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N-Terminus of a Major Allergen, *Alt a I*, of *Alternaria alternata* Defined to Be an Epitope

Key Words

Mould allergen
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Abstract

A 20mer peptide representing the N-terminus of a major allergen, *Alt a I*, of *Alternaria alternata* was synthesized and examined for its antibody binding and antibody induction activities. *Alt a I* peptide-BSA conjugate reacted with both human IgE and rabbit anti-*Alt a I* IgG in ELISA, albeit the binding of the peptide to IgE was relatively weak. Control conjugate showed no antibody binding. These results indicated that the N-terminus of *Alt a I* is an antibodybinding site. Moreover, peptide-KLH conjugate and nonconjugated peptide induced both IgG and IgE antibodies in Balb/c mice that recognized both native *Alt a I* allergen and peptide-BSA conjugate. Since the free peptide was able to induce antibodies in vivo, the peptide may also possess a T cell epitope.

Introduction

Alternaria alternata is one of the major inhalant allergen-producing moulds which induce IgE-mediated allergic diseases in atopic individuals [1]. Immunotherapy with mould allergen extracts is an accepted treatment for allergic disorders. However, successful immunotherapy with mould extracts has been inconsistent because extracts are often poorly standardized and characterized. To develop new therapeutic modalities, detailed knowledge of the structure-function relationships, particularly the epitopes of the defined allergens, is required. Efforts have been made to purify allergens from *A. alternata* extracts to better understand their molecular structure [2–5]. Recently, Deards and Montague [6], Matthiesen et al. [7], and our group [8, 9] have reported purification of similar major allergens, designated *Alt a I* or *Alt a-29*, from *A. alternata*. A sequence of 20 ami-

no acids from this purified protein allergen has been obtained using protein sequencing [8]. In the present study, a peptide representing the N-terminus of the *Alt a I* allergen was synthesized. The peptide was conjugated to carrier proteins and examined for its capacity to induce, and react with antibody. It was demonstrated by this study that the N-terminus of the allergen is not only an antibodybinding epitope, but also able to induce allergen-specific antibodies.

Materials and Methods

Peptides

Two 20mer peptides were synthesized using a solid phase peptide synthesizer with Fmoc chemistry (Core Laboratories, LSUMC, New Orleans, La., USA). One peptide (Pa: ADPVTTEGDYVVKISE-FYGR) represents the N-terminus of the *Alt a I* (also termed *Alt a-29*) allergen. Its hydrophobic index was analysed by the method of Kyte

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and Doolittle [10]. The other peptide is part of the deduced amino acid sequence of a ribosomal P₂ protein of *Cladosporium herbarum* (LGLAGNSSPSAEDIKTVLSS) [11]. This peptide was used as a control and termed Pr.

Conjugation of Peptides to Carrier Proteins

The peptides were conjugated covalently to carrier proteins using glutaraldehyde (Sigma, St. Louis, Mo., USA) by a modification of the previously described method of Coligan et al. [12]. Briefly, 1 mg key-hole limpet hemocyanin (KLH) or 0.5 mg BSA was dissolved in 0.1 M borate buffer pH 10, followed by addition of 1.5 mg of the synthetic peptide. Freshly prepared 0.3% glutaraldehyde was added slowly while the reaction was stirred. After 2 h, the reaction was stopped by addition of glycine to block nonreacted glutaraldehyde while continuously stirring for 30 min. All reactions were carried out at room temperature. The peptide-protein conjugates were washed 3 times with 0.1 M borate buffer, pH 8.0, and twice with Milli Q water through Microcon™ 30 (Amicon, Beverly, Mass., USA). The conjugates were concentrated in water, the concentration was determined by Biorad protein assay, and the conjugates were stored at -20°C.

Sera

Sera of 6 patients highly allergic to *A. alternata*, as determined by a skin test reaction of 4+ and a direct RAST with titer of 2+ to 3+, were pooled [13]. Sera from 6 nonatopic individuals were pooled and used as control.

