

Engineered Alt a 13 Fragment of *Alternaria alternata* Abrogated IgE Binding without Affecting T-cell Stimulation

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Received: 31 March 2008 / Accepted: 15 July 2008
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Abstract

Rationale Epitopes were delineated for allergenic proteins, but studies are required to identify residues mediating IgE binding. In the present study, the in silico approach was used to identify IgE-binding residues of Alt a 13¹⁻⁵⁰ fragment and confirmed by experimental approach.

Method and Results IgE-binding epitopes of Alt a 13 mapped computationally were cloned, expressed, purified, and characterized using various immunochemical and biophysical methods. Among four fragments of Alt a 13, Alt a 13¹⁻⁵⁰ demonstrated maximum IgE binding with two immunodominant regions and was mutated at these regions. The mutation in first region, Alt a 13¹⁻⁵⁰-K4A_S6F, did not show any change in immunological and biophysical properties of protein. However, mutations in the second region, Alt a 13¹⁻⁵⁰-T21F_N27I, caused reduced IgE binding, histamine release, and low IL-4 release on stimulation of

Alternaria alternata positive patients peripheral blood mononuclear cells in vitro.

Conclusion This suggests that residues T21 and N27 are important for the secondary structure. In conclusion, Alt a 13¹⁻⁵⁰-T21F_N27I with reduced Th 2 response and intact T-cell proliferation capacity has potential for clinical use.

Keywords Allergy · IgE · lymphoproliferation · mutants · recombinant allergen

Introduction

Fungi were implicated as causative factors in respiratory allergy and asthma [1]. Prevalence of fungal allergy is about 20% to 30% among atopic individuals and up to 6% in the general population [2]. They produce a complex mixture of species-specific or cross-reactive allergens [3]. Among fungi, *Alternaria alternata* is one of the most prevalent aeroallergens worldwide, and the allergenic proteins from this fungus were characterized using in vivo and in vitro methods [4, 5]. IgE-binding epitopes of various allergenic proteins were identified using synthetic peptides spanning the entire molecule and testing their IgE reactivity [6–8]. Gene fragmentation was used to identify the IgE-binding region of Lol p VB, Hol i 5, and Per a 3 allergen [9–12]. These studies suggest that allergens vary in their ability to induce effector-cell function and have differential numbers of spatially orientated IgE-binding epitopes [12, 13].

Biophysical techniques and bioinformatics tools were employed to identify residues involved in antigen–antibody interaction of Bet v 1 and Der f 13 allergens [14, 15]. Bet v 1 six-point mutant with substitutions at 10, 12, 13, 25, 30, and 57 amino acid positions displayed reduced IgE binding

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but conserved T cell activating capacity [15]. Similarly, substitutions of important IgE-binding residues identified by NMR (E41A, K63A and K91A) in Der f 13 revealed reduced allergenicity without affecting T-cell proliferation activity [13]. Mutations at C-terminus cysteine residues in Asp f 2 showed reduced allergenicity as compared to full-length protein [16]. Ole e 1, having deletion at 135Δ10, displayed reduced allergenicity, intact T cell reactivity, and induced blocking antibodies, indicating its suitability as a candidate vaccine [17]. Thus, identification of the epitope and, more importantly, amino acids involved in IgE binding would help in molecular characterization of allergens to improve diagnostic reagents and vaccines for allergic disorders.

Previously, Alt a 13, glutathione-S-transferase (GST) from *A. alternata*, was characterized as an allergen in many extracts [18–20]. GSTs were shown as cross-reactive allergens from fungi, house dust mite, cockroach, and *Sarcoptes scabiei* [21–23]. GST, being cross-reactive, has potential for therapy in a wide spectrum of allergy patients. In the present study, epitopes and residues of Alt a 13^{1–50} involved in IgE-binding were identified using a bioinformatics approach and validated by immunoassays and clinical tests. Mutant proteins were prepared by substituting the amino acids to reduce the allergenicity for therapeutic use.

Experimental

Plasmid Constructs Polymerase chain reaction (PCR) was used for the generation of fragment proteins and mutants using different oligonucleotide primers. Plasmid containing Alt a 13 construct was used as template. Alt a 13^{1–50} was generated using GS1F fwd: 5'*GAATT*CGATGTCAGACAAACCCTCTGAA3', GS1R rev 5'AAGCT TAACTCCATCGACAACGTAAATTT3' primer pair. Alt a 13^{51–101} using GS2F fwd: 5'*GAATT*CGATGCTGCTCCTGGTTTACCGAGA3', GS2R rev: 5'AAGCTTC CAAAGGTCCCCTCCTTGA CA3' primer pair. Alt a 13^{102–152} using GS3F fwd: 5'*GAATT*CGATGGCGGGCGTAACGTGCAAG3', GS3R rev: 5'*AAGCT*TTTATAGATCTTAACCTTTCAATCG3' primer pair and Alt a 13^{153–231} using GS4F fwd: 5'*GAATT*CGATGTCACGGTTCCTACGACA3'GS4R rev: 5'*AAGCT*TCCACCGCTCCAGAGCTACAACGA 3' primer pair (restriction sites are set in italics). PCR product was purified, digested with restriction enzymes (*EcoRI* and *XhoI*), and ligated into pET22b+ vector. The ligation mix was transformed into DH-5α cells and clones were picked for plasmid isolation. Inserts were characterized by enzyme digestion, amplified using PCR, and sequenced. PCR-based site-directed mutagenesis was performed for the generation of mutants having amino-acid substitution at 4, 6, 21, and 27 residue numbers.

