA Fast Protein Liquid Chromatography System (GE Healthcare). Protein concentration was determined by UV absorption at 280 nm in a Hitachi spectrophotometer. GST-fusion proteins were expressed similarly using a pGEX-4T1 vector (GE Healthcare) with the insert subcloned between *Bam*H I and *Xho*I and purified using a glutathione sepharose 4B column (GE Healthcare).

NMR experiments

Purified Blo t 5 protein (with His-tag removed) was concentrated to ~2 mM using Amicon Ultra 5 (Millipore) and exchanged into a buffer containing 50 mM phosphate buffer (pH 7) with 10% D_2O. NMR experiments were performed at 298 K on an 800 MHz Bruker NMR spectrometer. Backbone assignments were conducted by HNCACB (17) and CBCA-(CO)NH (18) experiments using an uniformly labeled ^{15}N and ^{13}C protein sample. Side chain proton assignments were obtained from H(CCO)NH and (H)CC(CO)NH experiments (19). ^{1}H-^{13}C HSQC experiment was recorded using a 10% ^{13}C-labeled protein sample to perform stereo-specific assignments of methyl groups. Proton distance constraints were obtained from 3-D ^{15}N-edited Nuclear Overhauser Effect Spectroscopy (NOESY) and ^{13}C-edited NOESY experiments with 100 ms mixing time. ^{1}H-^{13}C HSQC and ^{13}C-edited NOESY experiments were conducted using the protein sample with a similar buffer but in 100% D_2O. Hydrogen bond restraints were determined by recording ^{1}H-^{15}N HSQC of uniformly ^{15}N-labeled protein exchanged into 100% D_2O buffer for 3 h. NMR data was processed using NMRPipe (20) and analyzed by NMRView (21).

Structures were solved by using the software CYANA (22) with NOE-restraints from ^{15}N-NOESY and ^{13}C-NOESY experiments, hydrogen bond restraints, and dihedral angle restraints predicted by the program TALOS (23). NOE-restraints were assigned in CYANA automatically and subsequently verified manually. Structures were calculated using the standard torsion angle dynamic protocol and 10 conformers with the lowest target function values were selected for further energy minimization using AMBER 7.0 (24). The final ten structures were checked with Procheck-NMR (25) and deposited at the Protein Data Bank (PDB ID: 2jrk). The backbone and side-chain resonance assignments of Blo t 5 were deposited at the Biological Magnetic Resonance Bank with accession number 15345.

Site-directed mutagenesis

Selection of residues for site-directed mutagenesis was made based on solvent accessibility of residues on the NMR structure as well as sequence alignment of Blo t 5 with group 5 allergens from other dust mite species. Mutant constructs were constructed by PCR-based overlap extension using designed oligonucleotide primers (1st BASE) with mismatches (26). A DNA insert with mismatches was subcloned into pGEX-4T1 plasmid (GE Healthcare) to generate GST-fusion proteins to be used in ELISA experiments. GST-tag Blo t 5 and its mutants were purified using glutathione-Sepharose (GE Healthcare) and subjected to analysis by SDS-PAGE.

Circular dichroism (CD) spectropolarimetry

CD experiments were conducted with 10 μM of protein in PBS 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 190–260 nm with 0.1 nm resolution using a scan speed of 50 nm/min and averaged for ten scans. Each spectrum was corrected by subtracting buffer signal and normalized to mdeg = 0 at wavelength 260 nm.

Specific IgE binding ELISA experiment

For ELISA experiments, all proteins used were GST-tagged fusion proteins for maximum binding on ELISA plates. Sera from patients were diluted 1/3 with 1% BSA in PBS and pre-absorbed with 0.2 mg/ml of GST protein overnight at 4°C. Wild-type Blo t 5 or mutants were coated overnight at 4°C onto Maxisorp ELISA plate (Nunc) at 1 μg protein per well in volumes of 50 μl. Each well was washed three times with 200 μl of PBS-T (PBS, 0.05% Tween 20) and blocked with 100 μl of PBS-1% BSA for half an hour at room temperature. Plates were then washed with PBS-T and incubated with 50 μl of pre-absorbed sera for 2.5 h at room temperature. After washing three times with PBS-T, 100 μl of biotin conjugated anti-human IgE mAb (BD Pharmingen) diluted 1/250 in PBS-1% BSA was added and incubated for another 2 h. After washing with PBS-T, avidin conjugated HRP (BD Pharmingen) diluted 1/1000 in PBS-1% BSA was added and incubated for half an hour. The plates were then thoroughly washed with PBS-T before 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich) was added. Absorbance measurements were conducted at 655 nm using an ELISA plate reader. All experiments were repeated twice separately and results were reported as mean values. Percentage of IgE binding was calculated by the formula % IgE binding = 100 × M/WT where WT and M represent absorbance values obtained from wild-type and mutant Blo t 5, respectively.

Inhibition of IgE binding by mutant proteins

In a 50 μl reaction volume, 1 μg of GST-Blo t 5 protein was coated onto Maxisorp ELISA plate (Nunc) overnight at 4°C. Sera were pre-absorbed with 0.2 mg/ml of GST protein and various amounts (0.01, 0.1, 1, 5, 10, and 20 μg) of Blo t 5 or multiple mutants of Blo t 5 (double mutant 2A, 2B; triple mutants 3C, 3D, 3E, 3F; and quadruple mutant 4A). After an overnight incubation, plates were washed with PBS-T and blocked with PBS-1% BSA for 30 min at room temperature. Plates were washed again with PBS-T and incubated with sera pre-absorbed with Blo t 5 or its multiple mutants for 2.5 h at room temperature. Subsequent incubation with Abs and colorimetric development were performed as described above.

Mouse immunization

Mouse immunization experiments were conducted to compare the abilities of the wild-type, 3D, 3F, and 4A mutants of Blo t 5 to induce IgG Abs specific for Blo t 5. Eight-week-old female BALB/c mice were used in our studies. Groups of four mice were each injected i.p. with 200 μl of Ag containing 20 μg of purified Blo t 5, 3D, 3F, and 4A mutants conjugated with 1.25 mg/ml aluminum hydroxide gel (Sigma-Aldrich) every 2 wk. Buffer containing adjuvant aluminum hydroxide was similarly injected into another mouse as buffer control. Preimmune and postimmune blood samples after the sixth injection were drawn by orbital bleeding, and sera were kept at −20°C until analysis. Animals were maintained in the Animal Holding Unit of the Faculty of Medicine, National University of Singapore, according to local guidelines.

Inhibition of human IgE binding to Blo t 5 by specific mouse IgG Abs

The volumes of all samples and reagents used in ELISA experiments were 50 μl, whereas the washing steps were conducted three times with 200 μl

[4] Abbreviations used in the paper: NMR, nuclear magnetic resonance; CD, circular dichroism; NOESY, Nuclear Overhauser Effect Spectroscopy.