To produce murine antisera to the peptide, 6- to 8-week-old female Balb/c mice (Jackson Laboratories, Bar Harbor, Me., USA) were immunized with the antigens as described previously [14]. Briefly, each mouse received subcutaneously either 10 µg of the peptide-KLH, or 30 µg of the free peptide, plus 2 mg dextran sulfate as adjuvant for both antigens in 200 µl of saline. Groups of 4 mice were immunized with each antigen preparation. The mice were boosted twice at 3-week intervals using the same amounts of antigens and 1 mg dextran sulfate. Seven days after the second and third immunization, the mice were bled and the sera were collected and pooled from their respective groups.

Rabbit anti-*Alt a I* sera were prepared by immunization of the animals with the allergen homogenized with complete Freund's adjuvant as described previously [9].

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot

The *Alt a I* allergen was electrophoresed under either reduced or nonreduced conditions as reported previously [8]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a vertical slab gel apparatus using 12% polyacrylamide gel according to the method of Laemmli [15]. The separated proteins were either stained with Coomassie blue or electrotransferred onto polyvinylidene difluoride membrane. The membrane was probed with 1:200 diluted murine anti-peptide-KLH sera after saturation with 3% Blocking Reagent (Boehringer Mannheim, Montreal, Canada). Antibody binding to the allergen was detected with alkaline phosphatase-conjugated rabbit anti-mouse IgG and developed with the chromogenic substrates nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

ELISA

Peptide-specific murine and rabbit IgG as well as human IgE antibodies were detected by ELISA. Wells of microtiter plates (Nunc) were coated by incubating with 0.1 µg peptide-BSA conjugate or 5 µg peptide in 100 µl bicarbonate buffer (0.05 M, pH 9.6) overnight at room

temperature. After three washes with TBS-Tween (TBST) buffer and saturation of the plates with 3% skim milk, 100 µl of diluted sera was added into wells. The following dilutions of the sera were used: 1:5 for human serum pool, 1:10,000 for rabbit anti-*Alt a I* allergen, and serial dilutions (1:5 to 1:80) for murine anti-peptide antibodies. After overnight incubation at room temperature, followed by 3 washes of 10 min each in TBST, the plates were incubated with alkaline phosphatase-conjugated antibodies against either mouse IgG, rabbit IgG (Zymed, San Francisco, Calif., USA), or human IgE (Sigma, St. Louis, Mo., USA) for 2 h at room temperature. All alkaline phosphatase conjugates were used in 1:2,000 dilutions. The antibody binding to the peptide was determined by enzymatic reaction with the chromogen, *p*-nitrophenyl phosphate, and expressed as OD₄₁₀ units. Figures represent assays carried out in triplicate.

Passive Cutaneous Anaphylaxis

Peptide-specific IgE antibodies induced in the mice by peptide-KLH conjugate or free peptide were determined as passive cutaneous anaphylaxis (PCA) titers [14]. Wistar rats were sensitized with 50 µl of serially diluted mouse sera injected intradermally on the dorsal side. Forty-eight hours later, the rats were challenged by the intravenous injection of 200 µg peptide-BSA conjugate in 1 ml saline containing 0.5% Evan's blue. The PCA titer was the highest dilution giving a clearly visible skin reaction (> 5 mm in diameter).

Results

Hydropathicity of the *Alt a I* N-Terminus

Analysis of the hydropathicity of the *Alt a I* N-terminal 20 amino acids by the Kyte and Doolittle method (fig. 1) showed that the character of the sequence alternates from hydrophilic to hydrophobic every 6–7 residues. Amino acids with strongly polar side chains were also dispersed through the sequence at regular intervals, suggesting amphipathic character (positions 2, 7, 9, 13, 16, 20). These properties, potentially indicative of B or T cell epitopes, prompted us to examine (1) whether a peptide with this sequence contains IgG- and IgE-binding epitopes, and (2) whether the peptide can act as an immunogen or allergen in mice.

