

YOUNG INVESTIGATOR FACILITATED COMMUNICATION

Molecular cloning and expression of a recombinant *Aspergillus fumigatus* protein *Asp f II* with significant immunoglobulin E reactivity in allergic bronchopulmonary aspergillosis

BANANI BANERJEE, VISWANATH P. KURUP, SUHAS PHADNIS, PAUL A. GREENBERGER, and JORDAN N. FINK

MILWAUKEE, WISCONSIN, and CHICAGO, ILLINOIS

The cDNA of *Aspergillus fumigatus* encoding an allergen was cloned and expressed in *Escherichia coli*. The 987 bp long cDNA clone expressed a recombinant protein *Asp f II* of 34 kd. This protein exhibited binding to immunoglobulin E (IgE) in the serum samples from patients with allergic bronchopulmonary aspergillosis (ABPA). The patients with ABPA and central bronchiectasis demonstrated high levels of serum IgE antibodies, whereas patients with ABPA without central bronchiectasis, patients with asthma and *Aspergillus* skin test reactivity but no evidence of ABPA, and patients with aspergilloma showed only low levels of IgE antibody to *Asp f II*. In two-dimensional electrophoresis, a native antigen electroeluted from an *A. fumigatus* culture filtrate antigen preparation showed an isoelectric point and molecular weight similar to that of *Asp f II*. In a competitive enzyme-linked immunosorbent assay (ELISA), the IgE antibody reactivity of *Asp f II* with patient serum samples could be significantly inhibited by culture filtrate antigens of *A. fumigatus*. These results indicate that *Asp f II* has immunologic reactivities comparable to those of native *A. fumigatus* antigens. The recombinant *Asp f II* can be expressed in *E. coli* in large quantities and should prove useful as a standardized allergen for sensitive and specific immunodiagnosis of ABPA, especially in patients with central bronchiectasis. (J LAB CLIN MED 1996;127:253-62)

Abbreviations: ABPA = Allergic bronchopulmonary aspergillosis; ABPA-CB = ABPA with central bronchiectasis; ABPA-S = ABPA serology positive; *Af* = *Aspergillus fumigatus*; ELISA = enzyme-linked immunosorbent assay; IgE = immunoglobulin E; IgG = immunoglobulin G; IPTG = isopropyl- β -D thiogalactopyranoside; LB = Luria Bertani; PBS = phosphate-buffered saline solution; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

From the Departments of Medicine and Pathology, Medical College of Wisconsin; the Research Service, Veterans Affairs Medical Center, Milwaukee; and the Department of Medicine, Northwestern University Medical School, Chicago.

Supported by Veterans Affairs Medical Research funds and by the Ernest S. Bazley Grant to Northwestern Memorial Hospital and Northwestern University.

Submitted for publication July 27, 1995; revision submitted Oct. 10, 1995; accepted Oct. 18, 1995.

Reprint requests: Viswanath P. Kurup, PhD, VA Medical Center, Research Service 151-I, 5000 West National Ave., Milwaukee, WI 53295-1000.

0022-2143/96 \$5.00 + 0 5/1/70184

Aspergillus fumigatus causes a wide spectrum of human diseases ranging from invasive aspergillosis in immunocompromised patients, aspergilloma in pulmonary cavitary lesions, and ABPA in patients with allergic asthma.¹⁻³ ABPA complicates allergic asthma and results from colonization of *Af* in the respiratory tract, leading to immune injury and inflammatory responses.^{4,5}

The demonstration of serum IgE and IgG antibodies to *Af* in patients with ABPA is important in the diagnosis of the disease.^{3,6,7} The accurate diagnosis and clinical evaluation of the disease by immediate cutaneous reactivity, agar gel diffusion, radioallergosorbent test, ELISA, and Western blot requires purified, well-characterized, and biologically standardized antigenic preparations. In addition, it is essential to have antigens with known structural and functional properties to understand the immunopathogenesis of the disease.

Semipurified or purified antigens prepared by using conventional purification techniques often exhibit batch-to-batch variation and heterogeneity in their compositions.^{8,9} To circumvent the problems related to standardization of antigenic preparations, attempts are being directed to obtain biologically active recombinant antigens/allergens of *Af*. An 18 kd allergen of *Af* having immunodiagnostic specificity that can differentiate patients with ABPA from those without the disease has been cloned, expressed, and evaluated.^{10,11} Recently we have cloned a gene from *Af* encoding a heat shock protein (HSP-1) that binds to IgE and IgG *Af* antibodies in the serum samples of patients with aspergillosis.¹² Another *Af* recombinant protein, a 32 kd nonglycosylated alkaline protease recently isolated, failed to exhibit any significant allergenicity or antigenicity in the study reported by Moser et al.¹³ However, cloning of cDNA that encodes additional relevant allergens of *Af* would facilitate a more reliable diagnosis and should provide useful information in the understanding of *Af*-mediated disease. Such purified and standardized allergens would provide reliable results, leading to consistent diagnosis. In addition, cloning could be used to express large quantities of invariable allergens.

In this report we describe the molecular cloning of a cDNA clone from *Af* that expresses an immunologically active recombinant allergen *Asp f II* associated with ABPA and especially with ABPA patients with central bronchiectasis, the cardinal and unique feature of the disease.^{4,5} This purified recombinant protein exhibits specific IgE binding activity with serum samples from patients with ABPA and discriminates ABPA-S from ABPA-CB.

