

Cloning and expression of *Aspergillus fumigatus* allergen Asp f 16 mediating both humoral and cell-mediated immunity in allergic bronchopulmonary aspergillosis (ABPA)

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Summary

Background *Aspergillus fumigatus*, a ubiquitous fungus, is responsible for a number of lung disorders in atopic and non-atopic individuals. Standardized, pure, and relevant allergens are desirable for reliable immunodiagnosis of the disease and to understand the structural and functional properties of these allergens and the role they play in causing ABPA.

Objective Molecular cloning and characterization of a relevant allergen from *A. fumigatus* cDNA library.

Materials and methods A cDNA library was constructed from 96 h old mycelium of *A. fumigatus* using λ ZAP expression vector. A novel gene encoding an *A. fumigatus* allergen was identified by screening the library with sera from ABPA patients. The gene was cloned and the allergen over-expressed in *Escherichia coli*. This recombinant allergen, Asp f 16, was evaluated in ELISA and Western blots using sera from patients and normal subjects and peripheral blood mononuclear cells (PBMC) for antigen-induced stimulation.

Results Seventy percent of the patients with ABPA demonstrated high levels of serum IgE antibodies to Asp f 16, a 43-kDa protein, whereas patients with allergic asthma, *Aspergillus* skin test-positive asthmatics without clinical evidence of ABPA, and normal controls failed to show Asp f 16-specific IgE binding by ELISA. The deduced amino acid sequences of Asp f 16 showed extensive sequence homology to 30.6-kDa Asp f 9 at the N-terminal region of the protein. PBMC from the majority of patients with ABPA exhibited significant proliferation with the recombinant Asp f 16 allergen.

Conclusion Specific humoral and cell-mediated immune responses of Af-sensitized patients against Asp f 16 suggest its usefulness in the immunodiagnosis of hypersensitivity diseases due to Af and understanding the pathophysiology of ABPA.

Keywords: Af, Asp f 16, allergen, molecular cloning, cDNA library, ABPA, IgE antibody, ELISA, PBMC, stimulation index

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Introduction

ABPA is a disabling hypersensitivity lung disease detected in about 1–2% of asthmatic (15% asthmatic patients sensitized to Af) and 7–35% of cystic fibrosis

patients [1–4]. Unlike *Aspergillus* skin prick test-positive asthmatics, ABPA patients usually show significantly elevated total and specific serum IgE and IgG to Af proteins [5]. The clinical features of ABPA vary widely from episodes of mild asthmatic responses indistinguishable from uncomplicated asthma to severe asthma with fleeting radiological infiltrates, proximal bronchiectasis and lung fibrosis [6]. Diagnostic criteria such as skin prick test

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Table 1. Total serum IgE, Af-specific serum IgE and IgG, skin prick test of patients with ABPA and allergic asthma

Subjects ABPA	Total IgE ng/mL	IgE-Af	IgG-Af	Prick test to Af	Subjects Asthma	Total IgE ng/mL	IgE-Af	IgG-Af	Prick test to Af
RS	2568	ND	ND	ND	DA	1100	1	1.8	Negative
RD	3300	ND	ND	ND	MA	506	0.76	0.07	Negative
BH	4227	ND	ND	ND	DB	1166	0.27	0.2	Negative
JC	8592	ND	ND	ND	SB	490	0.71	0.54	Negative
RT	1465	6.21	3.52	Positive	AB	139	0.67	0.25	Negative
JT	1570	ND	ND	ND	DB	199	1.25	1.25	Positive
KN	3600	ND	ND	ND	JD	1997	0.12	0.14	Negative
KL	1853	8.05	3.55	Positive	DJ	128	0.17	0.17	Negative
GC	3300	ND	ND	ND	MM	463	1.08	0.18	Negative
RB	6744	ND	ND	ND	RM	283	0.88	0.62	Negative
FD	19 752	2.9	2.88	Positive	JP	418	0.5	0.7	Negative
PZ	808	2.59	2	Positive	FP	4056	1	0.87	Negative
RA	1096	2.89	2.75	Positive	CR	1838	0.5	1	Positive
JB	ND	3.33	3.6	Positive	DR	66	0.33	0.14	Negative
PC	992	3.7	2.92	Positive	RS	602	1.61	0.09	Negative
JD	ND	9.98	3.8	Positive	SS	445	0.95	0.43	Negative
SD	780	2.44	4.25	Positive	JT	1154	0.58	0.67	Positive
MD	ND	2.67	2.9	Positive	KS	24	0.87	0.94	Negative
EG	1718	2.21	2.21	Negative	AG	827	1.57	0.92	Negative
TK	3936	2.73	3.4	Positive	KZ	24	1.07	1.09	Negative
SM	800	8.52	2.27	Positive	DD	870	0.86	1	Negative
KM	696	6.02	3.26	Positive	GP	816	1.61	1.61	Negative
SS	4485	5.85	4.25	Positive	JB	232	2.28	2.32	Negative
DW	1440	5.6	4.81	Positive	RG	166	0.85	0.82	Negative
DW	2312	2.67	2.62	Positive	DT	37	0.88	1.55	Negative
TA	300	5.92	4.12	Positive	CY	10.7	0.81	0.91	Negative