Table I. *Structural statistics for the NMR solution structure of Blo t 5*

Data Collection	Statistics
Restraints used for calculation	
Total NOE restraints	1631
Intraresidue	686
Sequential	484
Medium range	305
Long range	156
Hydrogen bond restraints	96
Dihedral angle restraints	176
CYANA target function	1.83 ± 0.17
Maximum violation for upper limits (Å)	0.22 ± 0.02
Maximum violation for torsion angle (degree)	3.55 ± 0.18
Amber energies (kcal/mol)	
Total energy	−4750.7 ± 22.7
Ramachandran statistics (%)	
Most favored	82
Additionally allowed	16
Generously allowed	2
Disallowed	0
Root mean square deviation from mean structure (Å)	
Backbone atoms (residues 21–117)	0.67 ± 0.13
All heavy atoms (residues 21–117)	1.26 ± 0.21

of PBS-T each time. GST-tagged Blo t 5 was coated overnight at 4°C onto a Maxisorp ELISA plate (Nunc) at 1 μg of protein per well. Plates were washed with PBS-T and blocked with PBS plus 1% Tween 20 for 30 min at room temperature. The plates were washed again with PBS-T and preincubated with 1/5 diluted mouse serum from control mouse or from mice immunized with Blo t 5, 3D, 3F, or 4A mutants. The wells for control were incubated with PBS plus 1% Tween 20 alone. Plates were incubated for 2.5 h at room temperature. After incubation, plates were washed with PBS-T and incubated with 1/2 diluted sera from patient P1 in PBS for overnight at 4°C. After washing with PBS-T, biotin-conjugated anti-human IgE mAb (mouse IgG isotype, lot no. M075594; BD Pharmingen) diluted 1/250 in PBS-T was added and incubated for 2 h. Following washing with PBS-T, avidin-conjugated alkaline phosphatase (BD Pharmingen) diluted 1/1000 in PBS-T was added and incubated for another 30 min. The plates were then thoroughly washed with PBS-T, and 100 μl of p-Nitrophenyl phosphate (p-NPP, Sigma-Aldrich) was added. The color reactions were observed through absorbance measurements at 405 nm. Percentages of inhibition of human IgE binding to Blo t 5 after preincubation with mouse sera were determined with the following formula: percentage of inhibition of IgE binding = 100 − (*OD*I/*OD*C) × 100. *OD*I and *OD*C represent absorbance values after preincubation with mouse sera and PBS-T control, respectively.

IgE binding by peptides derived from Blo t 5

Twenty overlapping peptides, each of 16 residues, covering the whole sequence of Blo t 5 were synthesized in the format of "biotin-SGSG-peptide-amide" (GL Biochem). One microgram of each peptide was coated on Reacti-Bind streptavidin-coated high binding capacity clear 96-well plate (Pierce) at 4°C for 24 h. Plates were washed with PBS-T for three times and blocked with PBS-0.1% Tween 20 at room temperature for 1 h. Plates were washed again and incubated separately with sera (1/1 dilution in PBS) from 10 individual patients for 48 h at 4°C. After washing with PBS-T, alkaline phosphatase-conjugated anti-human IgE (Sigma-Aldrich) diluted 1/250 in PBS-0.1% Tween 20 was added and incubated for 6 h. Plates were then thoroughly washed with PBS-T, and absorbance was measured at 405 nm upon adding 100 μl of p-NPP (Sigma-Aldrich) as substrate.

PBMC proliferation and cytokine expression

PBMCs were isolated from fresh blood by using a general Ficoll-Hypaque gradient centrifugation technique. PBMCs were cultured in 100 μl of RPMI 1640 and 10% FBS medium in 96-well plates at 1×10^5 cells per well. One hundred microliters of wild-type Blo t 5, 3D, 3F, or 4A mutants was added to the cells at a final protein concentration of 0.025 μg/ml. PMA (0.3 μg/ml) was used as reference for 100% stimulation. PBMCs were incubated for 6 days in a 5% CO_2 incubator at 37°C. Cells were tested for proliferation by using the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium salt (WST-1) colorimetric method according to standard manufacturer's protocol (Roche). Absorbance was measured at a 440 nm wavelength with a reference wavelength of 600 nm. Proliferation was reported as the stimulation index using the formula $(S - C)/C \times 100$, where S is the absorbance of stimulated cells and C is the absorbance of control nonstimulated cells. For the measurement of cytokines, supernatants of the cell cultures at day 6 were harvested. Cytokine concentrations were determined by using human Th1/Th2 BioPlex cytokine assay (Bio-Rad) according to the standard protocols from manufacturer for Th1 and Th2 cytokines. Assay on each sample was conducted in duplicate, and the result was reported as mean value.

Skin prick test

Written consents were obtained from allergic patients before skin prick tests. Tests were conducted with crude extract of *B. tropicalis* (0.2 mg/ml), 0.001 μg/ml of recombinant wild-type or mutant Blo t 5 in PBS containing 50% glycerol. Histamine (10 mg/ml) and PBS buffer were included as positive and negative controls, respectively. All proteins and buffer used in these experiments were filter-sterilized with 0.22 μm filter membranes. Skin prick was conducted with a sterile lancet and measurement of wheal and erythema was conducted after a 15 min interval. The size of the wheal was calculated using the formula ($\pi \times$ radius^2) for circular ones and ($0.8 \times$ length $\times$ width) for elliptical ones.

Screening of sera of atopic patients by immunodot blot

To further determine the prevalence of IgE binding at the particular epitopes and effectiveness of the mutants of Blo t 5 as a hypoallergen, we screened a total of 55 and 34 consecutive atopic sera from two regional centers managing allergic conditions for IgE binding using immunodot blot. A total of 42 of these were found to have specific IgE to the Blo t 5 allergen. Twenty-three of the patient sera were from the KK Women's and Children's hospital in Singapore (patients KK1 to KK23), while the other 19 sera were from allergy clinic in Jakarta,

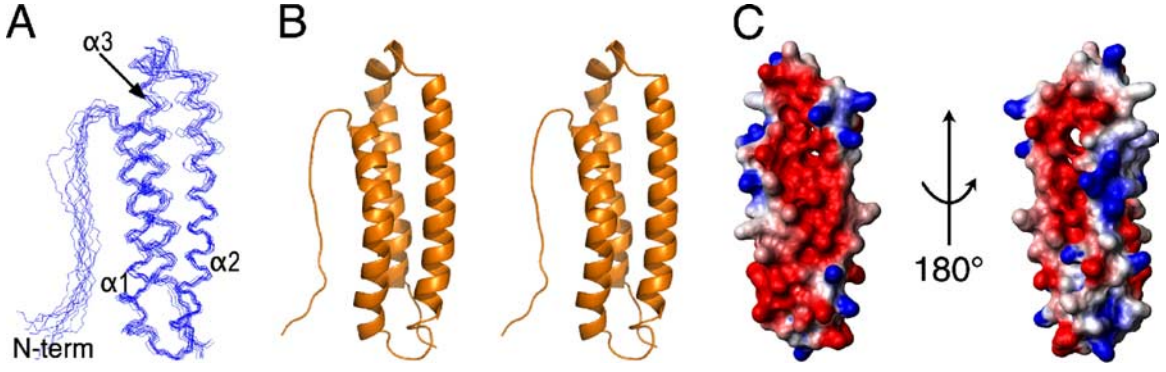

FIGURE 1. Solution structure of Blo t 5. *A,* An ensemble of 10 best structures of Blo t 5 solved by NMR. The N-terminus, helices α1, α2, and α3 are labeled in the diagram. *B,* Stereoview of the ribbon diagram of Blo t 5. Figure prepared with the program PyMOL (43). *C,* Surface diagram of Blo t 5 with electrostatic potential as calculated by the program MOLMOL (44). Positive and negative charged residues are colored blue and red, respectively. The N-terminal flexible region is removed for clarity. The orientation of the molecule in the left panel is the same as that shown in *B*.