METHODS

Construction of cDNA library. A strain of *A. fumigatus* (*AF* 102, ATCC 42202) originally isolated from the sputum of a patient with an aspergilloma was grown in synthetic broth (AOAC, Difco Laboratories Inc., Detroit, Mich.) under constant agitation at 37° C for 96 hours.¹⁴ The total *Af* RNA from the mycelium was extracted with guanidium isothiocyanate as previously described.¹⁵ The poly (A) RNA was obtained from the total RNA by oligo dT cellulose chromatography.¹⁶ A cDNA expression library was constructed in λ ZAP II vector (Stratagene, La Jolla, Calif.) after reverse transcribing the poly A RNA. A primary library of 2×10^5 recombinants was amplified to generate a high titer phage stock.¹²

Library screening and DNA sequencing. *E. coli* strain XL1-Blue was infected with 5×10^4 phages containing the cDNA and plated on LB agar (Difco) in 150 mm Petri dishes. The plates were incubated at 37° C for 4 hours and layered on nitrocellulose filters (Schleicher and Schuell, Keene, N.H.) presoaked with 10 mmol/L IPTG (Sigma, St. Louis, Mo.) on top of the agar. The plates were again incubated at 37° C for 4 hours before blocking with Tris-buffered saline solution containing 1% bovine serum albumin and *E. coli* lysate. The membranes were screened with anti-*Af* rabbit hyperimmune serum and pooled serum samples from patients with ABPA, as previously described.¹²

The positive clones were isolated and purified to homogeneity. One of the positive clones exhibiting strong reactivity with serum samples from patients with ABPA was selected for sequencing and characterization.¹⁷ Double stranded plasmid DNA was used as a template for DNA sequencing. The plasmid was isolated from an overnight culture of XL1-Blue cells by using a plasmid midi kit according to the protocol of the manufacturer (Qiagen Inc., Chatsworth, Calif.). The starting primers for sequencing were 5' ATGGAAACAGCTATGACCATG-ATTACG and 3' TTGTAAAACGACGGCCAGTGA-ATTG. The entire cDNA insert was sequenced by primer walking. Sequencing was carried out on a 6% polyacrylamide gel with a vertical gel sequencing apparatus (model S2; GIBCO/BRL, Life Technologies Inc., Gaithersburg, Md.). All sequences were read and recorded manually. The primers used at different stages of sequencing were obtained from Biosynthesis Inc., Lewisville, Tex. The sequence similarity searches were done with the BLAST program of the National Center for Biotechnology Information and with the Genetic Computer Groups (Madison, Wis.) package.^{18,19}

Subcloning of *Asp f II* gene and high expression of protein in *E. coli*. The cDNA clone of *A. fumigatus* was amplified by polymerase chain reaction with sense primer 5' GGATCCGTCGGCAGACGAGGCGGC 3' and antisense primer 5' CTCGAGCTAAGTGCAATGAAGCTGTCCACC 3'.²⁰ These primers were designed from the nucleotide sequences of the open reading frame of the cDNA clone. The amplified fragments were subcloned into PCR II vector (Invitrogen, San Diego, Calif.).

The isolated plasmid DNA was digested with BamHI and XhoI and ligated to similarly digested expression vector pET23b (pET expression system; Novagen, Madison, Wis.). The induction and analysis of the fusion protein was performed according to the pET expression protocol of the manufacturer. In brief, *E. coli* strain HMS174 was transformed with the recombinant plasmid vectors by electroporation with *E. coli* pulser apparatus (BioRad, Hercules, Calif.). The transformed cells were plated on LB agar containing ampicillin (100 µg/ml). Plasmid isolated from overnight culture was further transformed into BL21DE3pLysS by electroporation followed by plating on LB agar containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). When the cells reached the log growth phase ($A_{600} = 0.4$), IPTG was added to a final concentration of 1 mmol/L to induce the expression of fusion protein by the recombinant *Af* clone. Cells were harvested at intervals of 1 hour for 4 hours after IPTG induction and were sonicated. The cell lysates containing recombinant expression protein were analyzed on a 12% polyacrylamide gel containing 0.5% SDS under reducing and denaturing conditions. The gels were stained with Coomassie brilliant blue dye, and the expression at different periods of induction was compared.²¹ Because histidine was not incorporated in the fusion protein, electroelution of the band from the SDS-gels was used for its purification. The purity of the protein preparation was ascertained by two-dimensional gel electrophoresis. Eluted protein was lyophilized and stored at -20°C .

Rabbit hyperimmune serum against *Asp f II*. Rabbit anti-*Af* antibody was raised as previously described.¹² Polyclonal antibody against *Asp f II* was raised with the following modifications. In brief, 100 µg of purified protein was emulsified in an equal volume of Titer Max research adjuvant (CytRX, Narcross, Ga.) and injected subcutaneously at multiple sites. Four consecutive injections were given at weekly intervals. The fifth injection was given after an interval of another 15 days, and the animal was bled 1 week after the last injection. The serum titer for anti-recombinant protein antibody was checked by ELISA and immunodiffusion with *Asp f II* and culture filtrate preparations of *Af*.

Human serum samples. Serum samples from groups of patients were used for this study. They included 11 patients with ABPA-S, 9 patients with ABPA-CB, 8 patients with allergic asthma and immediate wheal and flare cutaneous reactivity to *Af*, and 9 patients with aspergilloma.⁵ The serum samples were collected from patients attending the Allergy-Immunology Clinics of the Medical College of Wisconsin affiliated hospitals and from the Allergy Clinics of Northwestern University Medical School. Serum samples from five normal control subjects with no clinical evidence of respiratory disease also were studied.

