

Role of C-Terminal Cysteine Residues of *Aspergillus fumigatus* Allergen Asp f 4 in Immunoglobulin E Binding

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Received 15 August 2003/Returned for modification 7 October 2003/Accepted 8 December 2003

Among the several allergens cloned and expressed from *Aspergillus fumigatus*, Asp f 4 is a major one associated with allergic bronchopulmonary aspergillosis (ABPA). The structure-function relationship of allergens is important in understanding the immunopathogenesis, diagnosis, and treatment of allergic diseases. These include the epitopes, conformational or linear, deletion of the N or C terminus or both N and C termini, and glycosylation or nonglycosylation, all of which affect immune responses. Similarly, the role of cysteine residues present in allergens may yield useful information regarding the conformational structure of allergens and the immunoglobulin E (IgE) epitope interaction. Such information may help in developing new strategies towards immunotherapy. In order to define the role of cysteine in the interaction of the antibody with Asp f 4, we have constructed mutants by selectively deleting cysteine residues from the C-terminal region of the Asp f 4. Immunological evaluation of these engineered recombinant constructs was conducted by using sera from patients with ABPA, *Aspergillus* skin test-positive asthmatics, and healthy controls. The results demonstrate strong IgE binding with Asp f 4 and two truncated mutants, Asp f 4₁₋₂₃₄ (amino acids [aa] 1 to 234) and Asp f 4₁₋₂₄₁ (aa 1 to 241), while another mutant, Asp f 4₁₋₁₉₆ (aa 1 to 196), showed reactivity with fewer patients. The result suggests that deletion of cysteines and the alteration of IgE epitopes at the C-terminal end resulted in conformational changes, which may have a potential role in the immunomodulation of the disease.

Allergic bronchopulmonary aspergillosis (ABPA) is a disabling hypersensitivity lung disease that primarily occurs in patients with asthma and cystic fibrosis (CF) and results from inhalation of *Aspergillus fumigatus* conidia present in the environment and their entrapment and subsequent growth in the airways (7, 8, 17). *A. fumigatus* contains a number of antigens with diverse biochemical properties. Several relevant allergens showing binding to human serum antibodies have been identified and purified (5, 12). In recent years, molecular cloning has been employed to obtain pure antigens relevant to the disease. Of the 22 recombinant *A. fumigatus* allergens, Asp f 4 is a major one demonstrating strong binding to serum immunoglobulin E (IgE) from ABPA patients (9, 11, 12). Earlier studies have demonstrated the presence of specific IgE antibody against Asp f 4 in a majority of patients with ABPA, including patients suffering from CF with ABPA (ABPA-CF) (6, 9, 12). Asp f 4 also elicited immediate skin test reactivity and induced stimulation of T cells from peripheral blood samples of ABPA patients (6, 9). This suggests that Asp f 4 is a major allergen involved in the pathogenesis of ABPA and plays a significant role in the regulation of T- and B-cell immune responses. The biochemical nature and immunological characteristics of Asp f 4 have not been elucidated completely. Although Asp f 4 is a major allergen, no information is available on the IgE binding epitopes or the structure-function relationship of this molecule.

The role of disulfide bonds formed between cysteines in IgE

binding has been investigated in studies with several other allergens, namely Lep d 2, Bet v 1, Der p 1, and Der p 2 (14, 16, 20, 22). Earlier studies have demonstrated that in Lep d 2, the disulfide bonds formed between cysteines 72 and 77 (Cys 72-77) was the single significant contributing factor in IgE binding (16). Mutants depleted of one or more of these cysteines showed various degrees of reactivity, whereas IgE binding was abolished completely when all six cysteines had been altered (16). In Birch pollen allergen Bet v 1 and house dust mite allergens Der p 1 and Der p 2, discontinuous IgE binding epitopes have been demonstrated and the IgE antibody binding was destroyed by fragmentation or mutagenesis of cysteine residues responsible for the formation of disulfide bonds (14, 20, 22). Our studies with another *A. fumigatus* allergen, Asp f 2, showed that alteration of cysteine residues at positions 204 and 257 induced marked differences in the IgE binding (2).

Since no information on the structure function of Asp f 4 is available, we undertook the present study to investigate the role of cysteine residues present in Asp f 4 in the immune responses in ABPA. We selectively deleted the cysteine residues at the C-terminal end of the Asp f 4 allergen and studied the IgE binding of the different mutants. The results indicate that deletion of cysteine residues at the C-terminal end of Asp f 4 showed weaker IgE antibody binding from fewer subjects, suggesting a significant role for certain cysteines. Some of these mutants may be of value for therapeutic and diagnostic purposes.