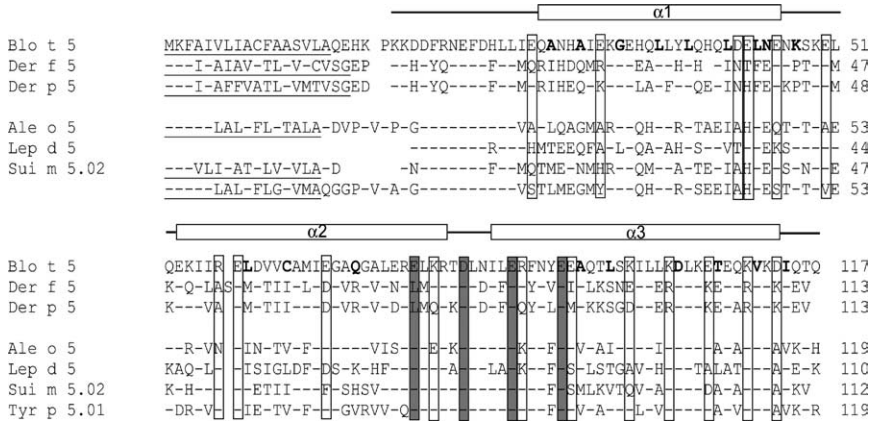

FIGURE 2. Sequence alignment of Blo t 5 with group 5 allergens from other house dust mites (Der f 5 and Der p 5) and less common storage product mites (Ale o 5 from *Aleuroglyphus ovatus*; Lep d 5 from *Lepidoglyphus destructor*; Sui m 5.02 from *Suidasia medanensis*; and Tyr p 5.01 from *Tyrophagus putrescentiae*). Residues that are identical to those in Blo t 5 are represented by "−". Residues that are determined to be buried based on the structure of Blo t 5 by the software GETAREA (27) are bold faced. Residues selected for site-directed mutagenesis in Blo t 5 are boxed, while the four major IgE binding epitope residues found are highlighted in gray (Glu76, Asp81, Glu86, and Glu91). Signal peptide regions predicted by the program "SIG-Pred" (http://www.bioinformatics.leeds.ac.uk/prot_analysis/Signal.html) are underlined. Boundaries of α-helices α1, α2, and α3 are shown as cartoon diagram above the sequences.

Indonesia (patients IN1 to IN19). All were doctor diagnosed with allergic diseases (asthma and/or allergic rhinitis) and has a positive skin prick response to the *B. tropicalis* extract. One microgram of each recombinant Blo t 5 protein (mutant or wild-type) was dotted onto nitrocellulose membranes, together with series-diluted IgE standard (National Institute for Biological Standards) as a positive control, 1 μg of BSA as a negative protein control, and 1 μl of buffer as a control. Membranes were air dried and blocked with PBS-0.1% Tween 20 and incubated with sera (1/1 vol/vol in PBS) at 4°C overnight, followed by 2 h in anti-human IgE Abs conjugated with alkaline phosphatase (1/1000 vol/vol in PBS; Sigma-Aldrich). The membranes were then developed with NBT/5-bromo-4-chloro-3-indolyl-phosphate (Promega) for 30 min, and spot intensities were measured with imaging software (Microimage v.3.01). Spot intensities (range, 0–255) were normalized by subtracting the local background, and intensities of >20 (equivalent to 2 SDs above the mean negative sera responses) were considered positive. The inter-assay and intra-assay concordances were >90 and 95%, respectively, demonstrating good reproducibility of the assay. A selection of these (where larger volumes of sera were available) were also validated and confirmed via allergen-specific ELISA as described earlier.

Approval

All human studies involved in this report had been reviewed and approved by the Institutional Review Board of the National University of Singapore and the Hospital Ethics Committee of the KK Women's and Children's Hospital.

Results

Solution structure of Blo t 5

A construct of Blo t 5 was generated with the N-terminal signal peptide and residue Pro21 removed to avoid interference with NMR chemical shift assignments. For NMR sample preparation, wild-type Blo t 5 protein was expressed as a soluble His-tagged fusion protein. Thrombin digestion was performed to remove the N-terminal His-tag and the protein was further purified using gel filtration chromatography. A highly purified protein sample that existed as a single major band on a 15% SDS-PAGE was obtained. The sample condition for NMR experiments has been optimized to 50 mM phosphate buffer (pH 7), while the protein sample is highly soluble even up to a concentration of 2 mM with no visible precipitation and degradation.

Heteronuclear multidimensional NMR experiments were conducted as described to obtain chemical shifts assignments as well as NOE distance restraints for structure determination. A total of 1631 NOE distance restraints, 176 TALOS angle restraints, and 96 hydrogen bond restraints were used for the structural calculation using CYANA software (Table I). An ensemble of ten high-resolution NMR structures was obtained with backbone and all atom root mean square deviation of 0.67 Å and 1.26 Å, respectively. The NMR structure of Blo t 5 consists of three α-helices forming a helix bundle as opposed to the predicted coiled coil structure (Fig. 1*A* and *B*). The first 15 residues at the N-terminal region are highly flexible and do not constitute any secondary structure. This finding agrees with our observation that several amide proton peaks (residues Asp15 to Glu20) from this region disappeared and could not be assigned in the ^{1}H-^{15}N HSQC spectrum. Following the flexible N-terminal region, three antiparallel α-helices form a helix bundle separated by tight turns. The three α-helices, α1, α2, and α3, are formed by residues Gln21 to Glu45, Gln52 to Thr80, and Asn83 to Asp113, respectively. Blo t 5, similar to most other inhalant allergens, also

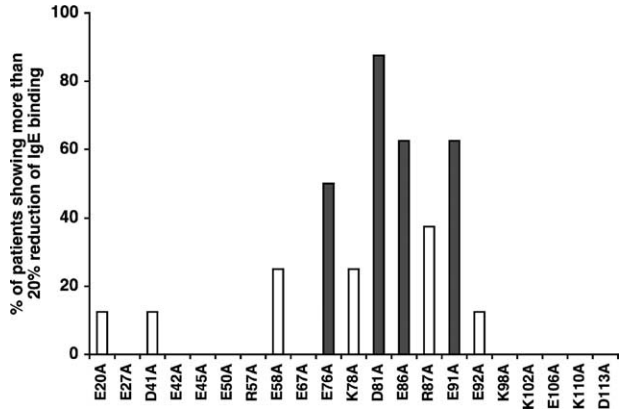

FIGURE 3. Prevalence of IgE binding reduction as induced by single site mutations on Blo t 5. Only when a mutation caused >20% of reduction in IgE binding are considered as significant reduction. The percentage of occurrence of such reduction in IgE binding caused by each of the 21 mutations among a total of eight patients is determined. The four major epitope residues, Glu76, Asp 81, Glu86, and Glu91, are highlighted in gray and mutation on each of these residues caused significant reduction in IgE binding in at least 50% of the patients.

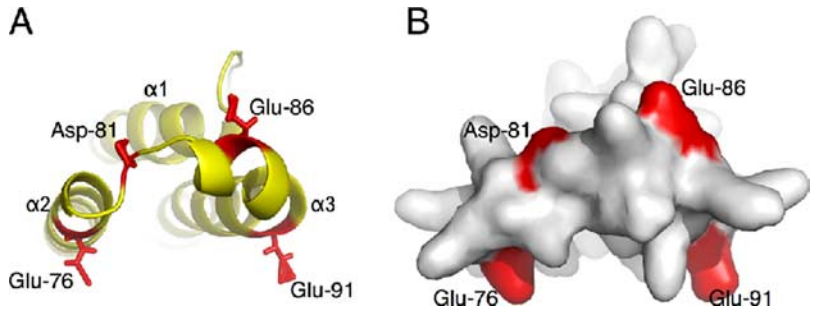

FIGURE 4. Locations of the major IgE binding epitope residues on Blo t 5. Ribbon diagram (*A*) and surface diagram (*B*) showing locations of the four major IgE binding epitope residues, Glu76, Asp 81, Glu86, and Glu91, at or around the loop region connecting helices α2 and α3 on one end of the Blo t 5 molecule. All four residues are highly exposed to solvent and originated from a short stretch of 16 residues.

contains a high percentage of basic (15.3%) and acidic (22.2%) residues. The surface of the molecule is mostly negatively charged with a high amount of exposed and charged residues (Fig. 1*C*).

