

SELECTIVE EXPRESSION OF A MAJOR ALLERGEN AND CYTOTOXIN, *Asp f I*, IN *Aspergillus fumigatus*¹

Implications for the Immunopathogenesis of *Aspergillus*-Related Diseases

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Asp f I is a major 18-kDa *Aspergillus fumigatus* allergen and a member of the mitogillin family of cytotoxins. The nucleotide sequence of the *Asp f I* gene was determined by sequencing polymerase chain reaction products amplified from *A. fumigatus* spore DNA. The entire 678-bp DNA includes an 81-bp leader sequence, preceding the N-terminal alanine codon, a 52-bp intron, and a 444-bp open reading frame, encoding a 149-amino acid protein (*M*_r 16,899), which is 99% homologous to mitogillin from *Aspergillus restrictus*. A mAb-based ELISA was used to compare *Asp f I* levels in spores, mycelia, and culture filtrate, and to determine the kinetics of allergen production. Disrupted hyphae or spore extracts had a 1000-fold lower level of *Asp f I* than culture filtrate, suggesting that germination of spores and growth of the fungus are essential for allergen production. *Asp f I* levels in *A. fumigatus* and *A. restrictus* peaked at day 3 (0.87 to 12.1 µg/ml), however, the allergen was not detected in *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans* cultures (<1.5 ng/ml) on either days 3 or 8. Northern analysis confirmed that *Asp f I* mRNA was detected only in *A. fumigatus* and *A. restrictus*, but not in the other four *Aspergillus* spp. *Asp f I*-specific DNA was generated after polymerase chain reaction amplification of genomic mycelial DNA obtained from *A. fumigatus* and *A. restrictus*, but not from the other *Aspergillus* spp. The results show that *Asp f I* is selectively expressed in *A. fumigatus*, and suggest that this cytotoxin could be a specific virulence factor for *A. fumigatus*.

Aspergillus spp. are among the most common fungi found in nature and are primarily saprophytes of dead organic matter (1). Inhalation of *Aspergillus* spores can cause a variety of human respiratory diseases, including

asthma, ABPA,³ aspergilloma, as well as severe invasive infection in immunocompromised persons, e.g., patients with leukemia undergoing chemotherapy or organ transplant recipients (2, 3).

Although the genus *Aspergillus* includes 132 species and 18 variants, *Aspergillus fumigatus* is the etiologic agent identified in 80% of human diseases caused by these fungi, and is the principal species causing infection in animals (1, 4). Other species that have been associated with aspergillosis include *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger*, and *Aspergillus terreus*. It has been suggested that *A. fumigatus* may be particularly resistant to lung defenses, and the production of virulence factors by *A. fumigatus* has been proposed as an explanation for its predominance as the causative agent of disease (5). However, the biochemical basis for these virulence factors has not been established. The small spore size of *A. fumigatus* (2.5 to 3.0 µm diameter) and its thermotolerance (12 to 52°C) enable *A. fumigatus* to reach terminal airways and alveoli and to grow at human body temperature. In addition, *A. fumigatus* produces low molecular weight toxic metabolites, including helvolic acid, gliotoxin, fumigatin, and fumagillin. Gliotoxin has been reported to cause immunosuppression and to inhibit phagocytosis by macrophages (5, 6). A 20- to 35-kDa proteinase from *A. fumigatus* has been shown to induce epithelial cell detachment from basement membrane, suggesting that proteolytic enzymes may also promote colonization (7).

We previously reported the purification of an 18-kDa *A. fumigatus* protein, *Asp f I*, that caused IgE antibody responses in 85% of patients with IgE antibodies to *A. fumigatus*, and showed homology to mitogillin, a cytotoxin produced by *Aspergillus restrictus* (8). Mitogillin is a member of a family of cytotoxins that inhibit protein synthesis in eukaryotic cells by cleaving the 3' end of the large RNA in the 60S ribosomal subunit (9). The 18-kDa protein is also a major Ag found in urine of patients with invasive aspergillosis (10).

In the present paper, we report the complete nucleotide sequence coding for *Asp f I*, compare *Asp f I* levels in spores and mycelia, and analyze the kinetics of *Asp f I* production in culture filtrates. The results show that this allergen is only expressed during fungal growth, is secreted in abundance in *A. fumigatus* culture filtrate, and

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³ Abbreviations used in this paper: ABPA, allergic bronchopulmonary aspergillosis; *Asp f I*, *A. fumigatus* allergen I; BBS, borate-buffered saline, pH 8.0; PCR, polymerase chain reaction; CD, circular dichroism.

is not encoded by either mRNA or DNA from *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans*. The results support the view that selective expression of Asp f I could promote colonization and contribute to the pathogenesis of *A. fumigatus*-related diseases.