MATERIALS AND METHODS

Cloning of the *A. fumigatus* gene encoding Asp f 4 and fragments. The cDNA library of *A. fumigatus* was constructed in a λ ZAP-II vector, and the protein was expressed in a pET 23b(+) vector as described previously (3). The primers used

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TABLE 1. Primer sequences used in the construction of Asp f 4 and its fragments

Construct (aa)	Molecular size (kDa) of protein	Primer sequences
Asp f 4 (1–286)	32	5'GGATCCGGGCGAGGTCGGCGACACTG3' and 5'GCGGCCGCGGCCAGATCAGCAGTGTAGGC3'
Asp f 4 (1–196)	22	5'GGATCCGGGCGAGGTCGGCGACACTG3' and 5'GCGGCCGCGCCCTTTGCGGCGCCCCAGGCAC3'
Asp f 4 (1–234)	26	5'GGATCCGGGCGAGGTCGGCGACACTG3' and 5'GCGGCCGCATACCCTGGACCTCA3'
Asp f 4 (1–241)	27	5'GGATCCGGGCGAGGTCGGCGACACTG3' and 5'GCGGCCGCCTCGCCGGCGTGATTGCAGATCTT3'

for constructing Asp f 4 and the three recombinant fragments of Asp f 4, namely, Asp f 4_{1–196}, Asp f 4_{1–234}, and Asp f 4_{1–241}, and the deduced molecular mass of the different recombinant proteins are shown in Table 1. The sense and antisense primers had *Bam*HI and *Not*I at their 5' ends. The PCR-amplified fragments were subcloned into PCR II vector (Invitrogen, San Diego, Calif.). The plasmid DNA was digested with *Bam*HI and *Not*I and ligated into similarly digested expression vector pET 23b(+) (Novagen, Madison, Wis.). The induction and analysis of the fusion protein was performed according to the pET expression protocol of the manufacturer (Invitrogen).

Isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cell lysates were analyzed by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% polyacrylamide) after being stained with Coomassie blue (3). The expressed proteins were affinity purified from cell extracts using an Ni²⁺-nitrilotriacetic acid agarose column (Qiagen, Valencia, Calif.). The purity, molecular size, and immunological reactivity of recombinant proteins were ascertained. The inserts of all the deletion mutants were sequenced, and the integrity and purity were confirmed.

Characterization of Asp f 4 and its mutants. Asp f 4 and three cysteine deletion mutants were studied for their immunochemical characteristics. The mutants were designed by deletion of cysteines from the C-terminal end one at a time. Asp f 4_{1–196} has only one cysteine at the N-terminal end, and the remaining three cysteine residues at the C-terminal end, namely, those at positions 198, 236, and 243, have been deleted. Asp f 4_{1–234} was constructed by deletion of the two cysteines at positions 236 and 243, while Asp f 4_{1–241} has only one cysteine deleted at the C-terminal end (C243). All the Asp f 4 recombinant proteins were characterized by SDS-PAGE and assayed for purity and molecular mass. The immunological reactivities of these recombinant mutants were studied by enzyme-linked immunosorbent assay (ELISA) and Western blotting by using sera from ABPA patients, skin test-positive asthmatics, and healthy controls (10, 13, 18).

Sera from patients and healthy controls. Sera were collected from 13 patients with clinical ABPA and 9 CF patients who also had ABPA diagnosed according to the criteria of Rosenberg et al. (19). Thirteen patients with asthma and immediate skin test reactivity to the *A. fumigatus* antigen and 12 healthy controls with no skin test reactivity to *Aspergillus* species were also studied. All sera were tested for specific IgE antibody by ELISA using Asp f 4 and the mutants. The institutional review committees of both participating institutions approved the study.