Site-directed mutagenesis and IgE epitope mapping

The solvent accessibility of residues was determined using GETAREA (27) based on the NMR structure of Blo t 5 and residues that are buried were excluded from mutagenesis study (Fig. 2). Site-directed mutagenesis was performed at 21 solvent accessible residues on Blo t 5 to identify those that are involved in IgE binding. These residues were selected based on sequence alignments with other known house dust and less common storage product mites (Fig. 2). As our previous findings on Der f 13 had shown that charged residues are more likely to be involved in IgE binding (3), only charged residues of Blo t 5 that are either highly conserved among all the group 5 allergens or those that are distinctly different from other house dust or storage product mites were selected for mutagenesis. Each of these residues was individually mutated to alanine and the corresponding mutant protein was tested for its ability to bind IgE in patient sera. ELISA experiments were conducted using different sera from eight individual allergic patients, P1 to P8, exhibiting positive skin prick reaction against the wild-type Blo t 5 protein.

The effect of mutation was analyzed based on the amount of reduction in IgE binding by the mutant protein. Only mutation at a particular residue that caused more than 20% of reduction in IgE binding was considered to be significant and the prevalence of these mutations among different patients is shown in Fig. 3. Out of 21 residues selected for mutagenesis studies, only four showed significant reduction in IgE binding in 50% or more of the patient sera. These four residues are Glu76, Asp81, Glu86, and Glu91 (Fig. 3). Mutation of the residue Asp81 to alanine seems to have the most drastic reduction in IgE binding and this was demonstrated in seven of eight (87.5%) serum samples tested. A total of five of eight (62.5%) sera showed IgE binding reduction in mutants E86A or E91A while half of serum samples showed significant reduction in IgE binding for the mutant E76A. Coincidentally, these four putative IgE binding epitopes are all acidic residues and are located on the same stretch of residues at around the turn region connecting α-helices α2 and α3 on one end of the Blo t 5

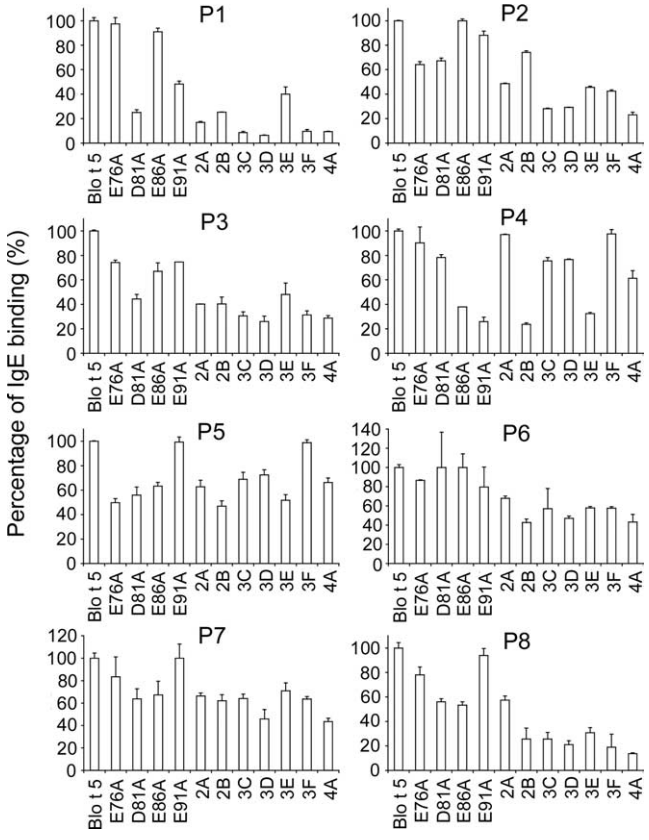

FIGURE 5. Percentage of IgE binding by wild-type and mutant Blo t 5 using sera from individual patients. Mutation in any of the four major epitope residues mostly resulted in some degree of reduction in IgE binding in all of the patients tested. Combination of these mutations for the preparation of double mutants (2A: E76A_D81A and 2B: E86A_E91A); triple mutants (3C: E76A_D81A_E86A, 3D: E76A_D81A_E91A, 3E: E76A_E86A_E91A and 3F: D81A_E86A_E91A); and quadruple mutant (4A: E76A_D81A_E86A_E91A) generally resulted in further reduction of IgE binding for most patients.

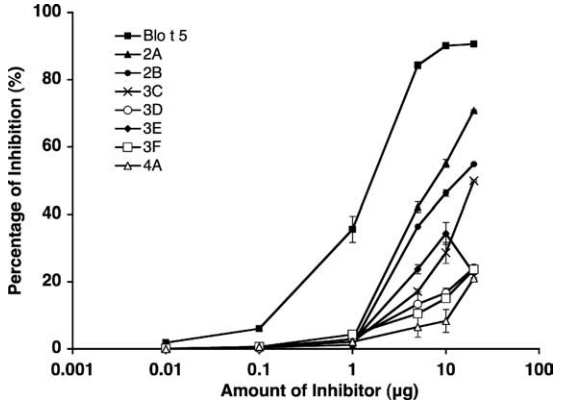

FIGURE 6. Inhibition IgE binding assay of wild-type and mutants of Blo t 5. The abilities of wild-type and mutant Blo t 5 to inhibit IgE binding by Blo t 5 adsorbed on the ELISA plate are tested using pooled serum from all eight patients. The wild-type Blo t 5 achieved almost 100% inhibition at 10 μg, but all the mutants are weaker in inhibition. The double mutants are consistently better than the triple and quadruple mutant in inhibition of IgE binding. The legends used for the different mutants are the same as those in Fig. 5.

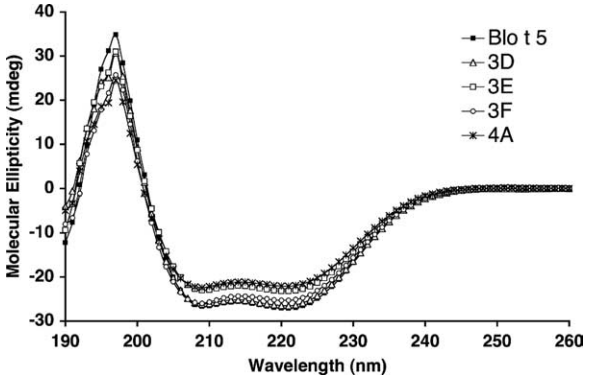

FIGURE 7. CD spectra of wild-type and selected mutants of Blo t 5. Far-UV CD spectra of the quadruple mutant 4A and the three triple mutants (3D, 3E, and 3F) indicate that these proteins still maintained a similar helical structure as wild-type Blo t 5 with two typical minima at 208 nm and 222 nm. The spectra shown are averages of 10 scans and are recorded at room temperature.

molecule (Fig. 4). Other mutants, such as E20A, D41A, E58A, K78A, R87A, and E92A showed reductions in IgE binding only in serum samples of particular individuals (Fig. 3).