Human IgE Reactivity

The two peptide-BSA conjugates and the native *Alt a I* allergen were coated on ELISA plates and examined for reactivity with human IgE antibodies. Figure 2a showed that human IgE bound both *Alt a I* allergen and Pa-conjugate, although the binding of the IgE to Pa-conjugate was weaker than to native *Alt a I* allergen. Since neither the control Pr-conjugate nor normal human serum pool showed a detectable reaction, the reactivity of the peptide to IgE antibodies was considered positive. Dot immunoblot analysis confirmed these results, as it was observed that IgE antibodies reacted faintly but positively with Pa-conjugate, but not Pr-conjugate [unpubl. results].

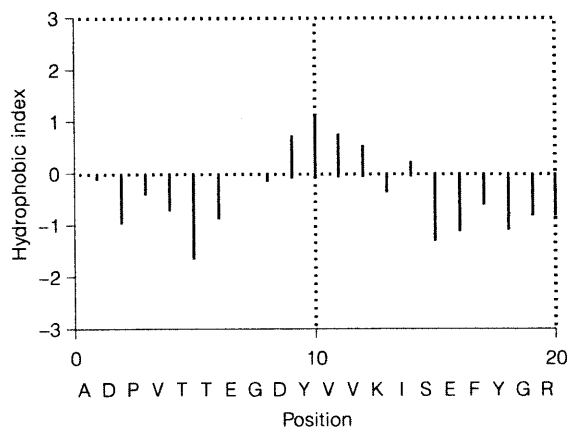


Fig. 1. Amino acid sequence of the *Alt a I* N-terminal peptide and hydropathic profile analysed using the Kyte and Doolittle method [10].

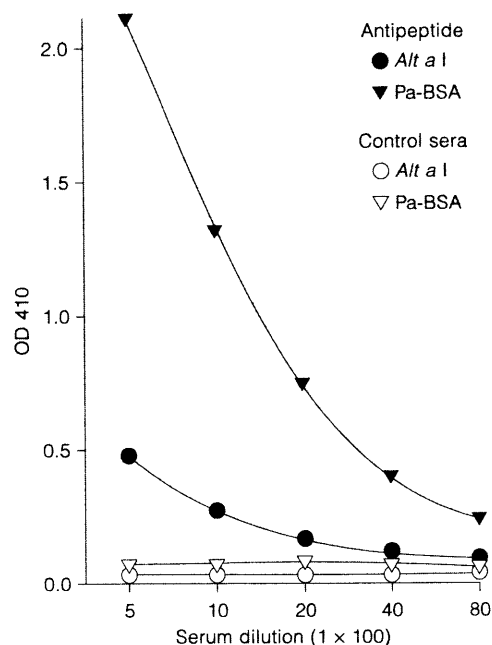


Fig. 3. Reactivity of the murine anti-Pa-KLH to the antigens. Sera from mice immunized with Pa-KLH and control murine sera were serially diluted and examined in ELISA.