ELISA for antigen-specific IgE in sera. The binding of IgE antibody in the sera of ABPA patients, skin test-positive asthmatics, and healthy controls against Asp f 4 and its three mutants was studied by a solid-phase ELISA as reported previously (17, 18). Immulon II polystyrene plates (Fisher Scientific, Hanover Park, Ill.) were coated with various recombinant proteins in phosphate-buffered saline (PBS) at a concentration of 10 µg/ml (pH 7.4) and left overnight at 4°C. The plates were washed with PBS containing Tween 20 (PBS-T), and the wells were blocked with PBS containing 3% bovine serum albumin. The plates were incubated for 1 h at room temperature and washed as before. The subsequent steps include incubation with serum, biotinylated goat anti-human IgE, and streptavidin peroxidase (4, 10, 18). In between the addition of reagents, the plates were washed three times with PBS-T. Finally, the color was developed with chromogen and orthophenylene diamine and the optical density at 490 nm was read in an ELISA plate reader (Dynatech, Chantilly, Va.) (4). All dilutions of reagents were selected empirically after checkerboard titrations with positive and negative control sera.

Western blot analysis of Asp f 4 and its fragments. Asp f 4 and its fragments were subjected to electrophoresis in a SDS–15% polyacrylamide gel as described earlier (13, 18). Antigen (10 µg) was applied to the gel, and after electrophoresis, the separated proteins were transferred to nitrocellulose membrane (Bio-Rad Labs, Hercules, Calif.) as described previously (13). The free binding sites on nitrocellulose membrane were blocked with PBS containing 5% skim milk powder. The blocked membranes were incubated overnight at 4°C with a 1:10

dilution of pooled sera from ABPA patients. The membrane strips were washed thoroughly and treated with biotinylated rabbit anti-human IgE antiserum for 1 h (1). This was followed by incubation with streptavidin alkaline phosphatase, and the color was developed with a 5-bromo-4-chloro-3-indolyl phosphate toluidine-nitroblue tetrazolium substrate (Boehringer Mannheim, Indianapolis, Ind.).

Statistical analysis. The optical density values of the ELISA obtained from different groups of subjects and controls were compared by using Student's *t* test (two-tailed independent samples) in the Statworks program (Cricket Software, Philadelphia, Pa.). A *P* value of <0.05 was considered significant.

RESULTS

The full-length Asp f 4 has 286 amino acid residues with four cysteines at positions 81, 198, 236, and 243 in the sequence. The seven IgE binding linear epitopes mapped by using overlapping decapeptides and the cysteine residues are shown in Fig. 1A and B. The three mutants Asp f 4_{1–196}, Asp f 4_{1–234}, and Asp f 4_{1–241} were constructed by the deletion of various numbers of cysteine residues and linear IgE binding epitopes from Asp f 4. All three IgE binding linear epitopes at the N-terminal end were present in all mutants, while the last two epitopes at the C-terminal end were absent in all three truncated proteins.

The immunochemical characteristics of Asp f 4 and its fragments. *Escherichia coli* expressed Asp f 4, appearing as a 30-kDa protein, while different fragments appeared at 22-, 26-, and 27-kDa regions. The sizes of the proteins resolved in the gel agreed with the calculated sizes of the respective deletion mutants. The SDS-PAGE profiles of Asp f 4 and its fragments are shown in Fig. 2. The sequence analysis of Asp f 4 and three cDNA constructs confirmed the expected nucleotide sequences encoding the proteins.

IgE binding of Asp f 4 and fragments. Asp f 4 and the three mutant proteins were studied for their IgE binding by ELISA using sera from ABPA and ABPA-CF patients, *Aspergillus* skin test-positive asthmatics, and healthy controls. The results are shown in Fig. 3. None of the 12 healthy control sera showed significant IgE against the various Asp f 4 proteins or crude *A. fumigatus* antigen studied. Although sera from all 13 subjects belonging to the skin test-positive asthmatics reacted weakly with all antigens, only one patient showed significantly elevated IgE responses to the recombinant allergens. There were significant differences (*P* < 0.05) between control sera and ABPA and ABPA-CF sera with all antigens except Asp f 4_{1–196} when tested for IgE antibody. This was also true with skin test-positive asthmatics and ABPA patients (*P* < 0.05). Asp f 4 and mutants Asp f 4_{1–234} and Asp f 4_{1–241} showed strong IgE binding with ABPA patients' sera compared to the binding obtained with Asp f 4_{1–196} (*P* < 0.05). The crude *A. fumigatus* antigen reacted with over 70% of ABPA patient sera, while the recombinant proteins reacted with a lesser number of sera, and the reactivity varied from 15 to 61%. The least reactivity (1 of