Although majority of serum samples showed reduction in IgE binding for the 4 major epitope residues mentioned above, the selectivity of these residues varied considerably among different individuals (Fig. 5). For example, IgE binding for patient sera P1 and P3 seems to involve mainly the residue Asp81, with more than 70% reduction in IgE binding for the single mutant D81A in serum P1. In contrast, the binding of IgE from serum of patient P4 to Blo t 5 mainly involved residues Glu86 and Glu91 on helix α3, with close to 70% reduction in IgE binding for both E86A and E91A mutations. In the case of patients P7 and P8, sera IgE primarily seem to recognize residues Asp81 and Glu86 of Blo t 5. Residues Glu76 and Asp81 on the other hand are the major epitope residues for patients P2 and P5 (Fig. 5). Despite the fact that an individual patient may have different preference for IgE binding epitope residues, all of these four major epitope residues lie within a cluster consisting of a short stretch of 16 residues around the turn region connecting helices α2 and α3. This also implies that the major IgE binding epitope of Blo t 5 is a putative linear epitope.

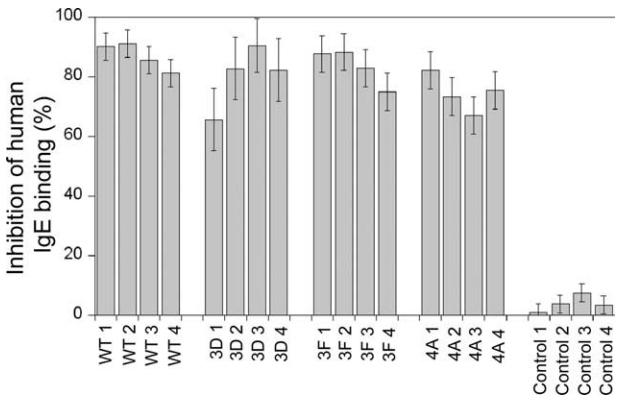

FIGURE 8. Percentage of inhibition of human IgE binding to Blo t 5 after preincubation with sera from mouse immunized with wild-type or mutant Blo t 5. Sera were used from four separate groups of four mice immunized with wild-type Blo t 5 (WT 1–4), 3D mutant (3D 1–4), 3F mutant (3F 1–4), and 4A mutant (4A 1–4). Sera from mice immunized with PBS were included as control (Control 1–4).

Peptide	Residue	Peptide	Residue	Peptide	Residue
1	1-16	8	41-56	15	81-96
2	9-24	9	49-64	16	85-100
3	17-32	10	61-76	17	89-104
4	21-36	11	65-80	18	93-108
5	25-40	12	69-84	19	97-112
6	29-44	13	73-88	20	102-117
7	33-48	14	77-92		

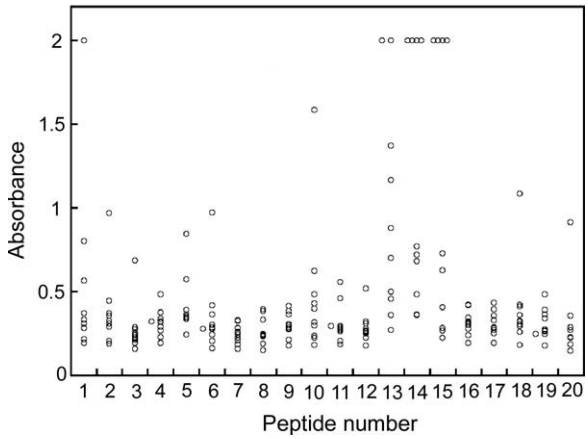

FIGURE 9. Prevalence of IgE binding by overlapping peptides derived from the sequence of Blo t 5. The ELISA absorbance caused by IgE binding with sera from 10 individual patients are determined for 20 overlapping peptides derived from Blo t 5. Note that an absorbance reading of 2.0 means that the value exceeded the linear detection range of the instrument. Data points having identical readings are scattered. Peptides 13, 14, and 15 show the highest prevalence and these peptides cover a region that contain the four major IgE binding epitopes, Glu76, Asp 81, Glu86, and Glu91.

Multiple mutations of epitope residues further reduce IgE binding

To investigate the effect of multiple mutations on the IgE binding by Blo t 5, we synthesized double, triple, and quadruple mutants of Blo t 5 based on the four major epitope residues. The double mutants 2A (E76A_D81A) and 2B (E86A_E91A) are prepared based on the fact that these residues are on opposite sides of the turn connecting helices α2 and α3. Four triple mutants, 3C (E76A_D81A_E86A), 3D (E76A_D81A_E91A), 3E (E76A_E86A_E91A), and 3F (D81A_E86A_E91A) and one

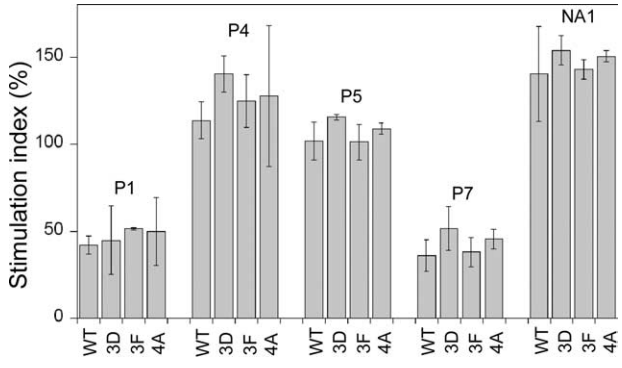

FIGURE 10. PBMC proliferation induced by the wild-type, 3D, 3F, or 4A mutants of Blo t 5. PBMCs from four Blo t 5-allergic patients P1, P4, P5, and P7 as well as a nonatopic individual (NA1) were stimulated with the wild-type, 3D, 3F, or 4A mutants of Blo t 5. Although the level of PBMC stimulation varied a lot between different individuals, the wild-type Blo t 5 and all the tested mutants caused similar levels of stimulation in the same individual. Proliferation was measured with the WST-1 colorimetric method and expressed as stimulation index (%) compared with control PBMCs without stimulation.

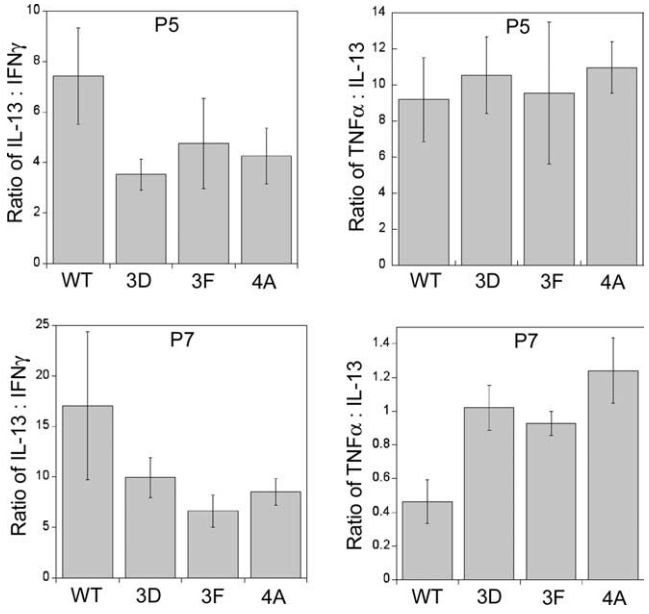

FIGURE 11. Profiles of cytokines secretion induced by the wild-type and 3D, 3F, or 4A mutants of Blo t 5. PBMCs from patients P5 and P7 were stimulated with the wild-type, 3D, 3F, or 4A mutants of Blo t 5 and the amounts of different cytokines secreted were measured. The cytokines IFN-γ and TNF-α are used as markers for Th1 type proliferation, whereas IL-13 secretion represented a Th2 type proliferation. Both patients P5 and P7 showed a decreased ratio of IL-13: IFN-γ when stimulated with the mutants of Blo t 5 compared with the wild-type protein. Patient P7 also showed an increased ratio of TNF-α: IL-13 when stimulated the mutants, but this trend is less obvious in patient P5.

quadruple mutant, 4A (E76A_D81A_E86A_E91A) were synthesized to cover all combinations of the four major epitope residues. In general, there were further reductions in IgE binding for the multiple mutants compared with the single mutants, and the triple or quadruple mutants seem to be more effective in doing so compared with the double mutants. This trend is observed in sera from most of the patients, e.g., P1, P2, P3, P6, P7, and P8. However, there are also individual cases where multiple mutants have a similar effect as the single mutants, e.g., in patient P5, or have even higher IgE binding compared with some of the single mutants, e.g., in patient P4 (Fig. 5). These variations suggest that there could be additional IgE binding epitopes and that the contribution to the overall IgE binding by the same epitope in different patients may be very different.