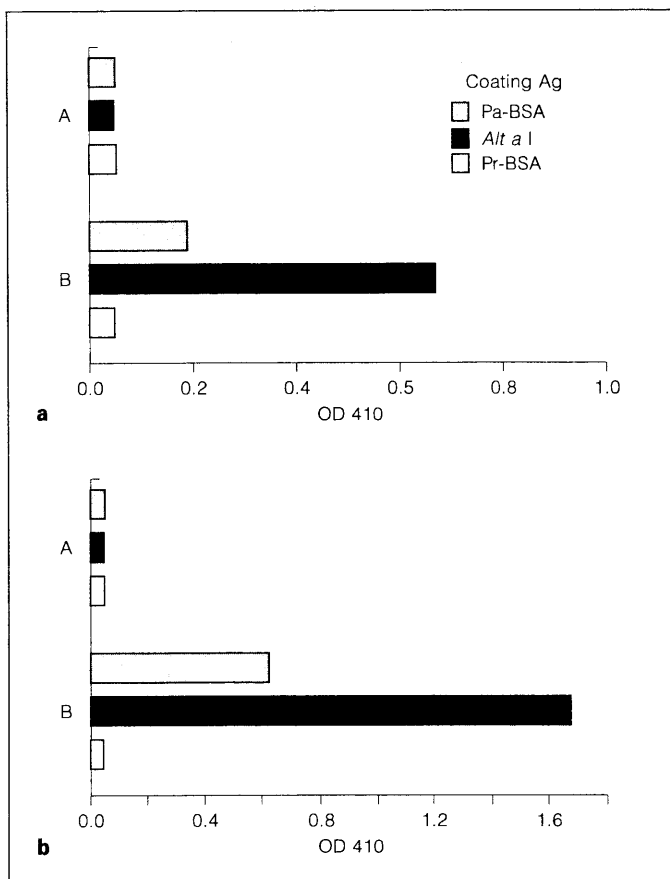


Fig. 2. a Human IgE binding to the antigens in ELISA. A = Control serum pool; B = atopic serum pool. **b** Recognition of the peptide (Pa) by rabbit anti-*Alt a I* allergen. A = Control rabbit sera; B = sera from allergen-immunized rabbit.

Recognition of the Peptide by Rabbit anti-*Alt a I*

The peptide was also examined for its reactivity with rabbit anti-*Alt a I* antibodies. The results demonstrated that the rabbit antisera recognized Pa-BSA, but not Pr-BSA conjugate (fig. 2B). The positive control *Alt a I* allergen reacted with the antiserum more strongly than the peptide conjugate. Preimmune serum was used as a negative control and showed no binding to either conjugate or to *Alt a I* allergen. Collectively, these results and the human IgE analysis revealed that the N-terminus of the *Alt a I* allergen is an antibody-binding epitope.

Murine IgG Antibodies Induced by the Peptide

Immunization of mice with Pa-KLH conjugate led to the induction of antibodies recognizing the peptide. Specific IgG antibodies in the antisera collected after the second immunization were detected by ELISA and the results are presented in figure 3. High titers of IgG antibodies were detected against Pa-BSA conjugate, whereas the titers of antibodies reacting with the native *Alt a I* allergen were relatively low. No detectable reaction was observed with the control Pr-BSA conjugate, and preimmune sera exhibited no antibody binding to any of the antigens. Thus, these results in-

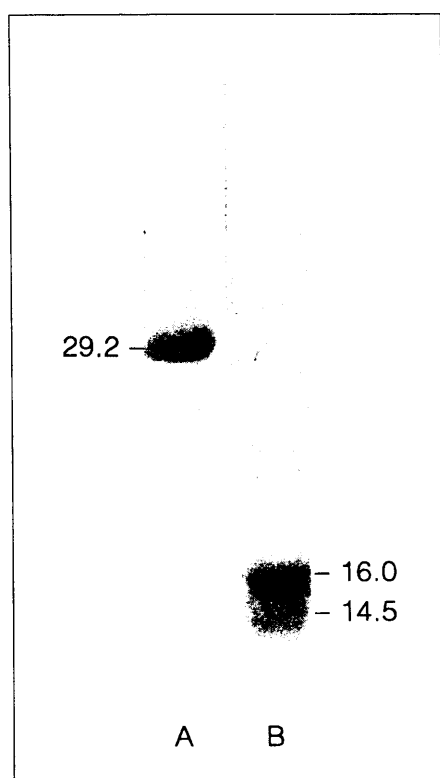


Fig. 4. Immunoblot analysis of allergen recognition by murine anti-peptide antibodies. The *Alt a I* allergen was treated under both non-reduced (**A**) and reduced (**B**) conditions and analyzed by Western blot. Values beside the bands represent molecular mass in kilodaltons.