reaction to Af extracts and serum IgG and IgE antibodies to Af proteins are often present in Af-sensitized asthmatics without ABPA and hence not specific. Central bronchiectasis, the characteristic feature of ABPA, could be detected only in the late stage of the disease and the predictive value of several other diagnostic criteria such as radiologic findings, eosinophilia and skin prick test reaction may depend on several factors including underlying diseases and age of the subjects studied. ABPA, if not detected at the early stage, eventually leads to pulmonary fibrosis and respiratory failure. In view of the risk associated with undetected ABPA, it is essential to develop sensitive and reliable *in vitro* immunodiagnostic methods for early detection of ABPA using well-defined Af allergens.

Until recently, *in vitro* tests to demonstrate serum IgE antibodies to specific allergens have been performed with crude extracts of Af. The crude extracts contain, in addition to the relevant allergens, a large number of non-allergenic components including carbohydrates, proteins, nucleic acids, and even toxins [6,7]. In recent years, a number of allergens have been obtained from Af by molecular cloning of genes [8,9]. The reactivity of these individual allergens

to the serum antibodies shows considerable variation, suggesting the need for a mixture of diagnostically relevant and purified *Aspergillus* allergens [9–14]. The results of serological analyses and skin test studies demonstrated that some of the recombinant allergens may prove useful in differentiating ABPA from other Af-induced diseases and can be of value in monitoring disease progression and response to treatment [15–17]. The current update of Af allergens by the International Union of Immunological Societies (WHO/IUIS) listed 18 allergens from *A. fumigatus* (Dr Wayne Thomas, Chairman, IUIS Nomenclature Committee, personal communication). The present study describes the molecular cloning and over-expression of a 43-kDa recombinant allergen (Asp f 16) from Af cDNA library. Among various subject groups studied, sera from ABPA patients demonstrated specific IgE antibody binding with Asp f 16, whereas asthma and normal subjects failed to show IgE binding with this allergen. Peripheral blood mononuclear cells (PBMC) from patients with ABPA also exhibited distinct proliferative responses against Asp f 16, indicating its involvement in cell-mediated immune responses in patients.

Materials and methods

Antigens

The crude culture filtrates and mycelial extracts from Af were prepared as described previously [11]. The antigen preparations were characterized for their protein content as well as for reactivity against specific Af antibodies as previously described [12].

Recombinant Af allergen

A cDNA expression library was prepared using poly (A⁺) RNA isolated from 96 h old mycelium of Af (AF 102; ATCC 42202) in λ ZAP expression vector. The primary library of 2×10^5 recombinants was amplified to generate a high-titre phage stock. *Escherichia coli* strain XL-1 Blue was infected with 5×10^4 phages with cDNA inserts and plated on LB agar plates. This cDNA library was screened with anti-Af rabbit hyperimmune serum and pooled serum samples from patients with ABPA as previously described [10]. The identified plaques were purified and rescued to pBluescript phagemids in XL-1 Blue. One of the positive clones exhibiting strong reactivity with IgE in the pooled ABPA sera was selected for sequencing and further characterization [14]. The sequence similarity searches were carried out using the BLAST program of the National Center for Biotechnology Information and with the Genetic Computer Groups package [18,19].

Over-expression of Asp f 16 in pET expression system

The cDNA clone of Af was amplified by polymerase chain reaction (PCR) with sense primer 5' GGATCCGGCA-GACTTGGTCCAAAGTGAA 3' and antisense primer 5' GCGGCCGCTCCAAACGATGGCGAAACCCC 3'. The amplified PCR product was purified from gel and subcloned at the Bam HI and Not I sites of pET expression vector 23b + (pET expression; Novagen, Madison, WI, USA). The expressed protein was eluted from preparative gel using electroelution apparatus (BioRad, Richmond, CA, USA) and the purified Asp f 16 was analysed in SDS-PAGE as described previously [11,12].

