

IgE binding synthetic peptides of Alt a 1, a major allergen of *Alternaria alternata*

Viswanath P. Kurup^{a,b,*}, Hari M. Vijay^c, Veena Kumar^c, Laura Castillo^{a,b}, Nancy Elms^{a,b}

^a Allergy-Immunology Division, Medical College of Wisconsin, Milwaukee, WI 53226, USA

^b VA Medical Center, Research Service 151-I, 5000 West National Avenue, Milwaukee, WI 53295, USA

^c Environmental Health Directorate, Health Canada, Ottawa, Ont., Canada K1A 0L2

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Abstract

Alternaria alternata protein, Alt a 1 is a major allergen associated with allergy in atopic patients. Although the molecule binds strongly to IgE antibody from patients, the epitopes involved have not been identified or defined. In the present study, we synthesized overlapping peptides spanning the whole sequence and evaluated their IgE binding with sera from patients with *Alternaria*-induced allergy. The results identified four IgE binding linear regions. Two of these regions K41-P50 and Y54-K63 showed consistent reactivity with all four patients studied. The specific epitopes involved in the immune response may be of value in the immunodiagnosis and probably also in specific immunotherapy.

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1. Introduction

Over 20% of the population in the industrialized nations are affected by IgE-mediated allergic diseases including asthma, rhinitis, and conjunctivitis [4,5,14]. A number of environmental contaminants such as pollen, mites, molds, and other soluble proteins have been implicated in these allergic diseases. Several species of plant pollens, insects, and fungi have been shown to cause IgE mast cell-mediated allergies [4,14]. Among the fungi associated with allergic disorders, *Alternaria* sp. is the one of the most frequently encountered species [9]. Crude antigen from the mycelial and culture filtrate extracts has been used to determine specific IgE in in vitro and in vivo testing [6,19]. Until recently, the antigen used for the diagnosis of *Alternaria*-induced allergy and asthma has been the crude culture filtrate and mycelial extracts of *A. alternata* [20]. The results obtained from such uncharacterized non-standardized preparations are not altogether dependable and reliable due to the complexity and irrelevancy of the components and to the differences in their production. However, in recent years a number of genes encoding the antigens have been genetically cloned

and the protein expressed [14]. On evaluation, several of the recombinant proteins were found to be useful in more reliable diagnosis [7,8].

About 10 genes encoding allergens from *A. alternata* have been cloned, sequenced, and expressed [3]. All these expressed allergens showed varying degree of reactivity with the IgE from patients with *Alternaria*-induced allergy and asthma. Among these allergens, Alt a 1 is a major allergen present in mycelial and culture filtrate extracts in appreciable quantities [6,8]. This allergen is not only the predominant component in the crude extract, but also is the most reactive against patients' sera.

Allergens isolated from molds or from other sources show diverse biochemical characteristics as some are proteins, while others are carbohydrates or glycoproteins [11,16]. It has been shown that several enzymes from fungi have markedly enhanced allergenicity. In addition to sharing similar enzyme activities, a number of allergens from fungi also share similar epitopes demonstrating comparable binding to IgE [2]. By identifying and understanding such epitopes, through immunochemical and other characterization may yield significant information for selecting better diagnostic and therapeutic reagents.

Antigen specific immunotherapy used currently has a number of drawbacks. They can possibly cause anaphylaxis. However, through saturating the IgE antibody bound to cell

* Corresponding author. Tel.: +1-414-384-2000x41459/41510;

fax: +1-414-382-5374.

E-mail address: vkurup@mcw.edu (V.P. Kurup).

surface receptors using synthetic peptide epitopes, the IgE cross-linking can be prevented and the activation of mast cells and basophils can be averted. Subsequent exposure to antigens by the immune system will then lead to enhanced production of blocking IgG antibodies, which in turn prevent or lessen the allergic response in the patients [18]. Recently, it has been reported that a 20 mer peptide representing the N-terminus of Alt a 1 is an antibody binding site [23].

In the present study, we evaluated *A. alternata*, Alt a 1, for the IgE binding linear epitopes. Overlapping decapeptides spanning Alt a 1 sequence have been synthesized and the reactivity with serum IgE from *Alternaria* allergic patients evaluated. The results identified five IgE binding regions in Alt a 1 molecule, of which two IgE antibody binding epitopes showed consistent reactivity with the *Alternaria* allergic patients.