A

1	GEVGDTVYAT	INGVLVSWIN	EWSGEAKTSD	APVSQATPVS	NAVAAAAAAS
51	TPEPSSSHSD	SSSSSGVSAD	WTNTPAEGEY	CTDGFGGRT	PSGSGIFYKG
101	NVGKPWGSNI	IEVSPENAKK	YKHVAQFVGS	DTDPTWTVVFW	NKIGPDGGLT
151	GWYGNSALT	HLEAGETKYV	AFDENSQGAW	GAAKGDELPK	DQFGGYSCTW
201	GEFDFDSKIN	HGWSGWVDSA	IQAENAHHEV	QGMKICNHAG	ELCSIISDGL
251	SKVIDAYTAD	LAGVDGIGGK	VVPGPTRLVV	NLDYKE	

B

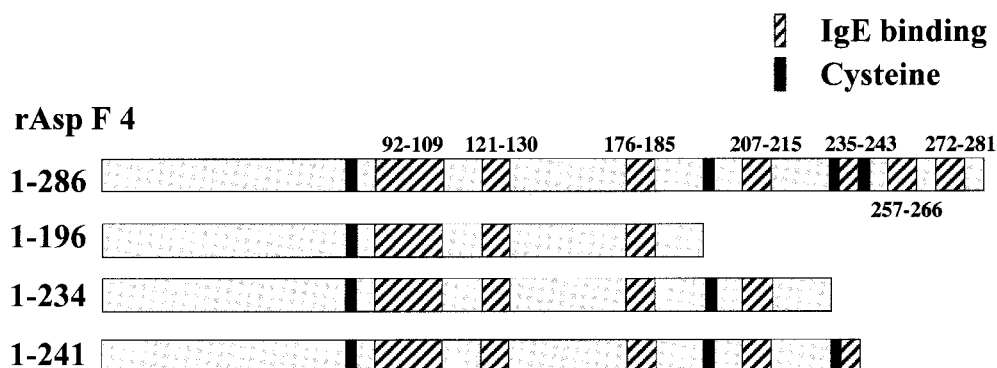


FIG. 1. (A) Deduced amino acid sequence of the allergen Asp f 4. (B) Schematic representation of Asp f 4 and three different cDNA constructs of Asp f 4 showing the seven IgE antibody binding linear regions and four cysteines at positions 81, 198, 236, and 243.

13 patients) was detected with Asp f 4₁₋₁₉₆. The other two mutants in which cysteines were deleted at positions 236 and 243 failed to show any major difference in their binding with IgE in the sera from ABPA patients. However, sera from patients with ABPA-CF reacted differently with these allergens. Asp f 4 reacted with over 55% of ABPA-CF sera compared to 77% with crude *Aspergillus* extract, while only 33% of the sera reacted with Asp f 4₁₋₂₃₄ and Asp f 4₁₋₂₄₁. In contrast, Asp f 4₁₋₁₉₆ showed very weak reactivity with only one ABPA-CF patient. These results indicate that C₁₉₈ and C₂₃₆ of Asp f 4 contribute towards the IgE binding of the allergen.

However, C₂₃₆ may not make significant contributions toward conformational integrity of the Asp f 4 epitopes, judging from the lack of differences in IgE binding to Asp f 4₁₋₂₃₄ or Asp f 4₁₋₂₄₁. Of interest, IgE binding to the full-length molecule increased to 55% based on restoration of C₂₄₃ to mutants lacking this amino acid (baseline binding, 33%).

Western blot analysis. IgE binding by Asp f 4 and its three mutants was assayed in Western blot analysis using pooled ABPA patient sera. The bands were resolved at 30-, 22-, 26-, and 27-kDa regions. The truncated fragments of Asp f 4, namely, Asp f 4₁₋₁₉₆, Asp f 4₁₋₂₃₄, and Asp f 4₁₋₂₄₁, contain three, four, and five of the seven IgE binding regions and one, two, and three of the four cysteines at the N-terminal region of the molecule, respectively. All three mutants demonstrated binding to IgE from pooled ABPA patients' sera in Western blot analysis, and the results suggest that all allergens share nonfunctional similarity among the engineered allergens (Fig. 4).