Subjects

Sera from three groups of subjects were selected for the study. Groups of 26 patients each from clinically confirmed ABPA and from asthmatics with or without immediate wheal and flare skin reactivity to Af, but no features of ABPA, were included [20–22]. All these 26 asthmatic patients were skin prick-positive to a mixture of different *Aspergillus* species, but only three were skin prick test-positive to Af. Patients

were seen at the Allergy Divisions of Northwestern University Medical School or the Medical College of Wisconsin. Serum samples from 26 normal control subjects with no clinical evidence of respiratory diseases were also included in the study. ABPA patients included in this study fulfilled at least six of the seven criteria proposed by Rosenberg *et al.* [21,22]. Human studies were carried out with the approval from Institutional review committees.

Total IgE estimation of serum samples

A fluorescence allergosorbent test (FAST; 3M Diagnostic Systems, Santa Clara, CA, USA) was used for the determination of total serum IgE. The threshold level for the detection of IgE was 2.4 ng/mL [23].

Reactivity of Asp f 16 with serum IgE

The binding of IgE in the sera of patients to rAsp f 16 was analysed by a solid-phase ELISA as previously described [12]. In brief, Immulon 2 polystyrene plates (Dynatech Labs, Chantilly, VA, USA) were coated with rAsp f 16 (10 μ g/mL) and crude culture filtrate (1 μ g/mL) in PBS for overnight at 4°C. The wells were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. This was followed by additional incubation of serum dilutions from various subjects. A 1:25 dilution with serum was used for IgE antibody. The plates were incubated for 3 h and washed three times with PBS containing 0.05% Tween 20 (PBS-T) for 5 min each. Biotinylated goat anti-human IgE (Vector Labs, Inc., Burlingame, CA, USA) diluted at 1:1000 was added to the respective wells. After incubation and washings, the wells were treated with 1:1000 dilution of streptavidin peroxidase (Zymed, San Francisco, CA, USA) for 1 h. The absorbance values of the colour reaction were read in an automatic ELISA plate reader (Dynatech) at a wavelength of 490 nm. The values were obtained by subtracting appropriate blanks and controls and the reproducibility was ascertained by comparing with known positive and negative sera.

Western blot analysis of the rAsp f 16 was carried out using pooled sera from ABPA patients, *Aspergillus* skin prick test-positive asthma group, and normal control subjects as described previously [12].

Asp f 16-specific PBMC proliferation

Proliferation of PBMC was performed in 96-well flat-bottomed microtitre plates in medium alone, in the presence of recombinant Asp f 16 or Af culture filtrate antigens. PBMC were obtained from six ABPA patients and four skin test-positive asthmatics. PBMC at a concentration of 2×10^5 /well were cultured in RPMI 1640

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      CTCGTGCCGAATTCGGCACAGCTTCCTGTCGTTTCTGAAGGGTTAAATCATTCATTTTC
1  -----+-----+-----+-----+-----+-----+-----+ 60
      AGTCGTTACTTTCAAAAGTCTTGACATATCATCATCTGCTATACAGTTCTCTTCCTTGAC
61  -----+-----+-----+-----+-----+-----+-----+ 120
      AAAACGCTCCTTTATCCTGCGTAGCGCAAACATGTATTTCAAGTACACAGCAGCAGCCCT
121 -----+-----+-----+-----+-----+-----+-----+ 180
              M Y F K Y T A A A L
      AGCTGCGGTGCTCCCTCTTTGCTCTGCACAGACTTGGTCAAAGTGCAATCCCCCTGCAGA
181 -----+-----+-----+-----+-----+-----+-----+ 240
      A A V L P L C S A Q T W S K C N P L A E
      GACCTGCCCCGCAACAAGGGTCTTGCTGCATCCACTTGCACCGCCGACTTCACCTCAGC
241 -----+-----+-----+-----+-----+-----+-----+ 300
      T C P P N K G L A A S T C T A D F T S A
      TTCAGCTTTGGATCAATGGGAAGTCACTGCAGGCAAAGTTCCCGTTGGCCACAGGGCGC
301 -----+-----+-----+-----+-----+-----+-----+ 360
      S A L D Q W E V T A G K V P V G P Q G A
      CGAGTTCACTGTCGCTAAGCAAGGCGACGCACCTACCATTGACACCGACTTCTACTTCTT
361 -----+-----+-----+-----+-----+-----+-----+ 420
      E F T V A K Q G D A P T I D T D F Y F F
      CTTGCGAAAGGCCGAAGTGGTGATGAAGGCCGCTCCTGGCACAGGTGTTGTTAGCAGCAT
421 -----+-----+-----+-----+-----+-----+-----+ 480
      F G K A E V V M K A A P G T G V V S S I
      CGTCCTGGAGTCGGATGATCTGGATGAGGTTGACTTGGTAAGATTGGGCGGTGACACCAC
481 -----+-----+-----+-----+-----+-----+-----+ 540
      V L E S D D L D E V D L V R L G G D T T
      TCAGGTTGAGACAACTACTTTGGCAAAGGAGACACCACCACATATGACCGAGGCACTTA
541 -----+-----+-----+-----+-----+-----+-----+ 600
      Q V Q T N Y F G K G D T T T Y D R G T Y
      CGTGCCCGTTGCCACTCCTCAGGAGACTTCCACACCTACACCATCGACTGGACCAAGGA
601 -----+-----+-----+-----+-----+-----+-----+ 660
      V P V A T P Q E T F H T Y T I D W T K D