2. Materials and methods

2.1. Serum samples

Atopic asthma patients showing skin test reactivity to commercial *A. alternata* antigens were selected for the study. After ascertaining the clinical symptoms the sera were studied for specific IgE against *Alternaria* antigen. Sera showing elevated IgE titers to *Alternaria* antigens by ELISA were selected for epitope mapping studies. Normal control sera were also used to determine the reactivity with the synthetic peptides, Alt a 1 and crude *Alternaria* antigen. All blood samples were obtained from patients after Institutional Research Review Committee approval.

2.2. Culture of *A. alternata*

The isolate 34-016 of *A. alternata* was obtained from Dr. E.G. Simmons, Mycological Services, Amherst, MA, USA and grown on synthetic revised tobacco medium (RTM) as reported earlier [21]. The culture was incubated for 17 days after which the pellicles were collected, washed with acetone and air-dried.

2.3. Preparation of spore and mycelial extract

The mold pellicles were homogenized and extracted as described earlier [21] with slight modifications. Briefly, the mycelial mat was extracted twice with 1:15 (w/v) Tris saline buffer for 48 h at 4 °C. The suspension was centrifuged at $1000 \times g$ at 4 °C for 20 min to remove traces of suspended particles. The resulting supernatant was concentrated and washed with water using Amicon YM-5 membrane. The retentate was filtered through 0.45 µm and lyophilized. Antigens were characterized for protein content by dye-binding assay and molecular weight by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as described earlier [19].

2.4. Native Alt a 1

Native Alt a 1 allergen was purified from the freeze-dried antigen prepared above from the spores and mycelium of *A. alternata* was further purified using ultra filtration and gel filtration using Sephadex G-100 columns [6]. The native antigen thus obtained was characterized by SDS–PAGE and by their reactivity with IgE in the sera of patients allergic to *Alternaria*, by ELISA and Western blot.

2.5. Recombinant Alt a 1

A. alternata was cultured in RTM as described previously [21]. From a 7-day-old culture the RNA was isolated using guanidine isothiocyanate method [7]. Purification of RNA and mRNA preparation as well as cDNA library in γ gt 11 construction was carried out by a commercial firm (Clontech, CA).

2.6. Antisera and immunoscreening

Polyclonal rabbit antiserum was prepared against Alt a 1 that was partially purified by Whatman DE-52 ion exchange chromatography and Sephadex G-100 gel filtration, and used in immunoscreening and immunoblots at 1:800 dilution in TBST buffer as reported previously [6,7]. In order to detect allergenicity of the clones, the library was screened using pooled atopic sera having specific IgE antibodies to *A. alternata* using standard protocols [7]. The cDNA encoding Alt a 29 (Alt a 1) was cloned and the major allergen expressed.

2.7. Synthesis of decapeptides

Decapeptides were synthesized on derivatized cellulose membrane using F-moc amino acids according to the procedure recommended by the manufacturers [2,3,13] (SPOTs, Genosys Biotechnologies Inc., The Woodlands, TX). SPOTs scan software program was used to generate amino acid sequence of the decapeptides and schedule for the coupling cycles. Initially the peptides were synthesized with five amino acid overlaps. The free amino functional group present on the spot was used in the synthesis of the various peptides and the carboxyl group of the N-terminal amino acid of the peptide was linked with the NH₂ group for the F-moc amino acid on the particular spot on the membrane. Based on published amino acid sequence of Alt a 1, decapeptides with five amino acid overlaps were synthesized covering the whole length of the 157 amino acid residues of the molecule [7,8]. Once the IgE binding regions were identified the peptides were synthesized with one amino acid offset and nine amino acids overlap to define the specific epitope. All the identified IgE binding epitopes were further evaluated using sera from atopic patients and control sera.