Using a pooled serum from 10 patients (P1 to P8 with two additional patients, P9 and P10), we have investigated the abilities of the different multiple mutants of Blo t 5 in inhibiting IgE binding to the wild-type Blo t 5 protein. Fig. 6 shows that the wild-type Blo t 5 can inhibit almost 100% of the IgE binding at an amount of 10 μg, indicating that the binding is due to specific interaction between IgE and Blo t 5. Double mutants, 2A and 2B, generally are better inhibitors of IgE binding than both triple and quadruple mutants. This agrees with the findings that double mutants usually have higher IgE binding than the other multiple mutants (Fig. 5). For triple mutants, mutant 3F is least able to inhibit IgE binding to Blo t 5, indicating that the more prevalent epitope residues could be Asp81, Glu86, and Glu91 (Fig. 6). These residues also appeared to be more prevalent during our analysis of IgE binding by single mutants of Blo t 5 and they are also more conserved among group 5 allergens than the residue Glu76. Among all the mutants, the quadruple mutant 4A showed the least ability to inhibit the IgE binding to the wild-type Blo t 5 protein.

To ensure that the loss of IgE binding by various different mutants were due to the loss of IgE binding epitopes instead of the overall structure of the protein, we acquired Far-UV CD spectra for every mutant used in this study. Fig. 7 shows the CD spectra of representative triple mutants and the quadruple mutant 4A. Results indicate that all these mutants retained their overall α-helical secondary structure with the characteristic minima at 208 nm and 222 nm closely resembling the CD spectrum of the wild-type Blo t 5. Monomeric states of these multiple mutants were also confirmed by the similar elution profiles from gel filtration chromatography as that of the wild-type Blo t 5 protein (data not shown).

Blocking of IgE binding by mice IgG induced from Blo t 5 mutants

Specific IgG responses were induced in all groups of mice immunized with the wild type, 3D, 3F, or 4A mutant of Blo t 5. To measure the inhibitory effect of the IgG raised on IgE binding, mouse antisera were assessed with inhibition ELISA using human serum from patient P1. All mouse sera from the wild-type group were able to inhibit the IgE binding to Blo t 5 at a percentage of $>$80%. Mouse sera from all of the mutant groups were able to achieve inhibition of greater than 65%, with more than half of the sera able to inhibit at greater than 80% (Fig. 8). Sera from the control group (immunized with buffer and adjuvant) were unable to block the binding of patient IgE to Blo t 5.

Inhibition of IgE binding by peptides (or binding of IgE to peptide) derived from Blo t 5

As we observed that all four major IgE binding epitope residues are located on the same stretch of short sequence, we postulate that this region could represent a putative linear IgE epitope of Blo t 5. To further confirm this, we prepared a set of 20 overlapping peptides covering the whole sequence of Blo t 5 and evaluated the ability of each peptide to bind sera IgE obtained from 10 individual patients (P1, P2, P3, and P11 to P17) allergic to Blo t 5. The prevalence of IgE binding to all the 20 peptides by sera from the

Table II. *Skin Prick tests for* B. tropicalis *crude extract, wild-type, or mutant Blo t 5*[a]

				Recombinant Proteins							
Patient	Histamine	Buffer	Crude	Blot5	2A	2B	3C	3D	3E	3F	4A
P1	12.6	0.0	7.1	7.1	3.1	4.9	12.6	1.8	9.6	0.8	1.8
P4	12.6	0.0	19.6	7.1	7.1	4.9	4.9	3.1	3.1	3.1	0.8
P5	9.6	0.0	12.6	19.6	4.8	12.0	7.1	1.8	8.0	1.8	1.8
P7	9.6	0.0	19.2	7.1	7.1	4.9	3.1	0.8	1.8	1.8	0.8
P18	12.6	0.0	24.0	9.4	7.1	5.9	3.1	0.8	0.8	0.8	0.8

[a] Patients P1, P4, P5, P7, and P18 were skin pricked with *B. tropicalis* crude extract (0.2 mg/ml), 0.001 μg/ml of wild-type or mutant Blo t 5 proteins. The numbers represent sizes of the wheals in mm^2 calculated using the formula ($\pi \times$ radius^2) for circular ones and (0.8 $\times$ length $\times$ width) for elliptical ones.

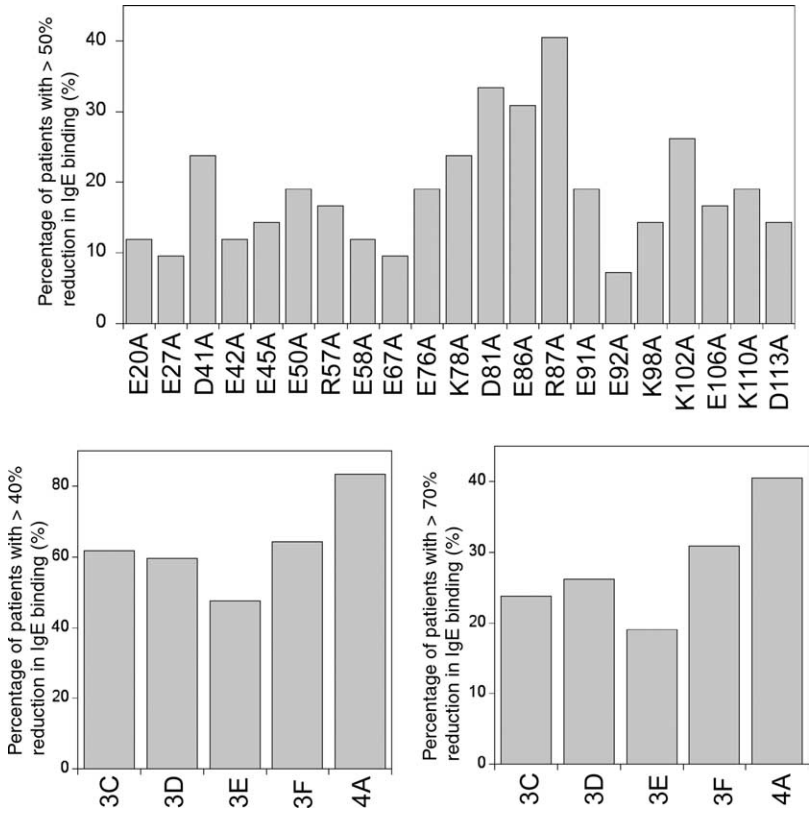

FIGURE 12. Mass screening of sera from patients for IgE binding to Blo t 5 mutants by immunodot blot. A total of 42 patient sera were used for the screening, with 23 from the KK Women's and Children's Hospital in Singapore and 19 from Indonesia. The percentage of patient that showed >50% reduction in IgE binding is plotted against the different single mutants of Blo t 5 used for screening. The percentage of patient that showed >40 or 70% is plotted against the different triple (3C, 3D, 3E, and 3F) and quadruple (4A) mutants used for screening.