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Fig. 1. Nucleotide and deduced amino acid sequences of cDNA clone encoding Asp f 16. The deduced amino acid sequence begins with the first potential in-frame initiation codon ATG (M) and ends with TGA stop codon denoted by the asterisk. The possible N-glycosylation site is underlined and the polyadenylation site upstream of poly (A) is italicized and underlined.

medium supplemented with 2 mmol/L glutamine, penicillin 100 U/mL, streptomycin 100 µg/mL, and 10% heat-inactivated human AB serum. The proliferative response of PBMC was determined using various concentrations of antigens and an optimum concentration of 5 µg/mL was selected for the antigen-induced PBMC stimulation [24]. The cells were incubated at 37°C in a humidified CO₂ incubator for 7 days before pulsing with 0.5 µCi/well of

³H-thymidine (specific activity of 6.7 Ci/mmol; DuPont Diagnostic Imaging Division, Billerica, MA, USA) for the final 18 h culture. The cells were harvested on glass fibre filter and the radioactivity counts were measured in a beta scintillation counter (Packard Instrument Co., Downers Grove, IL, USA). Stimulation indices (SI) were calculated as follows: S.I. = (counts in antigen-stimulated culture/counts in control culture without antigens).

	TGCCGTTACCTGGTCTATTGACGGTGCGGTCTGTCGTACGCTCACGTACAACGATGCCAA	
661	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	720
	A V T W S I D G A V V R T L T Y N D A K	
	GGGTGGCACTCGCTTCCCTCAGACTCCTATGCGCCTGAGACTGGCAGCTGGGCCGGCGGC	
721	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	780
	G G T R F P Q T P M R L R L A A G P A A	
	GACCCCAGCAACCCAGGGCACCATCGAGTGGGCCGGTGGCTTGACCGACTACAGCGCGG	
781	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	840
	T P A T P G H H R V G R W L D R L Q R G	
	GACCGTACACCATGTACGTCAAGTCCGTCCGTATCGAGAACGCCAACCCCGCCGAGTCTT	
841	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	900
	T V H H V R Q V R P Y R E R Q P R R V L	
	ACACCTACTCGGACAACCTCTGGCTCTTGGCAGAGCATCAAGTTCGACGGCTCCGTCGATA	
901	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	960
	H L L G Q L W L L A E H Q V R R L R R Y	
	CTCCTCCAGCTCTTCCGTGACCTCCTCCACCACCAGCACCGCCAGCTCCGCCAGCTCTAC	
961	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	1020
	S S S S S V T S S T T S T A S S A S S T	
	CTCGAGCAAGACCCCTTCCACCTCCACCCTGGCCACTTCCACCAAGGCGACTCCCACCCC	
1021	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	1080
	S S K T P S T S T L A T S T K A T P T P	
	GTCTGGAACCGACTCCGGCTCTAACTCGAGCTCCAGCGCGAACCTACTACCACCGGCGG	
1081	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	1140
	S G T S S G S <u>N S S</u> S S A E P T T T G G	
	CAGCGGCAGCAGCAACACCGGCTCTTGGCTCCGGCTCCGGCTCTGGCTCTGGCTCTACTC	
1141	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	1200
	S G S S N T G S W L R L R L W L W L Y S	
	TAGCACGGGCTCCTCCACTAGCGCCGAGCCTCCGCCACCCCCGAGCTCTCCAGGGCGC	
1201	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	1260
	S T G S S T S A G A S A T P E L S Q G A	
	CGCCGGCTCCATCAAGGGCTCGGTACAGCCTGCGCTCTGGTGTTCGGCGCCGTCGCTGCC	
1261	- - - - + - - - - + - - - - + - - - - + - - - - + - - - - +	1320
	A G S I K G S V T P A L W C S A P S L P	

Fig. 1. Continued

Statistical analysis

Mean + 2 S.D. of the normals were taken as significant. The mean titres of sera from ABPA and allergic asthma groups were compared with those of the normal controls by Student's *t*-test (two-tailed, independent samples). A *P*-value of < 0.05 was considered statistically significant.