2.8. IgE binding to the synthetic peptide

After the synthesis of various Alt a 1 peptides, the membranes were blocked overnight at 4°C with phosphate buffered saline (PBS, pH 7.4) containing 5% non-fat dry milk. These membranes were washed three times with PBS containing 0.1% Tween-20 and incubated with 1:10 dilution of the pooled sera. The serum pool was constituted by mixing three patients with allergic asthma demonstrating high IgE antibody against *A. alternata* allergens by RAST. After incubation, the membranes were washed three times with PBS Tween-20 (PBST) and allowed to react with biotinylated goat anti-human IgE. This reagent was diluted 1:1000 with PBST and the membrane was incubated for 1 h at room temperature followed by washing three times as mentioned earlier. The membrane was then incubated with 1:27,000 dilution of streptavidin alkaline phosphatase for 1 h. After washing, the reactivity was determined by developing the color using nitro blue tetrazolium-5-bromo-4-chloro 3-indolylphosphate (BioRad Laboratories, Richmond, CA) as described before [2,3,13]. The intensity of the color reaction on the SPOT membrane was analyzed semi-quantitatively using a densitometric scanner [2,13]. The optical density values of the SPOTs of asthmatics and normal controls were compared. The various epitopes recognized were again synthesized on the membranes as described earlier and their reactivity with patients and controls were repeatedly studied and the results ascertained.

2.9. IgE antibody to *Alternaria* allergens

All sera from normals and asthmatic patients were tested for specific IgE antibody against *Alternaria* antigens. Both crude extract and recombinant Alt a 1 were used in ELISA to determine the presence of specific IgE as described previously [2,12].

3. Results

3.1. Immunochemical characteristics of the allergens

Alt a 1 (Alt a 29), the major allergen of *Alternaria alternata* is a dimer of disulfide-linked subunits that migrate in SDS-PAGE under reducing conditions at 14,500 and 16,000 kDa regions. IgE antibody to Alt a 1 is present in the sera of 92% of *A. alternata* sensitive individuals. IgE from 46 out of 57 individuals sensitive to *A. alternata* reacted positively with recombinant Alt a 1 (82%) in immunoblots indicating that Alt a 1 is a major allergen of *A. alternata*.

The reactivity of recombinant Alt a 1 was compared with native Alt a 1, purified from crude *A. alternata* extract, in immunoblots under reduced and non-reduced conditions (Fig. 1). Both native and recombinant Alt a 1 showed molecular sizes of 29.2 kDa under non-reduced condition. Cloned Alt a 1 and the corresponding native protein reacted with *Alternaria* skin test positive asthmatic patients' sera while none of the normal controls showed any binding (Fig. 1).

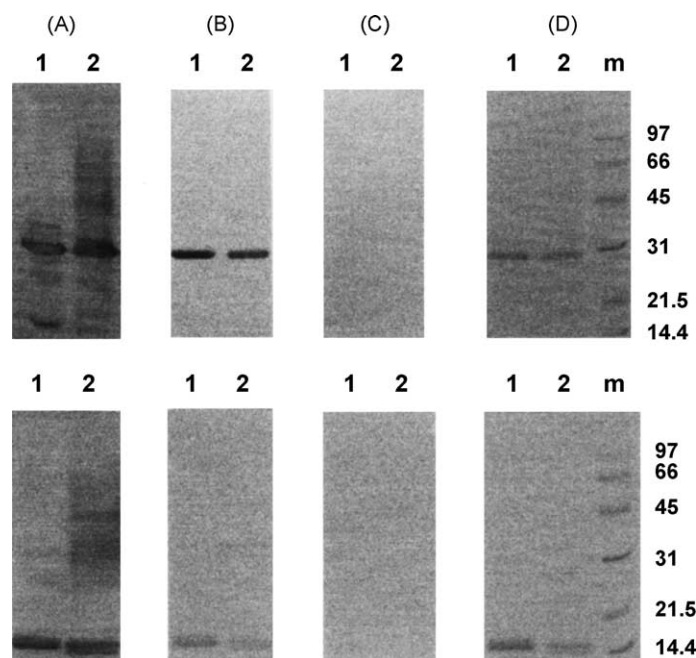


Fig. 1. Comparison of recombinant (lane 1) and native *A. alternata*-allergen (lane 2) Alt a 1 in immunoblots. Aliquots of 1 µg were run in 10% (non-reducing, top row) or 12% (reducing, bottom row) SDS-PAGE, transferred to PVDF membrane, and immunostained with rabbit IgG against partially purified Alt a 1 (A), IgE from *A. alternata* sensitive individuals (B), or non-atopic human serum pool (C), or stained with Coomassie brilliant blue R-250 (D). Position of molecular weight standards of known size (m) is indicated in thousands.

1 MQFTTIASLFAAAGLAAAAPLESRQDTASCPVTTEGDYVWKISEFYGRKP
51 EGTYYNSLGFNIKATNGGTLDFTCSAQADKLEDHKWYSCGENSFMDFSFD
101 SDRSGLLLKQKVSDDITYVATATLPNYCRAGGNPKDFVCQGVADAYITL
151 VTLPKSS

Fig. 2. The amino acid sequence of Alt a 1.