Double mutant Alt a 13^{1–50}K4A_S6F: A single-step PCR was carried out using the oligonucleotide GMF1 Fwd: 5'-*GAATT*CGAATTCGATGTCCGAGGCACCTTC GATCTT 3' and GS1R as reverse primer and Alt a 13^{1–50} as template. The resultant PCR product was with two-point mutations, Lysine⁴→Alanine (AAA {K}→GCA {A}) and Serine⁶→Phenylalanine (TCT {S}→TTC {F}), and the plasmid containing the insert was named as pGS5.

Mutant Alt a 13^{1–50}T21F_N27I: Three-step PCR reaction was performed to generate this mutant. Initial PCR was carried out with an internal sequence-specific oligonucleotide GMF3 Fwd: 5'AAAGGCTTCGCCACGTCCACACACA CATTAC CGTC3' and GS1R reverse primer. In the second step, GMR2 Rev: 5'GACGGTA ATGTGTGTGGACGT GGCGAAGCCTTT3' and GMF2 Fwd: 5'*T*CGAATTCGAT GTCCGAGCGACCCTCC 3' primer pair was used to obtain the PCR product. In the final step, PCR products of the above two steps were purified, mixed, and amplified using oligonucleotide GMF2 as forward and GS1R reverse primers leading to two point mutations threonine²¹→phenylalanine (ACA {T}→TTT {F}) and asparagine²⁷→isoleucine (AAC {N}→ATC {I}). Plasmid encoding for Alt a 13^{1–50} was used as template in all the above reactions. Resultant plasmid containing the insert was designated as pGS6.

Double mutant Alt a 13^{1–50}K4A_S6F_T21F_N27I: The mutant was generated by a single-step PCR reaction using pGS6 as template. GMF1 as forward and GS1R reverse primer pair was used to obtain the PCR product. The resultant PCR product was with four point mutations, lysine⁴→alanine (K4A), serine⁶→phenylalanine (S6F), threonine²¹→phenylalanine (T21F), and asparagine²⁷→isoleucine (N27I). Resultant plasmid containing the insert was designated as pGS7.

Protein Expression and Purification Alt a 13 was expressed and purified using glutathione Sepharose-4B affinity resin as described previously [22]. Fragment and mutant proteins were expressed as His-tag protein and purified using Ni-NTA resin.

Prediction of Epitope(s) The probable antigenic index of Alt a 13 fragment was predicted using PROTEAN module of DNASTAR software. A combination of algorithms was used to estimate flexibility, hydrophilicity, charge, and surface availability of the query sequence residues [24]. Amino acids in predicted epitope of Alt a 13^{1–50} were replaced by their opposite counterparts, e.g., neutral with charged residue, hydrophobic with hydrophilic, acidic with basic, and vice-versa to see the effect on net antigenic index. Substitutions that indicated lowering of predicted antigenic index were further confirmed by other softwares like ABCpred, BCIpep, etc. [25], and finally chosen for mutation.

Skin Testing and Sera Collection Intradermal tests were performed with *A. alternata* extract (1:500 w/v), Alt a 13 (10 µg/ml), Alt a 13¹⁻⁵⁰ (10 µg/ml), Alt a 13¹⁻⁵⁰K4A_S6F (10 µg/ml), and Alt a 13¹⁻⁵⁰T21F_N27I (10 µg/ml) on respiratory allergy patients at V.P.Chest Institute, Delhi. Phosphate buffer saline and histamine diphosphate (100 µg/ml) were used as negative and positive controls, respectively. Skin tests were graded as per the wheal size of positive control [22]. Three normal volunteers were also tested with *A. alternata* extract, purified protein, and fragments/mutants. Written informed consent was obtained from the subjects and the Institutional Ethics Committee approved the study protocol.

Circular Dichroism Circular dichroism (CD) was performed to determine the structure of Alt a 13 fragments and mutant proteins. It was measured on a spectropolarimeter (Jasco, Easton, MD, USA) using a 0.1-cm cell at 25°C over 190–300 nm wavelength [22].

IgE Reactivity of Alt a 13, Fragments, and Mutant Proteins Immunoblots of the fragments (Alt a 13¹⁻⁵⁰, Alt a 13⁵¹⁻¹⁰¹, Alt a 13¹⁰²⁻¹⁵², and Alt a 13¹⁵³⁻²³¹) and mutants (Alt a 13¹⁻⁵⁰K4A_S6F, Alt a 13¹⁻⁵⁰T21F_N27I, and Alt a 13¹⁻⁵⁰K4A_S6F_T21F_N27I) were performed as described earlier [22].

To determine specific IgE, ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 ng of Alt a 13, fragment proteins, mutant proteins, and 1 µg of *A. alternata* extract in 100 µl of 10 mM carbonate buffer (pH 9.6). The plates were developed and read at 490 nm [22].

To determine the binding affinity of the fragments and mutant proteins to the IgE antibodies, ELISA inhibition was performed. Alt a 13 (100 ng) was coated onto the wells. The plate was washed and kept overnight with preincubated sera (100 µl) and 100 µl of different concentrations of fragments and mutants (0.001–10 µg each). The plates were processed as described in ELISA.