Purification of native *A. fumigatus* antigen. The *Af* culture filtrate antigen was prepared as previously described.^{7,14,26} The antigen was analyzed on a 15% polyacrylamide gel under reducing and denaturing conditions by using a BioRad Mini Protean II electrophoresis unit

(BioRad, Richmond, Calif.) and staining with Coomassie brilliant blue.¹² The protein band in the same molecular range and showing the same reactivity with patient and rabbit anti-*Af* serum in Western blots as the recombinant protein was cut off from the gel. It was then subjected to electroelution (BioRad, model 422) and dialyzed overnight against distilled water to remove SDS from the eluted protein. The native antigen was lyophilized and stored at -20°C .

Two-dimensional electrophoresis of *Asp f II* and purified native antigen of *A. fumigatus*. Two-dimensional electrophoresis of purified recombinant protein and a native *Af* antigen was performed as previously described.²²⁻²⁴ Protein samples (100 µg/tube) were applied to prefocused 4% acrylamide gel in capillary tubes containing 9.2 mol/L of urea, 20% Triton X-100, and 3.5% pharmalyte 3-10 ampholyte (Pharmacia, Piscataway, N.J.). Electrofocusing was performed at a constant voltage of 500 V for 10 minutes and thereafter at 750 V for 5 hours. After isoelectric focusing, the second dimension separation was carried out on a 12% SDS polyacrylamide gel for 45 minutes at a constant voltage of 200 V. The resolved proteins on the second dimension gel were visualized by silver staining.^{21,22}

Western blot analysis of *Asp f II* and purified native *A. fumigatus* antigen using rabbit anti-*Asp f II* antibodies. Both *Asp f II* and native *Af* antigen proteins (10 to 20 µg per gel) were separated on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane (Schleicher & Schuell) by using a Trans-Blot apparatus (BioRad) as previously described.^{7,12} After blocking and washing, the membranes were incubated overnight at 4°C with a 1:250 dilution of rabbit hyperimmune serum against *Asp f II*. After overnight incubation at 4°C , the strips were treated with biotinylated goat anti-rabbit IgG antibody for 1 hour, followed by incubation with streptavidin alkaline phosphatase at room temperature for 1 hour. Color was then developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium-nitroblue tetrazolium substrate (Boehringer Mannheim, Indianapolis, Ind.) and the developed bands were compared.

Lectin (concanavalin A) binding property of native *A. fumigatus* antigen. The native *A. fumigatus* antigen was resolved on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane as described above. After washing and blocking, the membrane was incubated with a 1:100 dilution of biotinylated concanavalin A for 1 hour at room temperature. This was followed by treating the blot with streptavidin alkaline phosphatase for 1 hour at room temperature. The enzyme substrate reaction was carried out as described above.

Antigenicity/allergenicity of *Asp f II*

Antigen-specific ELISA. The binding of anti-*Af* antibodies to fusion protein was analyzed by a solid-phase ELISA.^{6,25,26} In brief, Immulon II polystyrene plates (Dynatech Laboratories, Chantilly, Va.) were coated overnight at 4°C with 10 µg per ml of recombinant protein in PBS, pH 7.4. The wells were then washed with PBS con-

taining 0.05% Tween 20. The coated plates were blocked with PBS containing 0.05% Tween 20 and 1% (wt/vol) nonfat dry milk powder (blocking buffer) for 1 hour at room temperature. After the wells were washed, patient serum samples diluted 1:50 in blocking buffer for IgE and 1:500 for IgG were added to the wells and incubated for 3 hours at room temperature. The wells were again washed, and biotin-conjugated anti-human IgE (Vector, Burlingame, Calif.) diluted 1:500 and biotin-conjugated anti-human IgG (Sigma) diluted 1:1000 were added and incubated for 1 hour. Streptavidin peroxidase (Zymed, San Francisco, Calif.) was then added and the color developed with the chromogen substrate (*O*-phenylene diamine, 0.015% hydrogen peroxide in citrate buffer). The color was read at 490 nm in an automatic ELISA plate reader (Dynatech). All dilutions of the reagents were selected empirically after checkerboard titrations with positive and negative control sera. Appropriate controls were included in all plates.

Competitive inhibition ELISA. Anti-*Af* rabbit serum (1:500 dilution) and a human serum sample (1:50 dilution) from a patient with ABPA were incubated with 100 μ g to 10 ng of the culture filtrate antigens. The serum samples were then centrifuged, and the supernatants were transferred to the washed and blocked *Asp f II*-coated plates. Further steps were carried out with various reagents as in the antigen-specific ELISA described above. The dose-dependent inhibition of titers was determined from the ELISA values.