On reduction, the bands appeared near 15 kDa region in the SDS–gel. The amino acid sequence of Alt a 1 is shown in Fig. 2.

3.2. Reactivity of the antigens with IgE in the sera

Of the 15 subjects studied, 8 had skin test positivity with *Alternaria* allergen extract, while 7 failed to show any dermal hypersensitivity reactivity. Of the eight patients, four showed strong IgE binding with both crude *Alternaria* and recombinant Alt a 1 (Fig. 3). Four patients showed only low levels of IgE to *Alternaria* crude allergen, while two of these patients showed very low levels of IgE to Alt a 1. Among the atopic patients with skin test reactivity to *Alternaria*, three and two patients each also showed strong IgE binding reactivity with *Aspergillus* and latex allergens, respectively. All three patients showed high levels of IgE in their sera to both *Aspergillus* and *Alternaria* allergens, while the patients reacted with latex allergy had negligible IgE to *Alternaria*. All except one serum from the normal controls showed no

reaction with any of the antigens studied. One normal subject reacted with Alt a 1 and showed significantly elevated IgE antibody (OD₄₉₀, 0.326) levels in the serum.

3.3. Binding of sera to overlapping Alt a 1 peptides

The peptides synthesized with five amino acid overlaps showed binding to the IgE from the serum pool of three *Alternaria* positive patients. The optical density of the spots plotted is shown in Fig. 4. Nine indiscrete IgE binding areas were demonstrable, which on further study using single amino acid offset and nine amino acid overlap demonstrated four significant IgE binding epitopes.

These selected peptide epitopes were further studied using selected *Alternaria* positive and negative sera. The results shown in Fig. 5 indicate that the two peptides KISEFYGRKP (K41–P50) and YYNSLGFNIK (Y54–K63) reacted strongly with all four patients studied. The peptide YSCGENSFMD (Y87–D96) showed weak inconsistent IgE binding, while VATATLPNYC (V119–C128) showed weak, but consistent

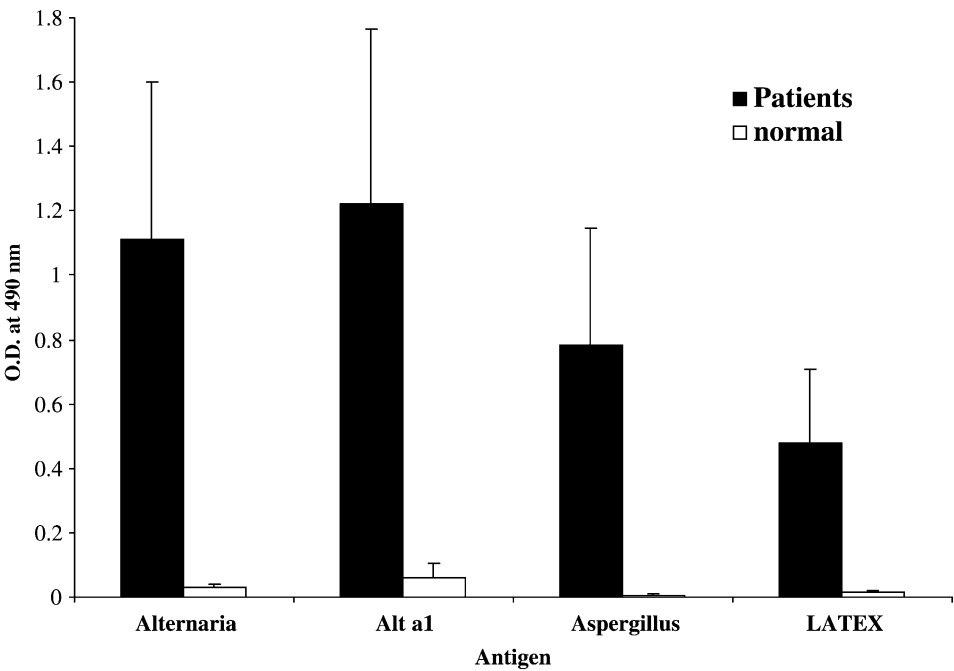


Fig. 3. The reactivity of the IgE from skin test positive asthmatics and normal controls against crude *Alternaria* extract, recombinant Alt a 1, *Aspergillus* and latex allergens. The optical density (OD₄₉₀) obtained from ELISA is given.