DISCUSSION

Earlier studies by various investigators have shown that among the other recombinant allergens, Asp f 4 is also a major allergen capable of demonstrating specific IgE in ABPA. Cramer and associates have shown that Asp f 4 has discriminating characteristics in its binding against antibody from ABPA patients and skin test-positive asthmatics (6). However, it is not known how this differential binding to IgE takes place. It has been shown that there are several IgE binding regions span-

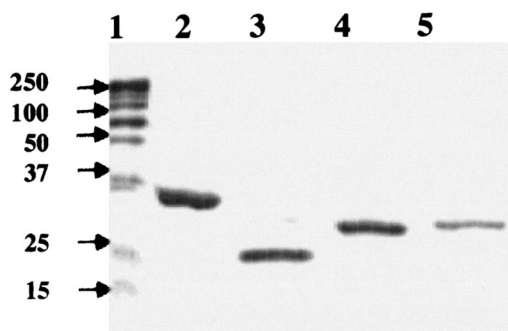


FIG. 2. SDS-PAGE analysis of Asp f 4 and its fragments. Ten micrograms of purified antigens was loaded and subjected to electrophoresis, and the gel was stained with Coomassie brilliant blue R250. Lane 1, molecular weight standard; lanes 2 to 5, Asp f 4, Asp f 4₁₋₁₉₆, Asp f 4₁₋₂₃₄, and Asp f 4₁₋₂₄₁, respectively.

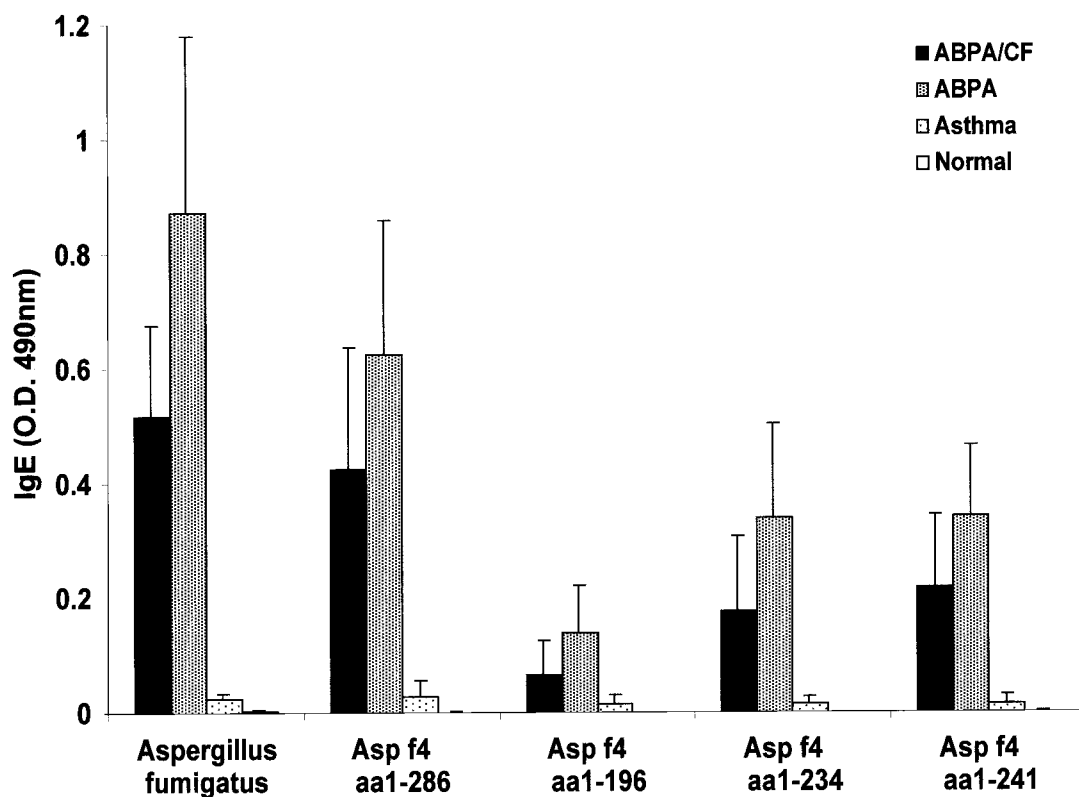


FIG. 3. Binding of *A. fumigatus* extract, Asp f 4, and three fragments with IgE in the sera from patients with ABPA, patients with ABPA-CF, skin test-positive asthmatics, and healthy controls by ELISA. Statistically significant differences between ABPA patients and controls and between Asp f 4₁₋₁₉₆ and the other truncated antigens in their IgE reactivity with ABPA sera were detected (for details, see Results).

ning the sequence of the purified recombinant allergens. In addition, cysteine residues frequently contribute to strong IgE binding through cyclic bond formation and through maintenance of conformational integrity of the epitopes. Hence, in order to ascertain this, we have systematically removed the cysteine residues and IgE binding linear epitopes from Asp f 4. Although sera from some ABPA patients reacted with all three fragments, there were differences in their immune responses, particularly in the realm of IgE antibody binding.