Histamine Release on Allergen Challenge Histamine assay was carried out using ELISA kit following the manufacturer's instructions (Immunotech, Marseilles, France). Briefly, heparinized blood samples from *Alternaria*-hypersensitive patients ($n=12$) were challenged with 5 ng of allergen extract, Alt a 13, Alt a 13¹⁻⁵⁰, or mutant proteins for 30 min at 37°C [22]. Heparinized blood from three nonallergic individuals was used as negative control.

Peripheral Blood Mononuclear Cells Proliferation and Cytokine Analysis on Antigen Challenge Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood ($n=12$) by Ficoll hypaque centrifugation (Sigma, St. Louis, MO, USA). For lymphoproliferation assays, 10⁵ cells/well were cultured in RPMI-1640 media supple-

mented with 10% FBS and challenged with 1 and 10 µg of allergen extract, Alt a 13, fragment, or mutant proteins for 72 h at 37°C. Phytohemagglutinin (5 µg) was used as positive control and RPMI-1640 alone served as negative control. Plates were incubated for 72 h at 37°C. Cells were washed, incubated for 2 h with MTT (100 µg/ml), lysed in acidic isopropanol, and read at 570 nm [22].

IL-4, IL-5, and IFN-γ were estimated by ELISA kits following the manufacturer's protocol (BD Pharmingen OptEIA kits). Standards (5–500 ng), 100 µl of sample (1:2), or controls were added to wells and incubated at 37°C for 3 h. The plates were washed, incubated with biotinylated detector antibody, and color-developed. OD values $\geq$ three times of the negative control were considered positive for both PBMCs proliferation and cytokine levels.

Structural Analysis of Alt a 13, Alt a 13¹⁻⁵⁰, and Mutant Proteins Various web-based servers were used to analyze the secondary structure and solvent accessibility of the Alt a 13, fragment, and mutant proteins [DPM, GOR1, GOR3, HNNC, MLRC, PHD, and SOPM, available at (<http://npsa-pbil.ibcp.fr>), PROTSKALE (<http://www.expasy.org/tools/protscale>), PREDICT (<http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html>), and PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/psiform.html>)].

Results

Physicochemical and Immunological Characterization of Alt a 13 and Fragment Proteins Alt a 13 purified using glutathione Sepharose-4B resin yielded 4.5 mg/l of bacterial culture. Yield of Alt a 13¹⁻⁵⁰, Alt a 13⁵¹⁻¹⁰¹, and Alt a 13¹⁰²⁻¹⁵² fragments purified using Ni-NTA resin was 5, 3.5, and 4 mg/l of bacterial culture, respectively. They migrated as homogenous single band with an apparent size of 6-kDa, whereas Alt a 13¹⁵³⁻²³¹ yield was 4.5 mg/l of bacterial culture and migrated at 8-kDa (data not shown).

All the 15 *A. alternata* hypersensitive patients' sera used for the study showed ≥ 3 times specific IgE values than controls. Out of these, nine patients showed high IgE binding (≥ 3 times of control) to Alt a 13, seven patients with Alt a 13¹⁻⁵⁰, and one patient with Alt a 13¹⁰²⁻¹⁵² by ELISA. Alt a 13⁵¹⁻¹⁰¹ and Alt a 13¹⁵³⁻²³¹ showed IgE values similar to control in all the patients (Table 1). Intradermal test and specific IgE values correlated very well. Alt a 13 and Alt a 13¹⁻⁵⁰ required 3.5 ng to achieve EC₅₀, whereas other fragments could not inhibit 50% binding of human IgE to Alt a 13 up to 10 µg protein concentration (Fig. 1a).

CD spectra of Alt a 13, Alt a 13¹⁻⁵⁰, and Alt a 13¹⁰²⁻¹⁵² showed minima around 210 and 265 nm, respectively, indicating ordered secondary structure. In contrast, Alt a

Table 1 Specific IgE Binding of *A. alternata* Extract, Alt a 13 Protein, Fragments and Mutants in Respiratory Allergy Patients