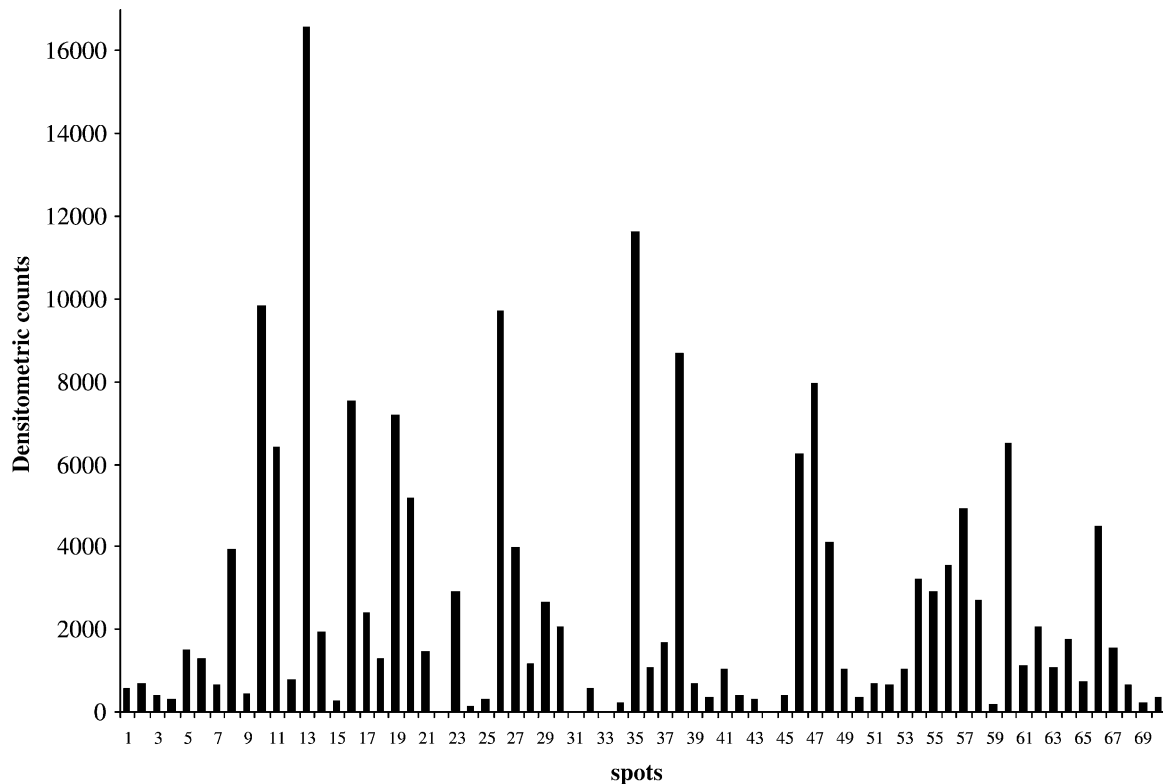


Fig. 4. The reaction of IgE with synthetic peptides on spot membrane measured as OD values are shown. The results demonstrate nine IgE binding epitopes including the four major epitopes are shown (see text).

binding with all four patients studied. The irrelevant peptide DLIAPGMNFQB showed very weak or no binding with the IgE from the sera studied. None of the sera studied from normal controls showed any significant binding to any of these peptides.

4. Discussion

Alt a 1, a major allergen of *A. alternata*, is associated with allergic diseases, particularly asthma [14,20]. Although a number of epitopes binding to IgE has been identified from