RESULTS

cDNA clones expressing *Aspergillus* proteins. A total of 136 positive clones were identified by screening the cDNA library with rabbit antiserum and serum samples from patients with ABPA. The pure recombinant λ ZAP phages that contained the cDNA inserts were treated individually by the in vivo excision protocol to subclone into pBlue script SK (+/–) plasmid. One of the recombinant phages was selected for further studies. The double-stranded plasmid DNA was used as template for nucleotide sequencing. The nucleotides and deduced amino acid sequences of cDNA encoding *Asp f II* are shown in Fig. 1. The insert is 987 base pairs in length and encoded a protein of 34 kd. Sequence analysis of cDNA revealed that the correct nucleotide sequence is in the same phase with the open reading frame of the β -galactosidase gene of the λ ZAP vector. The starting TCG codon represents serine at the 'N' terminal end and indicates lack of a start codon, suggesting that the 'N' terminal end is incomplete. The clone has a complete 3' untranslated region with a poly A tail. The possible polyadenylation signal AATATA was present 42 base pairs upstream of the poly A tail. Three potential N-glycosylation sites—27-29, 83-86, and 156-158—dis-

played Asn-x-Ser/Thr motifs. The T-sites program analysis with a window size of 11 amino acids revealed a major amphipathic region at residue 124 to 150 that also may have alpha helical structure.²⁷

The IPTG-induced *E. coli* cell lysate containing a recombinant expression vector was analyzed by SDS-PAGE. The high expression of the recombinant protein *Asp f II* was observed at the molecular weight range of 34 kd (Fig. 2, lanes b, d, f and h). A gradual increase in the concentration of the fusion protein was noticed, with an increase in the time of induction with IPTG from 1 hour to 4 hours. Uninduced cell lysate at identical intervals of growth failed to show the fusion protein (Fig. 2, lanes a, c, e, and g). Cell extract obtained after 4 hours of IPTG induction was used for purification of the fusion protein. The purified protein did not show any autodegradation as analyzed by SDS-PAGE but appeared to have an additional band at the higher molecular weight range of 60 to 65 kd (Fig. 3, A [lane c]). The culture filtrate preparation of *Af* exhibited a strong protein band at the molecular weight range of 30 to 40 kd (Fig. 3, B [lane b]) in the same molecular weight range as the recombinant protein (Fig. 3, B [lane c]).

Immunoreactivity against anti *Asp f II* antibodies. Recombinant *Asp f II* and native *Af* protein demonstrated immunoreactivity against anti *Asp f II* antibodies. The native antigen purified from *Af* culture filtrate preparation reacted strongly with rabbit hyperimmune serum raised against *Asp f II*. The native antigen showed size and isoelectric point identical to that of the *Asp f II* when studied by two-dimensional gel electrophoresis (Fig. 4). Both proteins exhibited isoelectric points in the range of 5.5 to 6.6 and a size of approximately 34 kd. In Western blot analysis, purified *Af* antigen from culture filtrate showed strong binding activity with rabbit hyperimmune serum raised against *Asp f II* (Fig. 5, lane a), comparable to that of *Asp f II* (Fig. 5, lane b). The Western blot analysis of concanavalin A binding of native *Af* antigen did not demonstrate glucose and mannose molecules in the purified antigen (result not shown).

Reaction of protein with antibodies in serum. Recombinant protein reacted with IgE and IgG antibodies in the serum samples of patients with ABPA. In the ELISA, when *Asp f II* was used, levels of serum IgE antibody were elevated in patients with ABPA-CB as compared with levels in patients with ABPA-S, allergic asthma, and aspergilloma and in normal control subjects (Fig. 6, A). The ELISA optical density values of *Asp f II*-specific IgE antibodies in ABPA-CB patients ranged from 0.076 to 1.44, in comparison with a range of 0.04 to 0.08 for normal

```

TCGGCACGAGACGAGGCCGGCCTGAACGAGGCGGTCGAGCTCGCCCGGCACGCCAAGGCC 61
SerAlaArgAspGluAlaGlyLeuAsnGluAlaValGluLeuAlaArgHisAlaLysAla

CACATCCTCCGCTGGGGCAACGAGAGCGAGATCTACCGGAAGTACTTTGGCAACCGGCC 121
HisIleLeuArgTrpGlyAsnGluSerGluIleTyrArgLysTyrPheGlyAsnArgPro

ACCATGGAGGCCGTCGGTGCCTACGATGTCATCGTGAACGGGGACAAGGCCAACGTGCTC 181
ThrMetGluAlaValGlyAlaTyrAspValIleValAsnGlyAspLysAlaAsnValLeu

TTCCGGTGTGACAACCCCGACGGCAACTGTGCTTTGGAAGGCTGGGGCGGCCACTGGCGC 241
PheArgCysAspAsnProAspGlyAsnCysAlaLeuGluGlyTrpGlyGlyHisTrpArg

GGCGCGAACGCCACCTCCGAAACCGTCATCTGTGATCGCAGCTACACCACCCGCCGCTGG 301
GlyAlaAsnAlaThrSerGluThrValIleCysAspArgSerTyrThrThrArgArgTrp

CTTGTCTCCATGTGCTCCCAGGGCTACACCGTCGCCGGCTCCGAGACCAACACCTTCTGG 361
LeuValSerMetCysSerGlnGlyTyrThrValAlaGlySerGluThrAsnThrPheTrp

GCTTCGGACCTGATGCACCGTCTGTACCATGTGCCTGCTGTGGGTCAAGGCTGGGTGCGAC 421
AlaSerAspLeuMetHisArgLeuTyrHisValProAlaValGlyGlnGlyTrpValAsp

CACTTCGCCGACGGCTACGACGAGGTGATTGCCCTGGCCAAGAGCAACGGCACCGAGTCC 481
HisPheAlaAspGlyTyrAspGluValIleAlaLeuAlaLysSerAsnGlyThrGluSer

ACGCATGACTCGGAGGCGTTCGAGTATTTGCCCTTGAGGCGTATGCGTTTGATATTGCT 541
ThrHisAspSerGluAlaPheGluTyrPheAlaLeuGluAlaTyrAlaPheAspIleAla

GCTCCCGGTGTGCGATGTGCTGGCGAGAGTCACGGCCCTGACCAGGGACATGACACCGGG 601
AlaProGlyValGlyCysAlaGlyGluSerHisGlyProAspGlnGlyHisAspThrGly

TCTGCCTCGGCGCCTGCGTCTACCTCCACCTCTAGCTCCAGCTCGGGCTCGGGCTCGGGC 661
SerAlaSerAlaProAlaSerThrSerThrSerSerSerSerSerGlySerGlySerGly

GCCACGACTACCCGACGGATTCTCCAGTGCCACTATTGATGTGCCGTCGAACTGCCAT 721
AlaThrThrThrProThrAspSerProSerAlaThrIleAspValProSerAsnCysHis

ACCCATGAAGGTGGACAGCTTCATTGCACTTAGCGGAGGACACGATATTTTATGACCGTA 781
ThrHisGluGlyGlyGlnLeuHisCysThr *

TGCTTGACGCCGTATGTTAACGCCGTATACTCCCGGTTGGACCGGCCCTGAGTAATTGGG 841

ATTAATATCTATTGTTTAAAAGGAAGTGTTATGGACGCGCGTTTACGTATTGGATTGCTT 901

GCCGAATATACATGCTATATAGCAATGTAAACCCAATAGAGACGTTCTTTATAAAAAAAA 961

AAAAAAAAAAAAAAAAAAAAAAAAAAAA 987