S. No.	Age/sex/ Clinical History	Sp. IgE values (OD _{490 nm}) ^a								Intradermal test reactions				
		<i>Alternaria</i> extract	Alt a 13	Alt a 13 ¹⁻⁵⁰	Alt a 13 ⁵¹⁻¹⁰¹	Alt a 13 ¹⁰²⁻¹⁵²	Alt a 13 ¹⁵³⁻²³¹	Alt a 13 ¹⁻⁵⁰ K4A_S6F	Alt a 13 ¹⁻⁵⁰ T21F_N27I	<i>Alternaria</i> extract	Alt a 13	Alt a 13 ¹⁻⁵⁰	Alt a 13 ¹⁻⁵⁰ K4A_S6F	Alt a 13 ¹⁻⁵⁰ T21F_N27I
1	40/F/AR	0.412	0.356	0.323	0.103	0.108	0.098	0.343	0.098	++	++	++	++	-
2	28/M/AR	0.486	0.412	0.113	0.103	0.182	0.082	0.103	0.082	++	++	++	-	-
3	28/F/AR	0.789	0.589	0.567	0.106	0.312	0.112	0.662	0.112	+++	++	++	++	-
4	25/F/AR,BA	0.478	0.232	0.207	0.133	0.148	0.118	0.133	0.118	++	++	++	-	-
5	23/M/BA	0.601	0.489	0.365	0.098	0.098	0.098	0.398	0.098	++	++	++	++	-
6	22/F/AR	0.498	0.409	0.389	0.173	0.156	0.056	0.373	0.056	++	++	++	++	-
7	22/F/AR,BA	0.382	0.21	0.098	0.101	0.216	0.076	0.101	0.076	++	++	++	++	-
8	42/F/BA	0.411	0.108	0.102	0.088	0.176	0.076	0.088	0.076	++	-	-	-	-
9	35/F/AR	0.297	0.256	0.105	0.124	0.098	0.098	0.124	0.098	++	++	-	-	-
10	60/M/AR	0.578	0.569	0.523	0.113	0.127	0.087	0.513	0.087	++	++	++	++	-
11	38/F/AR,BA	0.609	0.516	0.489	0.156	0.117	0.078	0.456	0.078	+++	++	++	++	-
12	18/F/AR	0.313	0.096	0.092	0.101	0.077	0.067	0.101	0.067	++	-	-	-	-
13	23/F/AR	0.689	0.408	0.158	0.107	0.066	0.056	0.107	0.056	++	++	-	-	-
14	29/M/AR	0.384	0.098	0.101	0.104	0.108	0.098	0.104	0.098	++	-	-	-	-
15	18/M/AR	0.402	0.345	0.356	0.096	0.087	0.098	0.296	0.098	++	++	++	-	-
C1	31/F	0.101	0.112	0.121	0.098	0.128	0.098	0.098	0.098	-	-	-	-	-
C2	27/F	0.098	0.101	0.088	0.078	0.076	0.076	0.078	0.076	-	-	-	-	-
C3	39/F	0.101	0.115	0.108	0.113	0.088	0.098	0.113	0.098	-	-	-	-	-

AR allergic rhinitis, BA bronchial asthma

^a Mean O.D. for C1–C3, *Alternaria* extract, 0.1; Alt a 13, 0.109; Alt a 13¹⁻⁵⁰, 0.106; Alt a 13⁵¹⁻¹⁰¹, 0.098; Alt a 13¹⁰²⁻¹⁵², 0.101; Alt a 13¹⁵³⁻²³¹, 0.0098; Alt a 13¹⁻⁵⁰ K4A S6F, 0.099; and Alt a 13¹⁻⁵⁰ T21F N27I, 0.097

13⁵¹⁻¹⁰¹ and Alt a 13¹⁵³⁻²³¹ showed substantial loss of structure (Fig. 2). Alt a 13¹⁻⁵⁰ showed immunological reactivity and biophysical properties equivalent to Alt a 13 and was chosen for mutational analysis.

In Silico Epitope Mapping Alt a 13¹⁻⁵⁰ showed two probable epitopes between positions 1–10 and 20–30 of Alt a 13¹⁻⁵⁰. These two immunodominant regions were predicted to be on a highly flexible region. These regions were hydrophilic, charged, and solvent-accessible as predicted. These regions were disrupted by double mutations to modify their predicted antibody-binding capacity. Changes from K4A, S6F and T21F, and N27I lowered the predicted antigenic index in both the epitopes while all four changes drastically reduced the predicted antigenic index of the entire fragment (data not shown).

Purification and CD Spectra of Mutant Proteins Alt a 13¹⁻⁵⁰ K4A_S6F, Alt a 13¹⁻⁵⁰ T21F_N27I, and Alt a 13¹⁻⁵⁰ K4A_S6F_T21F_N27I yielded 6.5, 5, and 6 mg protein/l of bacterial culture, respectively, and migrated as a single band at 6-kDa (data not shown). CD spectra revealed a similar structure for the mutant Alt a 13¹⁻⁵⁰-K4A_S6F and Alt a 13¹⁻⁵⁰ with minima around 210 and 265 nm. However, mutant protein Alt a 13¹⁻⁵⁰T21F_N27I and Alt a 13¹⁻⁵⁰K4A_S6F_T21F_N27I displayed loss of structure (Fig. 2).

Allergenicity Assessment of Alt a 13, Alt a 13¹⁻⁵⁰, and Mutant Proteins

ELISA with seven patients sera showed high IgE values to mutant Alt a 13¹⁻⁵⁰K4A_S6F, but Alt a 13¹⁻⁵⁰T21F_N27I or Alt a 13¹⁻⁵⁰K4A_S6F_T21F_N27I showed IgE values that were the same as the control, with all the patients' samples ($n=15$) demonstrating that these mutant proteins lack IgE-binding epitope. *In vivo* and *in vitro* reactivity of Alt a 13, Alt a 13¹⁻⁵⁰, and mutant Alt a 13¹⁻⁵⁰K4A_S6F correlated well except for patients 2, 4, and 15 (Table 1).

Binding affinity of Alt a 13¹⁻⁵⁰K4A_S6F (4.1 ng) was similar to Alt a 13¹⁻⁵⁰. Alt a 13¹⁻⁵⁰T21F_N27I or Alt a 13¹⁻⁵⁰K4A_S6F_T21F_N27I could not achieve EC_{50} with 10 μ g protein, suggesting that mutations have significantly lowered IgE binding capacity (Fig. 1a).