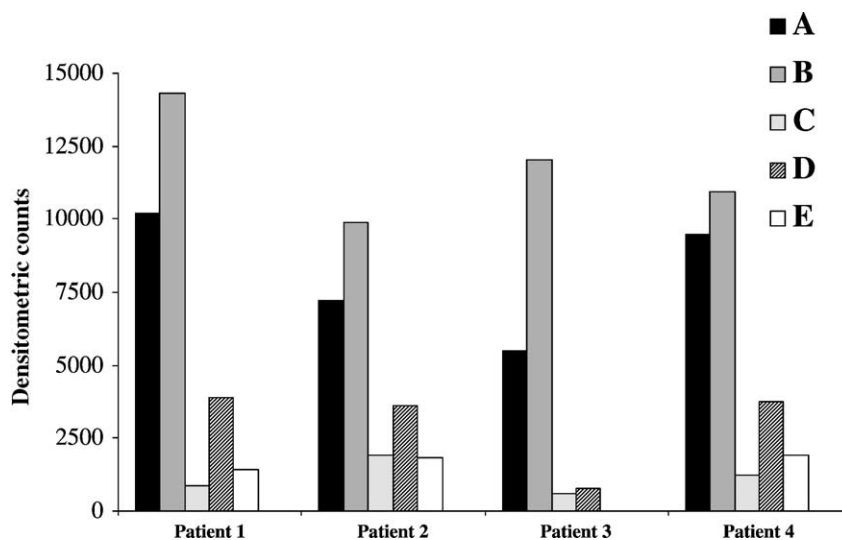


Fig. 5. The IgE binding of the four selected epitopes and an irrelevant peptide against four asthmatics with *Alternaria* skin test positivity. (A) KISEFYGRKP (aa 41–50); (B) YYNSLGFNIK (aa 54–63); (C) YSCGENSFMD (aa 87–96); (D) VATATLPNYC (aa 119–128); (E) DLIAPGMNFQB (irrelevant peptide).

Aspergillus and pollens, no such studies have been carried out to understand *Alternaria* epitopes [2,13]. By understanding the epitopes, the cross-reactivity with other allergens can be ascertained. It is also possible to select allergens with low or no cross-reactivity with related allergens to avoid false positivity in immunoassays. In the present study, we demonstrated four major IgE binding regions in Alt a 1 spanning the whole molecule. Although four linear decapeptides have been identified based on their reactivity with IgE from patients with *Alternaria* allergy, only two peptides demonstrated consistent reactivity with all four patients studied. However, none of the normal controls showed any reactivity indicating that these two peptides are specific epitopes of Alt a 1 and are associated with *Alternaria*-induced allergy.

It has been shown that a number of IgE binding epitopes are conformational [1,17]. However, competitive inhibition of IgE titer also demonstrated a substantial number of linear epitopes [2,13]. Similarly, mutants designed by deletion of epitopes through sequence deletion or point mutation of amino acids confirmed the presence of conformational epitopes and linear epitopes capable of binding to IgE in *Aspergillus fumigatus*-induced allergy. A number of epitope analysis of pollen and latex allergens further confirm this conclusion [2,3]. In Asp f 2, a major *A. fumigatus* allergen we have shown epitopes, which are strong IgE binders and showed cross-reactivity with related species of *Aspergillus*, but not with allergens from unrelated taxa [2]. On comparing sequences, the two major epitopes of Alt a 1 has no identity with any of the known allergens. Hence, these two epitopes demonstrate specificity in IgE binding, and hence, may be of value in the reliable demonstration of IgE against *Alternaria* in patients with *Alternaria* allergy and asthma.

Since none of the normal control sera reacted with these epitopes and all four patients investigated reacted to these two epitopes it can be concluded that they play a significant role in *Alternaria* allergy. As these peptides can be used directly in ELISA, the results can be used to compare crude *Alternaria* extracts for standardization of reagents used in the diagnosis of *Alternaria*-induced allergy. Although potentially useful, more patient and control sera need to be evaluated using these peptides before any final conclusions can be reached.

Synthetic peptides with IgE binding ability are useful as immunotherapeutic agents [15]. The conventional allergen specific immunotherapy has the inherent risk of possible anaphylaxis. A promising strategy is to select non-IgE binding altered allergens with propensity of T-cell stimulation [10,22]. Another plausible strategy is to use epitopes binding to IgE receptors on effector cells without causing cross-linking, thus preventing the release of mediators of anaphylaxis. This will prevent the whole antigen from binding to IgE and will be directed to induce blocking IgG antibodies. Of the four synthetic peptide epitopes, the two with consistent binding namely K41-P50 and Y54-K63 has no cysteine residues. However, the other two peptides with one cysteine each showed reduced binding to IgE or no re-

activity. The structural and functional responses and the role of these cysteines or in that respect all five cysteines in the molecule is not known. The role of oxidative effects or cyclic bonding in the antibody binding is currently not known.

In summary, we have mapped the IgE binding epitopes in the Alt a 1 molecule and identified two decapeptides with consistent IgE binding reactivity. However, the diagnostic significance and the potential use of these two peptides in immunotherapy are not known.

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