```

Fig. 1. The nucleotide sequence of the cloned DNA fragment showing the open reading frame encoding the protein *Asp f II*. The three possible glycosylation sites, 27-29, 83-86, and 156-158, are in boldface. The underlined sequence showed homology with a surface antigen of *Plasmodium falciparum*. Gene bank accession no. U20722.

control subjects. No differences were detected in the IgE antibody levels among patients with ABPA-S, asthma, and aspergilloma. The *Asp f II* serum IgE antibody in ABPA-CB showed significant differences from serum from patients with ABPA-S, allergic asthma, aspergilloma, and normal control subjects ($p < 0.05$). Serum IgG levels to *Asp f II* were elevated in patients with ABPA-S, ABPA-CB, and aspergilloma as compared with levels in patients with allergic asthma and levels in normal control

subjects (Fig. 6, B). However, when compared with samples from normal control subjects, samples from patients with allergic asthma had moderately elevated levels of IgG to *Asp f II*. Patients with aspergilloma demonstrated high levels of *Asp f II* serum IgG antibodies, ranging from absorbency values of 0.36 to 1.56 (Fig. 6, B).

In the competitive ELISA, a gradual dose-dependent decrease of over 35% binding of serum IgE antibody from a patient with ABPA was detected

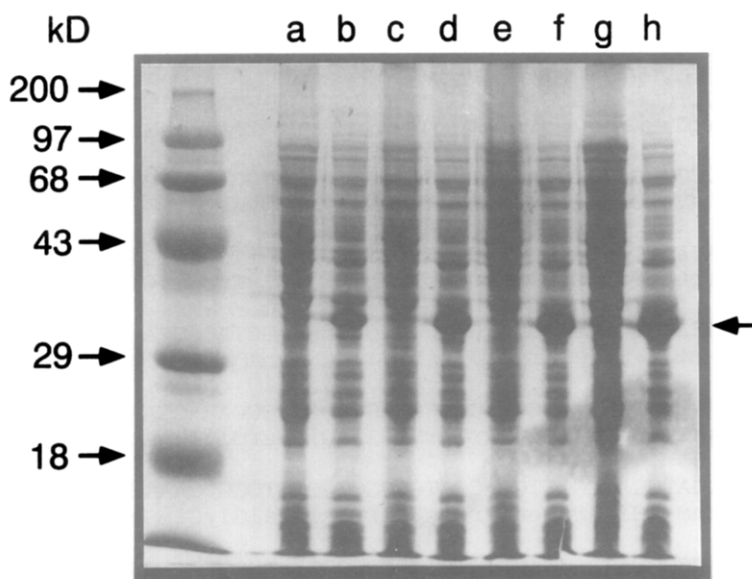


Fig. 2. SDS-PAGE gel (12%) stained with Coomassie brilliant blue showing expression of recombinant protein at different intervals of IPTG induction. Induced cell lysates at 1, 2, 3, and 4 hours of IPTG induction (lanes b, d, f, and h, respectively) and uninduced cell lysates at the same intervals of growth (lanes a, c, e, and g, respectively) are shown.