Out of 12 skin-test-positive patients, nine showed positive histamine release (>10%) with Alt a 13, whereas six of those patients showed positive release with Alt a 13¹⁻⁵⁰ and mutant Alt a 13¹⁻⁵⁰K4A_S6F. Histamine release with Alt a 13¹⁻⁵⁰T21F_N27I was similar to control, indicating that biological function of the mutant was inhibited (Fig. 3). Significant correlation was observed between histamine release and specific IgE for Alt a 13 ($R=0.787$, $p<0.01$), Alt a 13¹⁻⁵⁰ ($R=0.765$, $p<0.01$), and Alt a 13¹⁻⁵⁰K4A_S6F ($R=0.654$, $p<0.01$). Patient 3, 4, and 9 among the nine patients

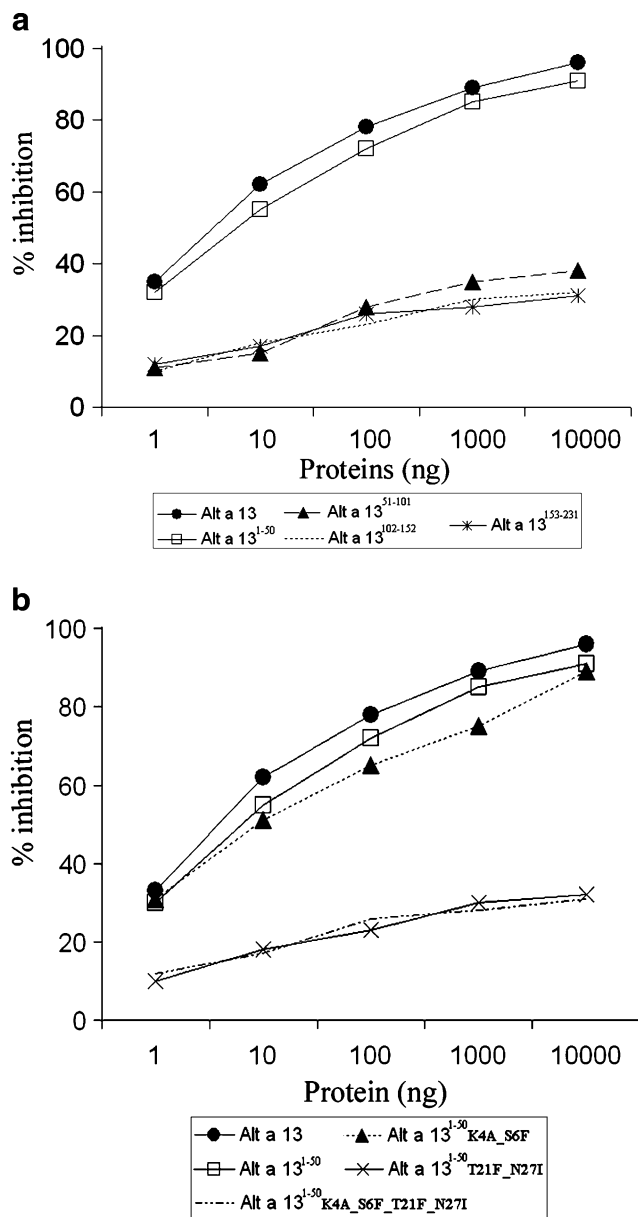


Fig. 1 ELISA inhibition: ELISA inhibition was carried using **a** fragments or **b** mutants as inhibitors to quantitate the binding affinity of fragments to patients' IgE as compared to Alt a 13. Alt a 13 (100 ng) was coated on solid phase and was incubated with the preincubated mixture of human sera (1:10) with different concentration of fragments (0.001–10 μ g each) overnight at 4°C. Plate was further incubated with anti-human IgE-HRP (1:1,000) at 37°C for 3 h. After incubation, plate was washed and developed using *o*-phenylenediamine dihydrochloride and read at 490 nm

giving positive histamine release to Alt a 13 showed variation between IgE reactivity and histamine release for Alt a 13¹⁻⁵⁰ and Alt a 13¹⁻⁵⁰ K4A_S6F. The normal controls ($n=3$) with negative skin test to fragments and mutants demonstrated 5–7% histamine release.

Alt a 13, Fragment, and Mutant Proteins Induced PBMCs Proliferation PBMCs of *A. alternata*-sensitized patients

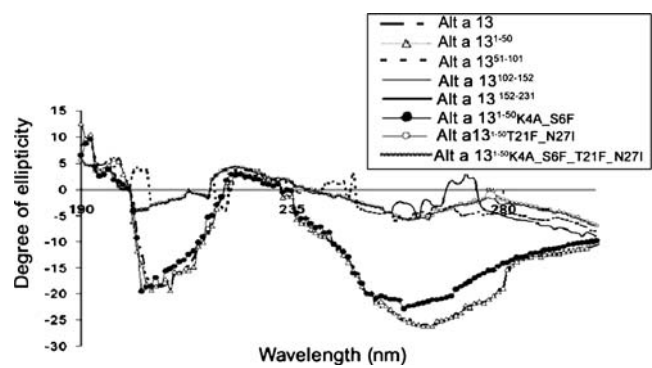


Fig. 2 CD spectra of Alt a 13, fragment proteins and mutant proteins: Proteins/fragments were taken at a concentration of 1 mg/ml and spectra recorded in 20 mM PBS (pH 7.4) in far UV range (190–300 nm) at 25°C. Values are expressed as mean residual ellipticity and is the average of 20 scans