Results

Characteristics of recombinant allergen Asp f 16

The open reading frame of the identified gene of 1546 bp from *Af* encodes a protein of 427 amino acids (Fig. 1). The stretch of hydrophobic amino acids at the N-terminal end

1321 GTGTTGGCATTCTAAACAAAATGACGATTTTGGTTTAAATGCACGATACACATCATGAAGG 1380
 -----+-----+-----+-----+-----+-----+-----+
 C W H S K Q N D D F G L M H D T H H E G

 1381 GGACGTCAGAACTATACATTTTCGGCATCGGGGTTTCGCCATCGTTTGGAGTATGACTACT 1440
 -----+-----+-----+-----+-----+-----+-----+
 D V R T I H F G I G V S P S F G V *

 1441 CCATCGTTCCTATTGATTTTCATTCTATGCCCATACCTATTCTTATACCCCGTACATACA 1500
 -----+-----+-----+-----+-----+-----+-----+

 1501 ACATGCAACATGTTACATATTTAATGAAAGTACGCTATTGTTCTGG 1546
 -----+-----+-----+-----+-----+-----+-----+

Fig. 1. Continued

indicates the presence of an 18 aa long signal peptide (GenBank Accession No. AF062651). The possible polyadenylation site AATGAA at the 3' end is found 18 bases upstream of the poly (A) tail. The amino acid composition of the protein indicates that it is rich in threonine (12.6%), serine (12.1%), alanine (9.6%), and glycine (8.6%). One possible glycosylation site (NSS) is present in the amino acid residues 318–320 of Asp f 16. The hydropathic profile

of the cloned allergen was analysed with a window of 11 amino acids [19]. The dominant hydrophilic regions of the allergen were demonstrated in the amino acid residues 108–124, 220–234, 237–253, 260–274, and 389–404. Further analysis with T-sites program revealed major amphipathic regions at amino acid residues 21–56 and 156–292, which might contribute to the α -helical structure of the protein [25].

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Asp f 9   11  myfkytaaalaavlp|lcsagtws|kcnplektcp|pnkglaastytadftsa 60
      |||||
Asp f 16  1   myfkytaaalaavlp|lcsagtws|kcnplaetcp|pnkglaastctadftsa 50
      |||||
Asp f 9   61  saldqwevtagkv|pvpgpqgaef|tvakqgdaptid|tdfyfff|gkaevvmka 110
      |||||
Asp f 16  51  saldqwevtagkv|pvpgpqgaef|tvakqgdaptid|tdfyfff|gkaevvmka 100
      |||||
Asp f 9   111 apgtgvvssivlesdd|ldevdwevl|ggdt|tqvqt|nyfgkgd|tttydr|gty 160
      |||||
Asp f 16  101 apgtgvvssivlesdd|ldevdlvr|lgd|tqvqt|nyfgkgd|tttydr|gty 150
      |||||
Asp f 9   161 vpvatp|qetfh|tytidwtkd|avtwsidg|avvr|tltyndak|ggtrfp|qtpm 210
      |||||
Asp f 16  151 vpvatp|qetfh|tytidwtkd|avtwsidg|avvr|tltyndak|ggtrfp|qtpm 200
      |||||
Asp f 9   211 rlrlgswaggdp|snpkgtiewaggl|tdysagpytm|yvk|svr.|ienan|pae 259
      |||||. . | . | | : : || |
Asp f 16  201 rlrlaagpaatpatpgh..hrvg|rwldrlqr|gtvh|hrqvr|pyrer|qprr 248
      |||||
Asp f 9   260 sytysdnsgswqsikfdgsvdiss|sssvtssttstassassts 302
      |||||
Asp f 16  249 vlhl|lgqlwll|laehqvr|rlrry|sssvtssttstassassts 291

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Fig. 2. Sequence homology between Asp f 16 and Asp f 9 was compared using GCG gap program. The N-terminal region of Asp f 16 from amino acids 1–204 exhibited high sequence homology with the corresponding amino acid sequences from Asp f 9. Thereafter, following a gap of 67 amino acid residues, complete homology was demonstrated for a region of 21 amino acids, predominantly serine and threonine from 271 to 291 of Asp f 16 sequence.