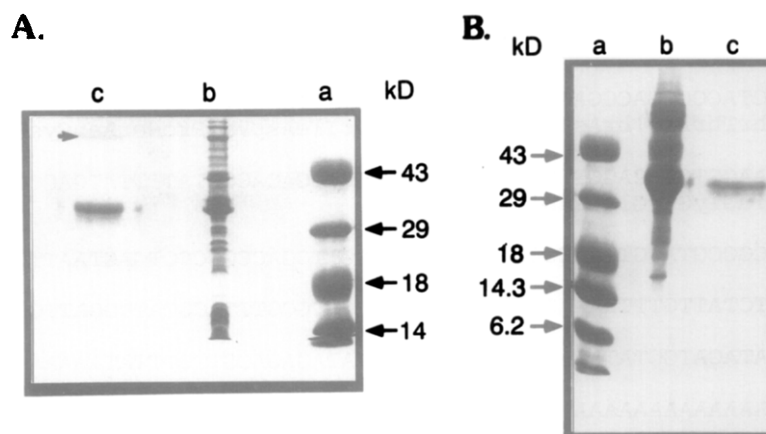


Fig. 3. A, SDS-PAGE gel (12%) stained with Coomassie blue, showing purified *Asp f II* sample (lane c) in comparison with *E. coli* extract (lane b) and molecular weight markers (lane a). B, SDS-PAGE gel (15%) stained with Coomassie blue, showing culture filtrate antigen preparation of *A. fumigatus* (lane b), purified *Asp f II* (lane c), and molecular weight markers (lane a).

(Fig. 7). The solid phase-bound *Asp f II* demonstrated a decrease in the serum titer of all *Af* culture filtrate antigen–preincubated serum. Rabbit anti-*Af* immune serum preincubated with *Aspergillus* antigens also showed a gradual decrease in IgG binding to *Asp f II*. Incubation of rabbit hyperimmune serum with culture filtrate antigens demonstrated 75% to 80% inhibition of antibodies to *Asp f II* in the reac-

tion mixture. These findings indicate that *Af* culture filtrate antigen contains *Asp f II*.

DISCUSSION

A diagnostically relevant *Af* allergen showing significant reactivity with ABPA was identified and expressed in *E. coli* by using molecular cloning. This recombinant allergen demonstrated specific IgE

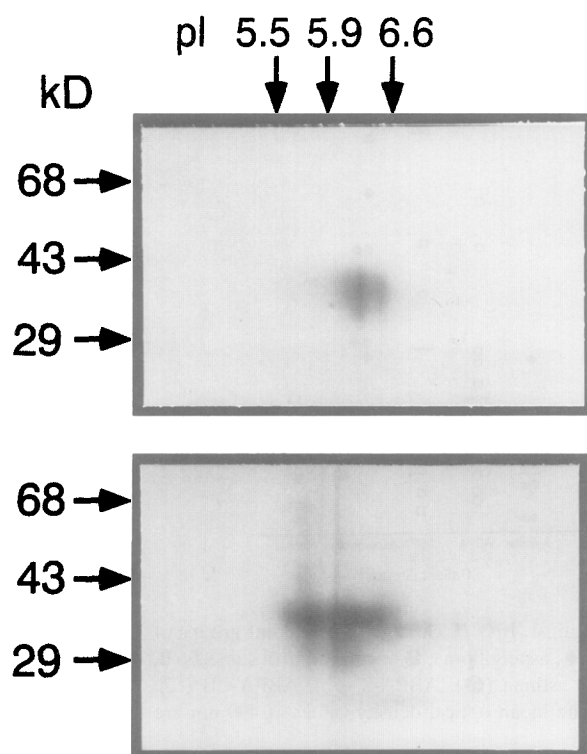


Fig. 4. Two-dimensional electrophoresis pattern of *Asp f II* (top) and a native protein purified from *A. fumigatus* culture filtrate antigen (bottom) by electroelution. In the first dimension, the proteins were separated by using ampholytes with an isoelectric point range from 3.0 to 10.0; in the second dimension, a 12% SDS-PAGE was used. Spots in both gels have comparable isoelectric points and molecular weights.

binding in the serum samples from patients with ABPA while binding to serum samples from patients with allergic asthma, and skin prick test reactivity to *Af* antigens was negligible.

A major 18 kD IgE binding protein, *Asp f I* has been purified and characterized as a member of the mitogillin family of cytotoxin and has been detected in several species of *Af*.^{26,28} A recombinant *Af* allergen, *Asp f I(a)*, which is of the same molecular weight as that of *Asp f I* and has a 99% sequencing homology to restrictocin (another ribotoxin from *A. restrictus*), has been reported by Moser et al.¹¹ This fusion protein demonstrated IgE binding activity and immediate cutaneous reactivity in 75% of patients with ABPA.²⁹ We previously have isolated a heat shock protein encoding gene from *Af*. This protein had binding activity to IgE in the serum samples of patients.¹² Although several other genes have been cloned and proteins expressed, no significant reactivity with IgE has been demonstrated with any of them.³⁰⁻³² Further, of the large number of antigens demonstrable in crude *Af* preparations,

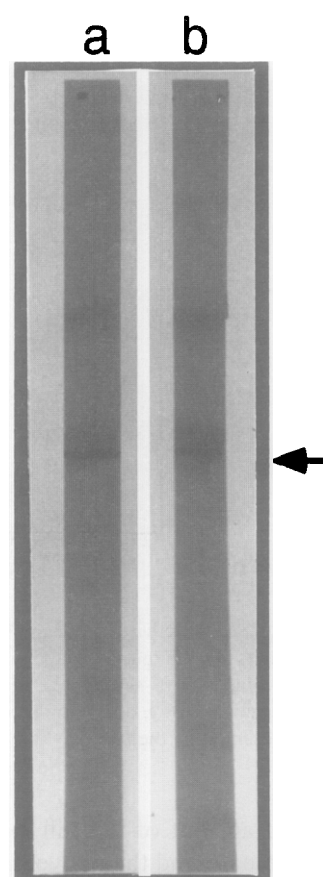


Fig. 5. Western blot of *Asp f II* and native *A. fumigatus* antigen when using rabbit hyperimmune serum raised against *Asp f II*. IgG binding of native antigen (lane a) and binding of *Asp f II* (lane b).

only a small proportion react with serum IgE from patients. The variability of the reactivity with *Asp f I*, mitogillin, and restrictocin has also been documented.²⁶ Therefore, because of the demonstrable wide spectrum of *Af* antigen reactivity, the use of several relevant antigens would be of value in the diagnosis of ABPA.