were stimulated with 1 and 10 μ g of full-length protein, fragments, or mutant proteins. Ten micrograms of these proteins/fragments/mutants gave higher stimulation as compared to 1 μ g. Alt a 13 showed higher T cell reactivity as compared to others, as demonstrated by proliferation of PBMCs isolated from *A. alternata*-sensitized patients (11/12). All the fragments were also able to stimulate PBMCs, with Alt a 13¹⁵³⁻²³¹ showing proliferation in a large number of patients (11/12), followed by Alt a 13¹⁰²⁻¹⁵² (10/12) and Alt a 13¹⁻⁵⁰ and Alt a 13⁵¹⁻¹⁰¹ (9/12). For mutants, Alt a 13¹⁻⁵⁰K4A_S6F showed T cell reactivity in nine out of 12 patients, while Alt a

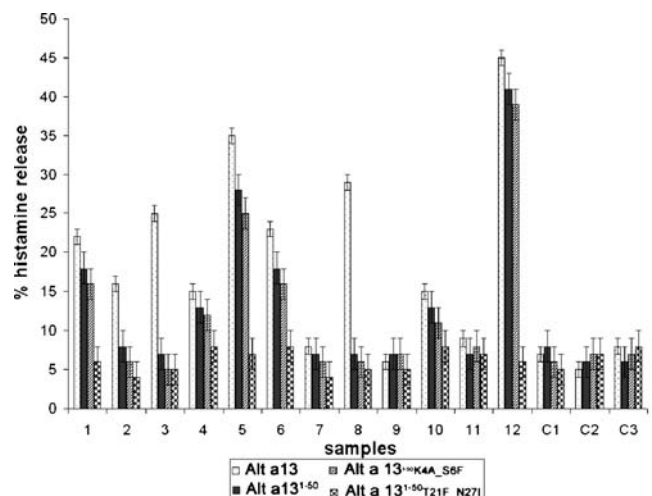


Fig. 3 Histamine release assay: Heparinized blood of *Alternaria*-hypersensitive patients ($n=12$) or healthy controls (C1–C3) were challenged with 5 ng of Alt a 13, Alt a 13¹⁻⁵⁰, Alt a 13¹⁻⁵⁰K4A_S6F, or Alt a 13¹⁻⁵⁰T21F_N27I for 30 min at 37°C. Histamine standards (0–100 nM) and released histamine from the samples were acylated and measured by ELISA. Spontaneous histamine release in unchallenged sample and total histamine was measured. The control samples were drawn from healthy individuals. Histamine release was calculated as percentage release from the standard curve. The graph represents mean $\pm$ SD (bars) of triplicate readings

13¹⁻⁵⁰T21F_N27I and Alt a 13¹⁻⁵⁰K4A_S6F_T21F_N27I showed stimulation in eight patients' PBMCs (Fig. 4). These data demonstrated that T cell reactivity of the mutants was retained even after replacement of the specific residues.

IL-4 release was high in most of the patients' samples with Alt a 13 (11/12), Alt a 13¹⁻⁵⁰ (9/12), or Alt a 13¹⁻⁵⁰K4A_S6F (7/12), whereas Alt a 13¹⁻⁵⁰T21F_N27I secreted low levels of IL-4 similar to controls (Fig. 5a). IFN- γ levels were low for Alt a 13, fragments, or mutants (Fig. 5b). The control subjects showed nondetectable levels of IL-4 and low levels of IFN- γ for Alt a 13, fragment, or mutants.

Structural Analysis of Alt a 13, Alt a 13¹⁻⁵⁰ and Mutants Predicted α -helical and β -sheet content of Alt a 13 (α , 44%; β , 43.3%), Alt a 13¹⁻⁵⁰ (α , 42.5%; β , 41.1%) and Alt a 13¹⁻⁵⁰-K4A_S6F (α , 39.5%; β , 39.8%) were similar with less random coil. However, random coil conformation (>50%) dominated the secondary structure of Alt a 13¹⁻⁵⁰-T21F_N27I and Alt a 13¹⁻⁵⁰-K4A_S6F_T21F_N27I.