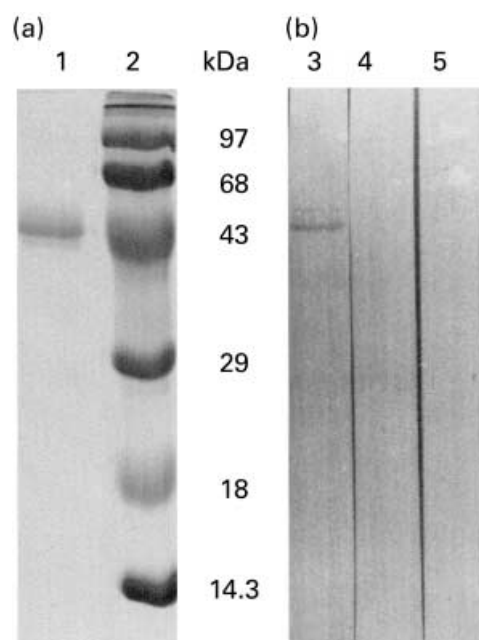


Fig. 3. (a) SDS-PAGE analysis of purified rAsp f 16. The purified protein was loaded at 10 μ g/protein per lane on a 12% SDS-polyacrylamide gel and stained with coomassie brilliant blue. Lane 1, Purified rAsp f 16; lane 2, molecular weight markers. (b) Western blots of purified rAsp f 16 for IgE antibody binding. Lane 3, With a positive ABPA serum pool; lane 4, an asthmatic serum pool; lane 5, pooled normal controls.

Asp f 16 failed to show significant sequence homology with any of the known functional and structural proteins; however, amino acid residues in the region of 99–214 exhibited 36% identity and 48% similarity with a probable membrane protein from the yeast *Saccharomyces cerevisiae*. In a stretch of amino acid residues 73–204, Asp f 16 shared 31% identity and 46% similarity with the hybrid endo- β -1,3-1,4 glucanase. Asp f 16 also demonstrated a distinct sequence homology with another Af allergen Asp f 9 at the N-terminal region of the protein (Fig. 2), whereas C-terminal region from amino acids 214–427 failed to show significant homology except in the region of a small stretch of amino acid residues from 271 to 291. The 43-kDa allergen reported here exhibited an overall sequence homology of 53% with the 30.6-kDa Asp f 9. The 1546-bp gene encoding the present allergen has a predicted size of 41.3 kDa, while Asp f 9 encodes a protein of 30.6 kDa, hence, differ also in protein size.

The high expression of Asp f 16 was observed at the molecular weight range of 40–43 kDa after 3 h IPTG induction. SDS-PAGE analysis of the purified rAsp f 16 is shown in Fig. 3a. The measured size of the rAsp f 16 is in close agreement with the predicted molecular size of 41.3 kDa.

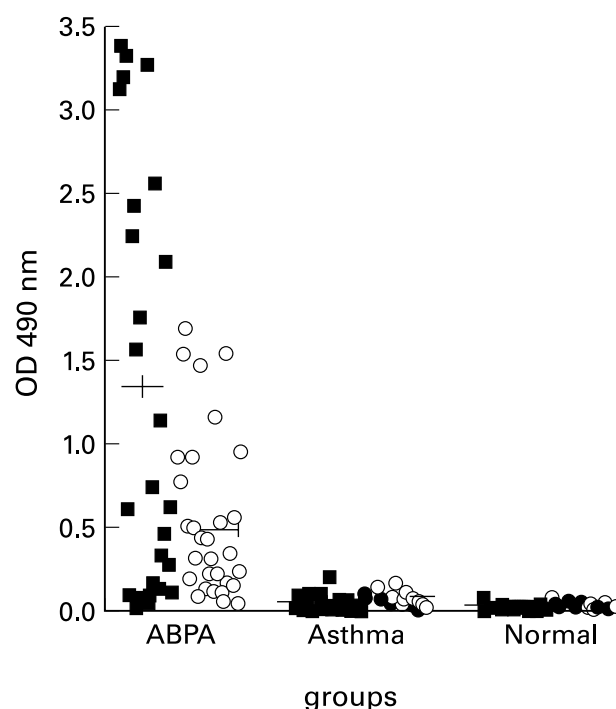


Fig. 4. Asp f 16 binds specifically to IgE antibodies in the sera of ABPA patients compared with that for *Aspergillus* skin prick test-positive allergic asthma and normal controls. IgE binding of recombinant Asp f 16 (■) and crude culture filtrate antigens of Af (○) were shown for 26 serum samples each from the indicated groups. The mean IgE absorbance of ABPA patients' sera measured at OD₄₉₀ was 1.3 ± 0.25 for Asp f 16, whereas for crude culture filtrate antigens, the mean absorbance at OD₄₉₀ was 0.50 ± 0.09 .

IgE binding of rAsp f 16 with sera from ABPA patients

Western blot analysis of the denatured rAsp f 16 on nitrocellulose membrane exhibited IgE antibody binding with sera from patients with ABPA, whereas pooled serum samples from asthma and normal controls failed to show antibody binding with rAsp f 16 (Fig. 3b).