The database search results indicate that the present allergen did not show significant homology to other proteins in the data bank, but a stretch of serine and threonine present at nucleotide positions 620-61 near the C-terminal end imparts a sequence homology to the surface antigen of *Plasmodium falciparum*. The possible polyadenylation site is represented by a hexamer AATATA at nucleotide position 906-911, followed by a CATG motif.³³ The sequence data indicate that the clone expressing *Asp f II* has a complete 3' untranslated region with a poly (A) tail, but it is not a full-length cDNA clone because of a lack of appropriate start codon at the 5' terminal end. The protein expressed by the cDNA

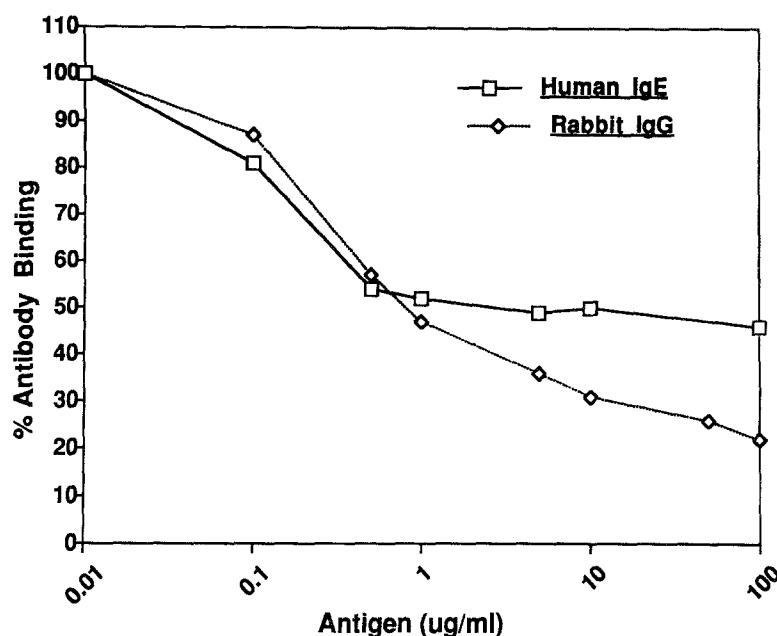


Fig. 7. Inhibition of antibody binding to solid phase coated with *Asp f II*. Rabbit antiserum and serum from a patient with ABPA were preincubated with different concentrations of crude culture filtrate antigens of *A. fumigatus*. Preincubated serum samples were then transferred to recombinant *Asp f II*-coated wells, and the remaining IgG and IgE binding activities were analyzed by ELISA. The absorbency values of serum samples without preincubation with crude culture filtrate antigens were set as 100%.

of the disease. In this context, the epitopes expressed by *Asp f II* might contribute to the reactivity of *Af* in producing bronchial wall injury. No specific IgG binding pattern of *Asp f II* was observed in the different patient groups, because all groups of patients and healthy control subjects demonstrated occasional increases in IgG antibody levels against *Asp f II*. This finding is not surprising, because *Af* is a ubiquitous fungus present in environments and thus results in frequent exposure leading to IgG production. Similar results were reported when using serum samples from healthy control subjects and different groups of patients when recombinant protein *Asp f(I/a)*, alkaline protease, and heat shock protein-1 were studied.¹¹⁻¹³

In conclusion, a recombinant protein was produced from *A. fumigatus* that has a molecular weight and isoelectric point similar to that of a native *Af* antigen, and it demonstrated reactivity with IgE antibody in the serum samples of patients with ABPA.

We thank Laura Castillo and Yiyong Wu for technical assistance and Donna Schrubbe for editorial assistance.

REFERENCES

1. Bardana EJ Jr. The clinical spectrum of aspergillosis. Part 1: epidemiology, pathogenicity, infection in animals and immunology of aspergillosis. *CRC Crit Rev Clin Lab Sci* 1980;13:21-83.
2. Bardana EJ Jr. The clinical spectrum of aspergillosis. Part 2: classification and description of saprophytic, allergic and invasive variants of human diseases. *CRC Crit Rev Clin Lab Sci* 1980;13:85-159.
3. Kurup VP, Kumar A. Immunodiagnosis of aspergillosis. *Clin Microbiol Rev* 1991;4:439-56.
4. Rosenberg MR, Patterson R, Mintzer BJ, Cooper BJ, Roberts M, Harris KE. Clinical and immunologic criteria for the diagnosis of allergic bronchopulmonary aspergillosis. *Ann Intern Med* 1977;86:405-14.
5. Greenberger PA. Allergic bronchopulmonary aspergillosis and fungoses. *Clin Chest Med* 1986;9:599-608.
6. Kurup VP. Enzyme-linked immunosorbent assay in the detection of specific antibodies against *Aspergillus* in patients' sera. *Zentral Bakteriell Microbiol Hyg [A]* 1986;261:509-16.
7. Kurup VP, Greenberger PA, Fink JN. Antibody response to low molecular weight antigens of *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis. *J Clin Microbiol* 1989;27:1312-6.
8. Longbottom JL. Antigens/allergens of *Aspergillus fumigatus*. II. Their further identification and partial characterization of a major allergen (Ag 3). *J Allergy Clin Immunol* 1986;78:18-24.
9. Wang JLF, Patterson R, Rosenberg M, Roberts M, Cooper BJ. Serum IgE and IgG antibody activity against *Aspergillus fumigatus* as a diagnostic aid in allergic bronchopulmonary aspergillosis. *Am Rev Respir Dis* 1978;117:917-27.
10. Arruda LK, Platts-Mills TA, Fox JW, Chapman MD. *Aspergillus fumigatus* allergen I, a major IgE binding protein, is a member of the mitogillin family of cytotoxins. *J Exp Med* 1990;172:1529-32.