It has been reported that the disulfide bonds formed between the different cysteine residues play an important role in the conformational structure of the allergens and this has con-

tributed to IgE antibody binding (2, 14, 15, 20–22). Earlier studies by Olsson et al. have demonstrated that in Lep d 2, the disulfide bond formed between cysteines 72 and 77 (Cys 72–77) was the single most important contributing factor in IgE binding (16). Similar reports studying Der p 2 and restrictocin indicate that cysteines are responsible for the retention of the overall antigenicity (15, 20). In the present study, Asp f 4₁₋₁₉₆ showed quantitatively decreased reactivity with IgE by ELISA compared with the full-length allergen and the two fragments. Interestingly, the Western blot analysis of the IgE antibody binding of denatured Asp f 4₁₋₁₉₆ exhibited comparable reactivities with Asp f 4₁₋₂₃₄ and Asp f 4₁₋₂₄₁. The reason for this discrepancy in antibody binding by ELISA and Western blotting may be due to the availability of N-terminal linear IgE epitopes of Asp f 4₁₋₁₉₆ after denaturation in Western blots. The native nondenatured Asp f 4 mutants used for ELISA may not have all the linear epitopes exposed on their surface for possible interaction with IgE antibodies.

In previous studies with the three deletion mutants of *A. fumigatus* allergen Asp f 2, it was noticed that one of the mutants, Asp f 2B (amino acids [aa] 68 to 203), with four cysteine residues at positions 81, 88, 109, and 123, failed to show IgE binding with sera from *A. fumigatus*-sensitized patients (2). However, Asp f 2A (aa 1 to 203) with an additional cysteine residue at the 14th position at the N-terminal region and Asp f 2C (aa 68 to 268) with three more cysteines at positions 204, 257, and 267 near the C-terminal region exhibited IgE antibody binding with ABPA sera (2). Recently, it was

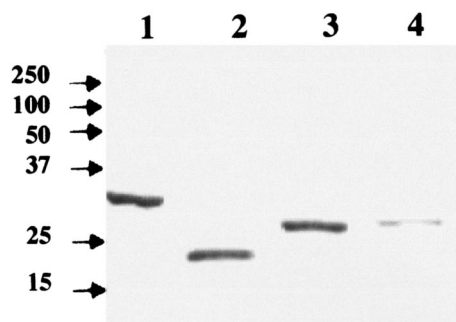


FIG. 4. Western blot of Asp f 4 and its fragments, showing reactivity with ABPA patient serum IgE. Lanes 1 to 4, Asp f 4 and the fragments Asp f 4₁₋₁₉₆, Asp f 4₁₋₂₃₄, and Asp f 4₁₋₂₄₁.

reported that in Asp f 2, there was a fourfold increase in IgE antibody binding when cysteine was added at the 267th position. However, a loss of binding was noted when C204 was mutated to alanine (2). The results of the present study also support the significance of some of the cysteine residues in their specific binding to IgE.

In the present study, we observed that the three mutants of Asp f 4 reacted differently with the IgE antibody from sera from patients with ABPA. Patients with ABPA-CF or ABPA had lesser reactivity (10 to 15%) with the mutant Asp f 4₁₋₁₉₆ by ELISA. The reactive sera from two ABPA patients and one ABPA-CF patient showed only weaker IgE binding (data not shown). Thus, the results suggest that N-terminal epitopes are essential for IgE binding whereas the deletion of C-terminal cysteines and epitopes results in quantitatively reduced reactivity. Engineered proteins with decreased IgE binding and significant cellular response may be of value in developing immunotherapeutic agents. Antigens with specifically removed cross-reactive epitopes may be useful in the precise diagnosis of the disease. The present study thus indicates that the N-terminal IgE epitope regions of the protein are crucial in maintaining the proper folding and three-dimensional structure of Asp f 4 whereas the C-terminal cysteines play a significant supporting role in IgE binding.

ACKNOWLEDGMENTS

This investigation was supported by the U.S. Veterans Affairs Medical Research and by the Ernest S. Bazley grant for Northwestern University and Hospital.

The editorial assistance of Donna Schrubbe and the technical assistance of Laura Castillo and Nancy Elms are gratefully acknowledged.

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