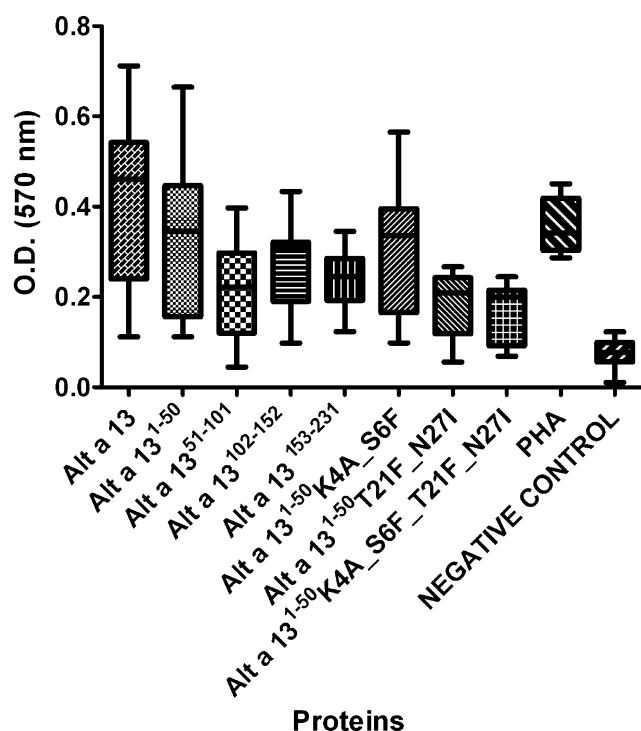


Fig. 4 Lymphoproliferation assay: PBMCs were isolated from heparinized blood of *Alternaria*-hypersensitive patients ($n=12$) and healthy controls ($n=3$) by Ficoll hypaque (Sigma) density gradient method. The 10^5 cells/well were cultured in RPMI media with 10% fetal calf serum and challenged with 10 μ g of Alt a 13, fragments or mutants. Phytohemagglutinin (PHA 5 μ g) was used as positive control for stimulation while cells grown in the absence of any stimulant were taken as negative control. Plates were incubated for 72 h. Cells were washed, incubated for 2 h with MTT (100 μ g/ml), lysed in acidic isopropanol, and read at 570 nm. Controls (mean) values were subtracted from the individual values of the patients and represented as box and whiskers plot. Means $\pm$ SEM of triplicate readings are presented. Values are plotted from minima to maxima

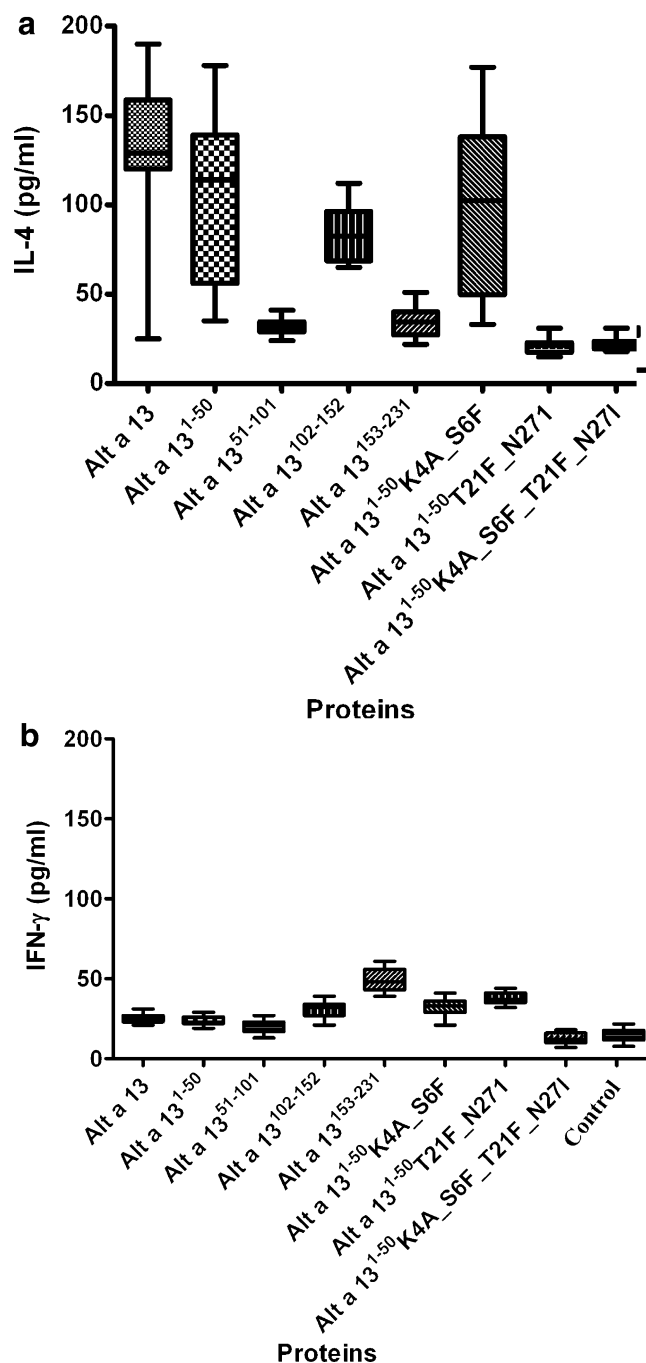


Fig. 5 Cytokines on allergen stimulation in PBMCs supernatant: **a** IL-4 and **b** IFN- γ were assayed in supernatants of PBMCs cultured in the presence of Alt a 13, fragments or mutants. The capture antibody (1:250 dil.) for each cytokine was coated on microtiter plates. Standards (5–500 ng), 100 μ l of diluted sample (1:2), or controls were added to wells and incubated at room temperature for 2 h. After washing, 100 μ l of biotinylated anti-human IL-4 antibody containing Streptavidin-horseradish peroxidase (1:250 dil.) was added to the well for 1 h at room temperature. After incubation, plate was developed using TMB and read at 450 nm. For IL-4, control (mean) values were subtracted from the patients' reading. Means $\pm$ SEM are presented as box and whiskers plot. For IFN- γ , control mean values are plotted along with the patients' mean values since the readings were almost equal for the patients and controls. Values are plotted from minima to maxima

Predicted solvent accessibility of Alt a 13¹⁻⁵⁰-K4A_S6F was 60%, whereas it decreased to 30% for Alt a 13¹⁻⁵⁰-T21F_N27I (data not shown).