11. Moser M, Cramer R, Menz G, et al. Cloning and expression of recombinant *Aspergillus fumigatus* allergen 1/a (*rAspf 1/a*) with IgE binding and type I skin test activity. *J Immunol* 1992;149:454-60.
12. Kumar A, Reddy LV, Sochanik A, Kurup VP. Isolation and characterization of a recombinant heat shock protein of *A. fumigatus*. *J Allergy Clin Immunol* 1993;91:1024-30.
13. Moser M, Menz G, Blaser K, Cramer R. Recombinant expression and antigenic properties of a 32 kilodalton extracellular protease, representing a possible virulence factor from *Aspergillus fumigatus*. *Infect Immun* 1994;62:936-42.
14. Kurup VP, Fink JN, Scribner GH, Falk MJ. Antigenic variability of *Aspergillus fumigatus* strains. *Microbios* 1978;19:191-204.
15. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. *Biochemistry* 1979;18:5294-9.
16. Aviv H, Leder P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc Natl Acad Sci USA* 1972;69:1408-12.
17. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977;74:5463-7.
18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403-10.
19. Genetics computer group. Program manual for the GCG package, version 7.2. Madison, Wisconsin: Genetics Computer Group, November 1992.
20. Saiki RK, Gelfand DG, Stoffel S. Primer directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science* 1988;239:487-91.
21. Kurup VP, Resnick A, Scribner GH, Gunasekaran M, Fink JN. Enzyme profile and immunochemical characterization of *Aspergillus fumigatus* antigens. *J Allergy Clin Immunol* 1986;78:1166-73.
22. O'Farrell P. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 1975;250:4007-21.
23. Alenius H, Kurup V, Kelly K, Palosuo T, Turjanmaa K, Fink JN. Latex allergy: frequent occurrence of IgE antibodies to a cluster of 11 latex proteins in patients with spina bifida and histories of anaphylaxis. *J LAB CLIN MED* 1994;123:712-20.
24. Merrill CR. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 1981;211:1437-8.
25. Brummund W, Resnick A, Fink JN, Kurup VP. *Aspergillus fumigatus* specific antibodies in allergic bronchopulmonary aspergillosis and aspergilloma. Evidence for a polyclonal response. *J Clin Microbiol* 1987;25:5-9.
26. Kurup VP, Kumar A, Kenealy WR, Greenberger PA. *Aspergillus* ribotoxins react with IgE and IgG antibodies of patients with allergic bronchopulmonary aspergillosis. *J LAB CLIN MED* 1994;123:749-56.
27. Delisi C, Berzofsky JA. T cell antigenic sites tend to be amphipathic structures. *Proc Natl Acad Sci* 1985;82:7048-52.
28. Arruda LK, Mann BJ, Chapman MD. Selective expression of a major allergen and cytotoxin, *Asp fl* in *Aspergillus fumigatus*. Implications for the immunopathogenesis of *Aspergillus*-related diseases. *J Immunol* 1992;149:3354-9.
29. Moser M, Cramer R, Brust E, Suter M, Menz G. Diagnostic value of recombinant *Aspergillus fumigatus* allergen 1/a for skin testing and serology. *J Allergy Clin Immunol* 1994;93:1-11.
30. Sirakova TD, Markaryan A, Kolattukudy PE. Molecular cloning and sequencing of the cDNA and gene for a novel elastinolytic metalloproteinase from *Aspergillus fumigatus* and its expression in *Escherichia coli*. *Infect Immun* 1994;62:4208-18.
31. Kolattukudy PE, Lee JD, Rogers LM, et al. Evidence for possible involvement of an elastinolytic serine protease in aspergillosis. *Infect Immun* 1993;61:2357-68.
32. Jatou-Ogay K, Suter M, Cramer R, Falchetto R, Fatih A, Monod M. Nucleotide sequence of a genomic and a cDNA clone encoding an extracellular alkaline protease of *Aspergillus fumigatus*. *FEMS Microbiol Lett* 1992;71:163-8.
33. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 1982;157:105-32.
34. Birnstiel ML, Busslinger M, Strub K. Transcription termination and 3' processing: the end is in site! *Cell* 1985;41:349-59.
35. Greenberger PA, Miller TP, Roberts M, Smith LL. Allergic bronchopulmonary aspergillosis in patients with or without evidence of bronchiectasis. *Ann Allergy* 1993;70:333-8.