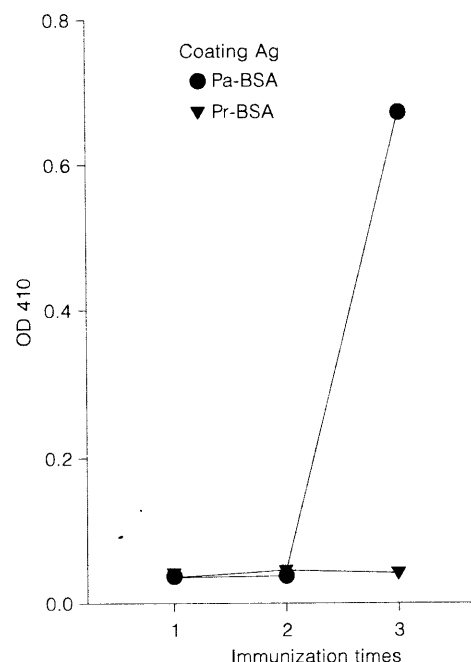


Fig. 5. Specific IgG antibody induction by free peptide (Pa). The murine sera were collected after each immunization and examined by ELISA. Sera were used at 1:500 dilution.

dicated that peptide-specific IgG antibodies were induced by Pa-KLH conjugate in mice, and that these antibodies also reacted with the native allergen.

The murine anti-Pa-KLH peptide antibodies were further examined by Western blot for their reactivity to *Alt a I* allergen after SDS-PAGE, under either reduced or nonreduced conditions. The dilution of the sera used in the immunoblot was 1:500 in TBST. The results demonstrated that allergen treated under reduced or nonreduced conditions was recognized by the antibodies (fig. 4).

The ability of the free peptide to induce IgG antibodies in mice was also examined by ELISA. Sera from peptide-immunized mice were collected after the second and third immunizations, and tested in serial dilutions against PA-BSA by ELISA and PCA assays. The results in figure 5 show that murine IgG antibodies specific to the peptide could not be detected after the first or second immunizations, but were elicited after the third immunization. The reactivity of the antisera to the control Pr-BSA conjugate remained negative. Thus, the peptide itself was able to induce antibodies in vivo.

Specific IgE Antibodies Induced by the Peptide

To determine whether IgE antibodies were also induced by the peptide-KLH conjugate or free peptide, PCA tests were used to detect specific IgE titers. Similar to IgG antibodies, high titers of peptide-specific IgE antibodies were induced by Pa-KLH while relatively low levels of the antibodies were induced by free peptide (fig. 6). Sera employed for this test were from mice immunized twice with Pa-KLH conjugate and 3 times with the free peptide. Use of Pa-BSA as a challenging antigen led to strong PCA reactions, whereas the Pr-BSA control antigen triggered no observable response. Sera from the mice prior to immunization were also used as controls; no responses were observed upon challenge with Pa-BSA [results not shown]. These results demonstrate that both the peptide conjugate and peptide itself were able to induce IgE antibodies.

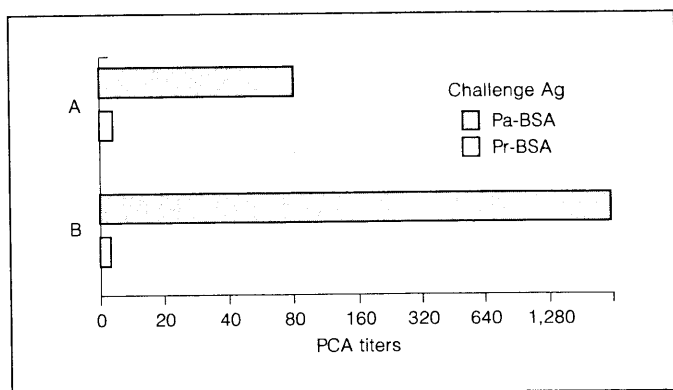


Fig. 6. Murine IgE antibody titers as determined by PCA. Rats were sensitized by antisera induced by free peptide (A) and Pa-KLH conjugate (B) and challenged with Pa-BSA and control conjugates. The open bars represent the controls used; actual PCA values obtained were essentially zero.

Discussion

Alt a I is a major allergen of *A. alternata* present in both mycelial and broth extracts. The allergen is not only one of the most prominent components in the extracts, but also one that is recognized by more than 90% of individuals allergic to the mould [7–9]. Although the allergenicity and immunogenicity of the allergen have been studied previously, the antibody-binding epitopes of the allergen have not yet been explored. The primary aims of this study were to determine whether the N-terminus of the *Alt a I* allergen is an epitope and to investigate the ability of the N-terminal peptide to induce antibodies in mice. This report has provided evidence that the peptide representing the N-terminus of the allergen is an epitope recognized by antibodies and is also able to induce antibodies in vivo.