Discussion

Various approaches have been used to map IgE-binding epitopes and identify residues involved in antigen–antibody interaction. These include epitope mapping by chemically synthesized overlapping peptides derived from the primary sequence, resolution of the structure of antigen–antibody complexes, site-directed mutagenesis, selection of peptide mimics, and use of computer-based algorithms [26, 27]. The knowledge about IgE binding epitopes and residues involved in antibody interaction helps in molecular characterization of allergen and delineation of allergic mechanism for better therapeutic options. In the present study, IgE-binding epitopes of Alt a 13¹⁻⁵⁰ fragment were identified using bioinformatics approach and validated experimentally.

Allergen molecules with reduced IgE-binding have less risk of IgE-mediated adverse reactions during immunotherapy. Previously, hypoallergenic variants of Bet v 1, a major allergen of *Betula verrucosa* were used successfully for immunotherapy [28]. The folding variants developed had >100-fold reduced allergenicity than wild-type Bet v 1. In the present study, fragmentation of Alt a 13 into four fragments led to the identification of N-terminal fragment (Alt a 13¹⁻⁵⁰) as maximum IgE binding region followed by fragment 3 (Alt a 13¹⁰²⁻¹⁵²). Alt a 13⁵¹⁻¹⁰¹ and Alt a 13¹⁵³⁻²³¹ fragments did not exhibit IgE-binding maybe due to the disruption of structure by fragmentation or changes in sequential/conformational epitopes.

Amino acid(s) involved in antigen–antibody interaction has been determined using computer-based algorithm, biophysical methods and by 3-dimensional structural analysis of antigen–antibody complex [13, 14]. Mutation in antibody interacting residues of Der f 13 (E41A_K63A_K91A) showed reduced IgE binding and histamine release and exhibited properties of a third-generation vaccine [14]. Similarly, IgE-binding residues of Bet v 1 were identified computationally and the mutant(s) synthesized showed reduced in vivo and in vitro reactivity [13]. In the present study, two predicted immunodominant regions in Alt a 13¹⁻⁵⁰ were mutated by two amino acids each. Mutations at amino acids 4 and 6 caused no effect on IgE-binding and other biological functions whereas replacement of amino acid 21 and 27 in Alt a 13¹⁻⁵⁰ significantly reduced IgE binding, histamine release, skin reactivity, and IL-4 levels. However, these substitutions did not affect the lymphoproliferative activity of Alt a 13¹⁻⁵⁰T21F_N27I as it could stimulate PBMCs proliferation in approximately 67% (8/12) of *A. alternata* sensitized patients' samples. IL-4 inducing capacity of Alt a 13¹⁻⁵⁰T21F_N27I

was significantly reduced in culture supernatant of patients' PBMCs. The observations suggest that mutations have reduced its IgE-affinity without affecting the T cell reactivity. Alt a 13¹⁵³⁻²³¹ also represents a potential fragment lacking IgE-binding but with intact T-cell stimulating activity. Furthermore, in vitro exposure with this fragment released low IL-4. In natural conditions, the potential threats of adverse reactions are from the IgE-binding epitopes of Alt a 13, and thus, a molecule having reduced IgE-binding would be clinically significant.

Verdino and Keller (2004) had shown that disruption of structure reduces the IgE binding capacity of allergen [29]. Mutations in one epitopic domain of Hev b 5 showed no effect on IgE binding, while mutation in the second domain significantly reduced the immunological reactivity of the protein [30]. In the present study, substitutions in the first region of Alt a 13¹⁻⁵⁰ did not affect the structure, as evident from the CD spectra, whereas mutants having substitutions in the second region displayed secondary structure completely different from the original fragment. Computational analysis also showed that both Alt a 13¹⁻⁵⁰K4A_S6F and Alt a 13¹⁻⁵⁰ have high percentages of α -helix and β -sheet elements with less of random coil, thereby imparting them an ordered structure. Alt a 13¹⁻⁵⁰ with mutation at positions 21 and 27 demonstrated secondary structure completely different from the original fragment and had reduced solvent accessibility. These observations suggest that Thr21 and Asn27 might be important residues for maintaining the structural integrity and the conformation of Alt a 13¹⁻⁵⁰ fragment. Some of the IgE-binding epitopes of Alt a 13 might be lost due to fragmentation possibly by disruption of IgE-binding domains.

The purified allergens have shown potential for diagnosis and therapy of allergic disorders but have risk of anaphylactic reactions at high concentrations [31, 32]. Proteins with reduced IgE binding and maintained T-cell activity were suggested for safe and effective immunotherapy [33, 34].

In conclusion, Alt a 13¹⁻⁵⁰T21F_N27I with reduced Th 2 response and potent T-cell proliferation capacity seems safe for allergen vaccine. Further studies with Alt a 13¹⁻⁵⁰ fragments and mutants in humans will have a potential for therapy of fungal allergy.

Acknowledgements Jay Shankar is an SRF from Council for Scientific and Industrial Research, New Delhi. Thanks are due to Sh. Dinesh Chandra, V.P.Chest Institute, Delhi for help in skin tests on patients. We sincerely thank the Council for Scientific and Industrial Research for the financial support for the study.

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