10 patients were determined (Fig. 9). The results show that peptides 13, 14, and 15 are the most prevalent peptides, with each of them interacting significantly with sera IgE from close to 60–70% of the patients tested. These three peptides cover the sequence of Blo t 5 from residues Leu73 to Leu96 located on the turn region between helices α2 and α3 as well as the N-terminal half of α3 and included all of the four major IgE binding epitope residues (Glu76, Asp81, Glu86, and Glu91). The common sequence between these three peptides is "DLNILERF" which contained two of the four major epitope residues, Asp81 and Glu86, with Asp81 being the most prevalent epitope residue in our study. These two residues are also absolutely conserved among a total of 16 group 5 and the homologous group 21 dust mite allergens (28).

Although most of the sera IgE interacted with peptides 13, 14, and 15, there are distinct variations among individual patients. Serum from one patient exhibited low levels of responses to five different peptides other than peptides 13, 14, and 15. One patient responded to up to six different peptides, but there was one individual patient that did not respond to any of the peptides (data not shown). We conclude that the region covered by peptides 13, 14, and 15 represent a major putative linear IgE binding epitope of Blo t 5, but other minor epitopes also exists that could contribute to the specific allergenicity of Blo t 5 in individual patients.

T cell reactivity and cytokine release profile

The immunogenicities of wild-type, 3D, 3F, and 4A mutants of Blo t 5 were determined by measuring cell proliferation in PBMCs of patients when challenged with these proteins. The levels of PBMC proliferation varied significantly among allergic patients. When stimulated with the Ags, cells from patients P4 and P5 could be proliferated at rates around 100% of the rate when these cells were stimulated with 0.3 μg/ml PMA, while P1 and P7 only attained ~50% proliferation. PBMCs from a nonatopic individual showed the highest level of proliferation of close to 150% (Fig. 10). This suggests that the level of PBMC proliferation is not necessarily correlated with the allergenicity toward a particular allergen. Nevertheless, the levels of stimulation caused by the mutant proteins were observed to be consistently similar to the wild-type Blo t 5 protein for each individual (Fig. 10). This indicates that the mutants of Blo t 5 have similar immunogenicities as compared with the wild-type protein.

Although the degrees of T cell proliferation stimulated by the wild-type and the mutants of Blo t 5 were similar, the cytokines induced were observed to be different. The pattern of T cell proliferation can be determined by measuring the amount of Th-1 cytokines, such as IFN-γ and TNF-α, compared with that of Th-2 cytokines, such as IL-13, secreted from PBMCs after stimulation with the allergen. Results showed that there was a switch from Th-2 toward Th-1 proliferation pattern in atopic individuals, P5 and P7, when stimulated with the mutants of Blo t 5 instead of the wild-type protein (Fig. 11). The switch is indicated by a reduced ratio of IL-13: IFN-γ and an increased ratio of TNF-α: IL-13, although the latter one is not as obvious in patient P5 as compared with patient P7 (Fig. 11). Similar experiments conducted using PBMCs from a nonatopic individual showed similar ratios of IL-13: IFN-γ and TNF-α: IL-13 when stimulated with the wild-type or mutant proteins of Blo t 5 (data not shown).

Skin prick test

To determine the respective abilities of the wild-type and various multiple mutants of Blo t 5 to cross-link IgE in vivo to release inflammatory mediators, we conducted skin prick tests on selected patients who were allergic to Blo t 5. Table II shows that majority of the patients (four of five) had lower allergic response to wild-type Blo t 5 protein compared with crude extract of *B. tropicalis* that contains multiple allergens. In general, the mutants of Blo t 5 showed lower allergenicity than the wild-type protein. The triple

and quadruple mutants could further reduce allergic response compared with the double mutants, with mutants 3D, 3F, and 4A more effective than mutants 3C and 3E. This agreed with the IgE binding inhibition assay in which mutants 3C and 3E generally showed higher inhibitions compared with mutants 3D, 3F, and 4A.

Screening of sera of atopic patients for IgE binding to Blo t 5 mutants

To determine the prevalence of IgE binding to the identified epitopes in a larger population and effectiveness of the mutants as hypoallergens, we screened a total of 42 patient sera for IgE binding using immunodot blot. Twenty-three of the patient sera were from the KK Women's and Children's hospital in Singapore (patients KK1 to KK23), while the other 19 sera were atopic patients from Jakarta, Indonesia (patients IN1 to IN19). Results showed that mutation at residues D81, E86 or R87 caused the highest percentage (>30%) of patient sera to have a significant reduction in IgE binding (>50% reduction) (Fig. 12). This result agrees with our findings that a major putative linear IgE epitope is located at the loop region between helices α2 and α3. There could be possibilities of other minor IgE binding epitopes in Blo t 5, such as residue D41 at the loop between helices α1 and α2 and residue K102 on helix α3. Further mutations and IgE binding studies using ELISA will be required to confirm the results of this larger screen.

To test whether the triple and quadruple mutants were effective hypoallergens for most of the patients allergic to Blo t 5, we screened these mutants for binding to IgE using the sera of all 42 patients. Results showed that all the triple mutants (3C, 3D, 3E, and 3F) and especially the quadruple mutant (4A) had effectively reduced IgE binding in majority of the patients. Triple mutants 3C, 3D, 3E, and 3F achieved up to 40% reduction in IgE binding in close to 60% of the patients, while the quadruple mutant 4A caused the same level of reduction in 80% of the patients (Fig. 12). Alternatively, the quadruple mutant 4A caused up to 70% reduction in IgE binding in close to 40% of the patients. This shows that the mutant 4A is an effective hypoallergen for majority of the patients tested and the IgE binding epitopes identified are prevalent in most of the Blo t 5 allergic patients.

Discussion

B. tropicalis is a common dust mite in tropical regions, usually found together with either *D. pteronyssinus* or *D. farinae*, or both, and predominates in Singapore, Malaysia, Columbia, and Brazil (29–32). Sensitization to *B. tropicalis* is highly prevalent in terms of occurrence and is strongly associated with allergenic diseases in these countries (33, 34). The group 5 allergen, Blo t 5, is the major allergen in *B. tropicalis* and a study has shown that 43% of *B. tropicalis*-sensitive patients were allergic to Blo t 5 (9). In contrast, Der p 1 and Der p 2 are two major allergens in *D. pteronyssinus* and bound 50–65% of the IgE Ab, with only around 10% IgE bound to each of Der p 4, 5, and 7 (8). To map the exact location of IgE binding epitopes on Blo t 5, we have determined the NMR solution structure of Blo t 5 and performed site-directed mutagenesis on selected charged residues. The structure of Blo t 5 is a helical bundle comprised of three α-helices in an antiparallel fashion. This is unexpected as the homologous Der p 5 protein is predicted to have coiled-coil helical structure (10). Helical bundle structures have also been observed in major timothy grass pollen allergens Phl p 5b (35) and Phl p 6 (36). These allergens, however, form a right-handed four-helical up-and-down bundle topology instead of the three-helical bundle structure in Blo t 5. While Blo t 5 is monomeric in solution, Phl p 5b is reported to form a dimer stabilized by one intermolecular disulfide bridge (35). Attempt to predict the biological function of Blo t 5 through the search of homologous structures using the DALI server (37) has also failed. Among 987 alignments, only five have a Z-score higher than 6.0 and the highest Z-score obtained is 7.8. Percentual identicalness (%id) of majority of the alignments is also very low and below 15%. We conclude that Blo t 5 represents a novel structural fold and its physiological function cannot be determined based just on the structure. However, Blo t 5 possesses an N-terminal signal sequence and is likely to be a secretory protein rather than a structural protein, although Blo t 5 tends to aggregate at acidic pH (data not shown) similarly to the homologous Der p 5, which assemble to form a filament under acidic conditions (10). During revision of the manuscript, the NMR structure of Blo t 5 has also been reported by another research group and the two structures are essentially identical to each other (38).