Our previous studies have demonstrated that the *Alt a I* allergen is a 29 kDa protein comprising two polypeptides that can be separated under reducing conditions. The ability of the Pa peptide derived from the *Alt a I* N-terminus to bind antibody suggests that the N-terminus of the allergen contains a sequence-determined epitope. It has been suggested that at least some IgE-binding epitopes may be largely peptide sequence-determined in nature, although in this study, the native protein seemed to react with the antibodies much more strongly than the denatured allergen [8, 9]. However, the question of whether the peptide represents a major epitope of the allergen needs to be further examined, as the peptide reacted rather weakly with human IgE or rabbit IgG antibodies against *Alt a I* allergen, as compared to the full length allergen.

Initially, we examined IgE antibody binding to the unconjugated peptide. There was no detectable binding to the peptide as determined by both ELISA and dot immunoblot analyses. Since the conjugated peptide showed IgE-binding activity, it appears that the peptide requires flanking amino acids or carrier protein support to maintain a suitable conformation for its reactivity with the antibodies. The weakness of the peptide in reactions with IgE antibodies may possibly be attributed to its short length, which might not encompass the entire IgE-binding epitope.

To investigate the allergenicity and immunogenicity of the allergen, several different animal species have been tested in our laboratory. High titers of IgG antibodies were induced in rabbits. However, at least two strains of rats and six strains of mice failed to induce detectable IgE antibodies to the allergen [2, 9]. By using the synthetic peptide, either alone or conjugated to the carrier protein, we were able to induce both murine IgG and IgE antibodies specific for the allergen. Thus, this study demonstrated the immunogenicity and allergenicity of the N-terminus of *Alt a I*, which may lead to generation of useful probes for further analysis, identification, and characterization of this allergen.

A number of laboratories have isolated and purified the *Alt a I* allergen from *A. alternata* and reported the same dimeric property in SDS-PAGE under reduced conditions [8, 9]. The N-terminal sequence of the allergen reported by Matthiesen et al. [7] is highly homologous to the N-termini of both subunits of the allergen, which we have purified and sequenced [8]. In the present study, we used the murine anti-peptide sera as a probe in Western blots and demonstrated that these highly homologous dimer subunits with respect to sequence may also share the same antigenicity.

It was noted that there was a conspicuous disparity between the reported titers of the Pa-KLH-induced murine IgG antibodies in ELISA reactions using Pa-BSA conjugate as opposed to native *Alt a I* allergen. However, it should be pointed out that this titer disparity might reflect differences in antibody affinity, since the antibodies were induced by the synthetic peptide. Although both coating antigens contain the same epitope, differences in the epitopes' tertiary conformations will affect their capacity for binding to the ELISA plate or to antibody, making direct comparisons between the titers inappropriate. Other antibodies raised against synthetic peptides have been reported to recognize the peptide but not the native antigen [16].

In order to induce antibody production in vivo, a short foreign peptide has to possess three essential features, i.e. a B cell epitope, a T cell epitope and a site recognized by major histocompatibility complex molecules. Analysis of the hydrophobic profile of the peptide using the Kyte and Doo-

little method has shown that the peptide used in this study contains alternating groups of 6–7 hydrophilic and hydrophobic amino acids. Antigenic determinants are often associated with sites with the greatest local hydrophilicity [17]. Regularly spaced amino acids with highly polar side chains can indicate amphipathic structures capable of acting as T cell epitopes [18]. The experimental results showed that the peptide itself was able to induce antibody production which,

in consensus with structural features, suggests that this 20mer peptide possesses both B and T cell epitopes. Generating a series of peptides with systematic substitutions within the N-terminal sequence will serve to delineate the exact sites of B and T cell stimulation. Since IgE antibodies were also induced by the peptide, it will be interesting to investigate the role of the peptide in the activation of different subsets of T cells.

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