The results of IgE binding by sera from patients and normal subjects to Asp f 16 are shown in Fig. 4. In an antigen-specific ELISA, rAsp f 16 demonstrated specific IgE binding with sera from ABPA patients. The mean ELISA IgE absorbance (OD₄₉₀) for ABPA patients ($n = 26$) was 1.3 ± 0.25 compared with 0.03 ± 0.01 for allergic asthma and 0.018 ± 0.002 for normal controls ($P < 0.05$). The mean optical density of rAsp f 16-specific IgE in the sera of 26 ABPA patients was almost 2.5-fold higher than for the Af culture filtrate allergens, as shown in Fig. 4. Sera from allergic asthmatics without ABPA and normal control subjects failed to demonstrate IgE binding with rAsp f 16. Among various subject groups studied, Asp f 16 exhibited 100% specificity as well as 70%

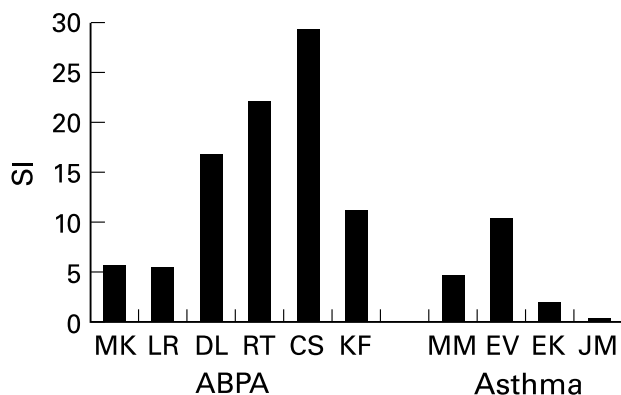


Fig. 5. Increased proliferative responses of peripheral blood mononuclear cells (PBMC) from patients with ABPA to Asp f 16 in comparison with the responses of Af-sensitized skin prick test-positive asthma patients. PBMC (2×10^5 /well) were stimulated with an optimum concentration of 5 μ g/mL of Asp f 16, and the proliferative responses of PBMC from different patients were measured by the ^{3} H-thymidine uptake. Four out of six ABPA patients showed significant proliferation in the range of 10–30 stimulation index (SI). Only one asthma patient with Af skin test positivity showed significant stimulation. For details see Materials and methods.

sensitivity in IgE binding with sera from ABPA patients.

Total IgE in the sera

In this study, total IgE levels in 26 subjects from each group were compared with the respective Af culture filtrate-specific IgE antibodies. No correlation was demonstrable in the levels of total serum IgE and Af-specific IgE in ABPA and *Aspergillus* skin prick test-positive and -negative asthma patients. As shown in Table 1, ABPA patient (patient TA) with total IgE as low as 300 ng/mL demonstrated a significantly high level of rAsp f 16 IgE antibody with an ELISA unit of 5.92. In contrast, Af-sensitized allergic asthma patient (patient FP) with a total IgE as high as 4056 ng/mL failed to show significant IgE antibodies (1.0 ELISA unit) against rAsp f 16 protein.

Stimulation of PBMC by Asp f 16

The PBMC from six ABPA and four Af-sensitized asthma patients were stimulated *in vitro* with various concentrations of Asp f 16 (Fig. 5). Four out of six ABPA patients showed significant stimulation indices when 5 μ g/mL of Asp f 16 was used in PBMC stimulation. A SI in the range of 10–30 was seen in these patients, while three of four allergic asthma patients showed lower indices compared

with ABPA (Fig. 5). Two of the six ABPA patients exhibited a low SI in the range of 5–6.

Discussion

In the present study, we report the cloning, sequencing, and expression of a recombinant Af allergen designated as Asp f 16. This allergen showed extensive sequence homology with Asp f 9 at the N-terminal region, but the C-terminal region of the protein representing amino acid residues from 214 onwards failed to show significant sequence homology with Asp f 9. The presence of complete 3' region of the protein was ascertained by the nucleotide sequences AATGAA 18 bases upstream of poly (A). Similar polyadenylation sites have also been reported for other fungal allergens [26]. The less conserved sequence homologies have been seen also with enzyme endo- β glucanase and a probable membrane protein of *S. cerevisiae* at the N-terminal region of the protein. Although there is a possible N-linked glycosylation site at the amino acid range of 318–320, the significantly high antibody binding of non-glycosylated recombinant Asp f 16 indicates that carbohydrate moieties may not be involved in allergen–antibody interaction.