Based on the structure of Blo t 5 and sequence alignment with other group 5 allergens from the house dust mite and the less common storage product mite, we have identified 21 surface exposed and charged residues for site-directed mutagenesis to map the IgE binding epitopes. By comparing the abilities of various mutants to bind to sera IgE in vitro and to induce skin prick response in vivo, we have identified four major IgE epitope residues of Blo t 5, namely, Glu76, Asp81, Glu86, and Glu91. Among these four residues, Asp81, Glu86, and Glu91 are more conserved among group 5 allergens, and they also appear to be more prevalent than residue Glu76 in IgE binding. These residues are located on a continuous stretch around the turn region connecting helices α2 and α3 on one end of the Blo t 5 molecule. The locations of these four epitope residues agrees with the previous finding that BtM, the C-terminal 72 residues fragment of Blo t 5, has almost all the allergenic epitopes of the natural Blo t 5 in the *B. tropicalis* extract (39). Furthermore, radioallergosorbent test inhibition with BtM-derived synthetic peptides showed that a peptide from residues 75–90 can inhibit 37% of IgE binding to BtM (14), indicating that the major IgE binding epitope residues could be located in this C-terminal region of the protein. A more recent study also found that 75% of Blo t 5-sensitive patients were allergic to a fragment of Blo t 5 from residues 70–117 of the protein (9).

Based on inhibition of IgE binding to the full length Blo t 5 protein by a mAb mAb 4A7, a conformational IgE binding epitope has been identified at the loop region between helices α1 and α2 (38). The epitope contains two surfaces, with residues Asn46 and Lys47 on one surface and residues Lys54 and Arg57 on the other, three out of the four residues are positively charged. Two overlapping fragments of Blo t 5, Blo t 5_{1-80} and Blo t 5_{46-117}, have been generated during the course of epitope mapping. Blo t 5_{1-80} was found to be unfolded, while Blo t 5_{46-117} existed as a dimer and structurally perturbed, both fragments were beyond recognition by mAb 4A7 (38). Blo t 5_{1-80} reacted poorly with Blo t 5-specific IgE and had very low inhibition of IgE binding to Blo t 5 (38). This agrees with our epitope mapping data as all the epitope residues, except Glu76, are not present in this fragment. In contrast, Blo t 5_{46-117} bound a substantial amount of Blo t 5-specific IgE and inhibited up to 50% IgE binding to Blo t 5. Interesting, IgE binding by Blo t 5_{46-117} could not be blocked by mAb 4A7 (38). This also agrees with our epitope mapping data and supports the presence of a linear epitope in this region of Blo t 5 that does not require intact structure for IgE binding. We had also included mutations at or around the conformational epitope as proposed by Naik et al., e.g., E45A, R57A, and E58A, but the reduction in IgE binding was not as significant and prevalent as the ones identified at the putative linear epitope. More extensive mutational study on the conformational epitope should be conducted.

Our finding that these four major epitope residues are located on a continuous stretch of the sequence leads us to believe that this is

a major putative linear epitope. Indeed, when short peptides derived from Blo t 5 were used for IgE epitope mapping, we found that three overlapping peptides covering the region Leu73 to Leu96 are most prevalent ones in IgE binding. Each of these peptides contains three of the four major epitope residues and together they constitute a single major putative linear IgE binding epitope at the C-terminal region of Blo t 5. A linear epitope for IgE binding is not uncommon in allergens. The crystal structure of the major bee venom allergen, Hyal, in complex with the Fab fragment of a mAb shows that the B cell epitope is a linear array of nine residues of Arg138 and His141 to Arg148. Surprisingly similar to Blo t 5, this linear epitope from Hyal is also located at the tip of a helix-turn-helix motive and charged residues, e.g., Arg138, Asp145, Asp146, and Arg148, are involved in direct contact with the complementarity determining regions of Fab. However, the short peptide (Arg138 to Glu152) derived from Hyal is neither recognized by mAb nor by human IgE (40). Instead of using a single linear epitope, other allergens can also employ multiple linear epitopes for IgE binding. Using overlapping peptides, the linear IgE epitopes of β-globulin, a major allergen of sesame seeds, have been mapped to residues Ser46 to Trp57 and residues Asn76 to Cys86 of the protein (41).

Another interesting finding is that both the putative linear epitope identified in this study and the conformational epitope proposed by Naik et al. (38) are clustered on two opposite ends of the elongated Blo t 5 molecule. The spatial clustering of IgE binding epitopes has also been observed at the C-terminal domain of the major timothy grass pollen allergen Phl p 1 (42). The C-terminal fragment of Phl p 1 represents a sterically oriented portion of the Phl p 1 structure and bound most of the allergen-specific IgE. This geographic distribution should facilitate the cross-linking of IgE and lead to efficient aggregation and focusing of FcεRI molecules on the cell surface (42). We propose that the clustering of IgE binding epitopes at the ends of the Blo t 5 molecule may serve a similar role in facilitating IgE cross-linking.

As the four major IgE binding epitope residues on this putative linear epitope of Blo t 5 are highly conserved among the group 5 allergens of dust mite, one should expect a high degree of cross-reactivity among these allergens. Instead, an extensive study on the IgE cross-reactivity of Blo t 5 and Der p 5 showed that Blo t 5 exhibits only low levels of IgE cross-reactivity with Der p 5 (12). In vitro fluorescent allergosorbent (FAST) inhibition and in vivo skin prick studies also show that Blo t 5 has a low cross-reactivity to Der p 5 (11, 13). The low cross-reactivity between Blo t 5 and Der p 5 may be explained by the fact that there are numbers of substitutions at this region of Der p 5. In Der p 5, the residue corresponding to the residue Glu76 of Blo t 5, one of the major epitope residue, is Leu73. Three of the less prevalent epitope residues in Blo t 5, Lys78, Arg87, and Glu92 are also replaced with Gln75, Gln84, and Met89, respectively, in Der p 5. Residues Lys78 and Arg87 of Blo t 5 were identified as prevalent epitopes during mass screening of sera from patients using immunodot blot experiments. These subtle differences are not observed in most of the other group 5 dust mite allergens. The low cross-reactivity between Blo t 5 and Der p 5 is also reported by Naik et al. as two of the epitope residues are not conserved between these homologues. Residue Lys47 in Blo t 5 is replaced with proline residues in Der p 5, whereas residue Arg57 is replaced with an alanine in Der p 5, respectively (38).

In conclusion, we have determined the solution structure of the major allergen, Blo t 5, from the dust mite *B. tropicalis*. Epitope mapping shows that Blo t 5 has a major putative linear IgE binding epitope at around the turn region connecting helices α2 and α3 and contains four charged residues as the major IgE epitope residues. The findings provide the basis for the rational design of Blo t 5 mutants with reduced allergenic activity, which could be used in the development of safer allergen-specific immunotherapy.

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Disclosures

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