The antibody binding sites of the allergen as predicted by its hydrophobic profile identified several hydrophilic regions throughout the molecule. These surface exposed regions may be the probable location of B cell epitopes in the allergen [19]. This is evidenced by high IgE antibody binding of Asp f 16 with sera from patients with ABPA. Indirect ELISA using Asp f 16 demonstrated specific IgE in 70% of sera from ABPA patients, while none of the 26 asthmatic sera or controls showed IgE binding to this allergen. In competitive ELISA inhibition assay, 100 ng of Asp f 16 exhibited >90% inhibition in IgE antibody binding sera from patients with ABPA to solid-phase coated Asp f 16 (data not shown), indicating the specificity of antigen–antibody reaction. However, Asp f 9 had been reported to recognize IgE in 89% of ABPA and 31% of Af skin test-positive asthmatic patients [9]. The reasons for the antibody binding differences of Asp f 9 and Asp f 16 may be due to the differences in the amino acid residues at the C-terminal region of the proteins or due to the differences in the patient groups used in these studies. This may also be due to the experimental conditions used in the assays reported from two different laboratories using diverse conditions.

However, the extensive homology between Asp f 9 and Asp f 16 at the N-terminal end suggests that both proteins are encoded by the same gene, and the difference in nucleotide sequences and molecular sizes might be attributable to the splicing and rearrangements of mRNA during transcription. A similar mechanism has been

reported for various isoforms of both secreted and membrane-bound IgE molecules originating from the same gene [27]. In addition, a recent antigenic characterization of Af culture filtrate proteins has clearly indicated the same parental origin for three proteins of different molecular weights such as p60, p40 and p37 [28]. The differences observed in the molecular mass and immunological reactivities among these proteins are attributed to the post-translational modification and proteolytic processing. Similarly there is a report of amino acid sequence differences between Af allergens Asp f 2 and gp55 near the N-terminal regions of the proteins [28,29]. In contrast, the allergens Asp f 16 and Asp f 9 exhibited amino acid sequence differences near the C-terminal ends, suggesting possible rearrangement and processing near the 3' region of the same gene.

Recently, Fontaine *et al.* purified an endo-1,3- β glucanase of 73 kDa from a cell wall autolysate of Af [30]. This glycosylated enzyme is reported to hydrolyse linear 1,3- β -glucan chains and to be possibly involved in cell wall morphogenesis. Although we have not yet studied the glycolytic properties of Asp f 16, the sequence similarities between the allergen and bacterial hybrid endo-1,3-1,4-beta-glucanase may be an indication of intracellular or membrane localization of Asp f 16. The specificity of IgE binding of Asp f 16 in ABPA patients may be attributed to the expression of this protein during the fungal colonization of the lung, a feature seen only in ABPA, but not in asthma. In addition, unavailability of Asp f 16 as a common aeroallergen may explain the lack of specific IgE in Af-sensitized asthmatic patients against this allergen. Previous studies have reported several intracellular Af allergens such as Asp f 2, Asp f 4 and Asp f 6 with specific IgE binding with sera from ABPA patients [10,11,16,31].

It has been reported that exacerbation of ABPA results in high total serum IgE and successful treatment of patients on long-term or short-term relapses are often associated with decline in total serum IgE [20,32,33]. However, we could not correlate the total serum IgE in ABPA patients to the presence of Af-specific IgE antibodies. In ABPA patients with a total serum IgE < 500 ng/mL, a significant level of allergen-specific IgE could be demonstrated indicating that allergen-specific IgE in this patient is independent of the total IgE in serum.

Several semipurified and purified recombinant Af allergens have shown distinct proliferative responses to the PBMC isolated from ABPA patients, indicating their involvement in cell-mediated immune responses in patients [24,34,35]. To delineate the Af-mediated allergic reaction in the pathogenesis of ABPA, allergens with known structure and functional properties are needed. Allergen-specific proliferative responses of PBMC from ABPA

patients to Asp f 1, a ribotoxin, and Asp f 6, a manganese superoxide dismutase enzyme, have recently been reported [31,34]. In another study, Asp f 1-specific T cell lines established from ABPA patients have been characterized as a CD4⁺ Th2 cell based on their cytokine secretion pattern [35]. In agreement with the previous observations using recombinant Af allergens, non-glycosylated Asp f 16, expressed in prokaryotic system, exhibited distinct cellular responses in patients with ABPA. Involvement of Asp f 16 in cell-mediated immune responses in patients is further substantiated by the prediction of distinct T cell epitope regions in the protein. Of the six ABPA patients, PBMC from four exhibited SI of > 10, whereas only one allergic asthma patient without ABPA showed a SI of 10.3. This patient with low levels of Af-specific serum IgE and no radiologic features of ABPA could be classified as a case of ABPA-S [10]. Taken together, the results suggest a major role of Asp f 16 in inducing a specific cellular immune response in ABPA patients, and this response positively correlates with the Asp f 16-specific IgE antibodies in sera of patients. In conclusion, the recombinant allergen Asp f 16 isolated from cDNA library of Af demonstrated significant roles in both humoral and cell-mediated immune responses in patients with ABPA, and the results indicate that Asp f 16 has diagnostic specificity for the *Aspergillus*-induced diseases.

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