MATERIALS AND METHODS

Aspergillus strains. *A. fumigatus* used throughout the present study was cultured from nasal washing of a patient (O.S.) with chronic sinusitis and a positive immediate skin test to *A. fumigatus*. This strain is termed *A. fumigatus* O.S. Six *A. fumigatus* strains were isolated by the Mycology Laboratory at the University of Virginia from sputum specimens or from bronchial-alveolar lavage fluids (strains 17.65, 17.66, 17.68, 17.69, 17.71, and 17.74). *A. fumigatus* 34475 and 36607, *A. restrictus* 34506, *A. flavus* 10124, *A. niger* 16404, *A. terreus* 10690, and *A. nidulans* 10074 were obtained from American Type Culture Collection (Bethesda, MD). *A. restrictus* 17070 was provided by Dr. F. Saad (Division of Biologic Products, Michigan Department of Public Health, Lansing, MI).

Aspergillus spore and mycelial extracts. *Aspergillus* spores were taken from Sabouraud agar Petri dishes and cultured for 7 days at either 37°C (*A. fumigatus*) or 25°C (all other *Aspergillus* spp.). *A. fumigatus* spore extract was obtained by extraction of 2.5-g spores in 100 ml PBS containing 0.02% Na-azide, rotating at room temperature for 48 h. The extract was dialyzed against distilled water for 48 h, freeze-dried, and reconstituted in 1 ml BBS. Spores or mycelia were disrupted using a Polytron apparatus, followed by vortexing with 0.45- μ m diameter glass beads, as described by Hazen (11). By microscopic observation, we estimated that 60% of spores were disrupted by this method. Supernatants from spores or hyphae after disruption were dialyzed against distilled water, freeze-dried, and reconstituted in 0.5 ml BBS. Protein concentration of the extracts was determined by Bradford assay (12).

Kinetics of Asp f I production. Aliquots of 75 ml Sabouraud broth were inoculated with 10 mg *Aspergillus* spores (100 μ l of a 1:10 w:v spore suspension in PBS containing 0.5% Tween 80) and cultured at 37°C or 25°C, shaking at 200 rpm. For time course experiments, *A. fumigatus* cultures were harvested daily (days 1 to 7) and at days 14 and 21. Other *Aspergillus* cultures were harvested at days 2 or 3, and 8. Culture filtrates were obtained by passage through filter paper (Whatman International, Ltd., Maidstone, United Kingdom) under vacuum, and were either dialyzed against distilled water, freeze-dried and reconstituted in 1 ml BBS, or assayed unconcentrated. The pH of each culture filtrate was measured and the mycelial weight was determined after washing of the hyphae with 1 liter PBS. A mAb-based ELISA was used to measure Asp f I in culture filtrates and spore and mycelial extracts (13). Briefly, microtiter plates were coated with an anti-Asp f I mAb, 4A6 (8), and incubated for 1 h with *Aspergillus* extracts. Bound Asp f I was detected by the addition of a rabbit anti-*A. fumigatus* antiserum (1:1,000), followed by incubation with peroxidase-conjugated goat anti-rabbit IgG. Plates were developed with 0.01 M 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in 0.07 M citrate-phosphate buffer, pH 4.2, containing 0.03% H₂O₂, and A₄₀₅ was determined by an ELISA plate reader. Values were calculated from a control curve (concentration range, 0.2 to 270 ng Asp f I/ml).

In immunoadsorption experiments, *A. fumigatus* culture filtrate on day 3 (100 μ l) was absorbed with Sepharose beads coated with mAb 4A6, rotating overnight at 4°C. Extracts were analyzed by SDS-PAGE, using silver stained high density gels and low molecular weight protein markers (Diversified Biotech, Newton Centre, MA), in a Phast System (Pharmacia, Piscataway, NJ).

PCR. *A. fumigatus* spore DNA and *Aspergillus* sp. mycelial DNA were extracted as described by Lee and Taylor (14), and amplified by PCR using Taq polymerase (Gene Amp amplification kit; Perkin-Elmer Cetus, Inc., Rockville, MD) or Pfu DNA polymerase (Stratagene, La Jolla, CA). Reactions were performed in 50 μ l volume, with denaturation at 94°C for 1 min, annealing at 45°C or 55°C for 1 min, and extension at 72°C for 3 min, for 30 cycles. An initial 3-min incubation step at 94°C was performed, and cycles were terminated by incubation at 72°C for 15 min. Initially, degenerate primers were used in PCR, based on the partial protein sequence of Asp f I (8). Primers were synthesized as follows: 5' TGG-AC[C,G,T,A]-TG[C,T]-AT[C,T,A]-AA[C,T]-CA[G,A]-C(A) 3', based on amino acid residues 3 to 9; and 5' AC[C,T]-TT[G,A]-TT[C,G,T,A]-GG[G,A]-TA[C,G,T,A]-GT[G,A]-T(A) 3' (anti-sense), based on residues 123 to 128. In addition, the oligonucleotide primers 161 (5' CGT-TCT-GGC-TGT-GCT-C 3' (antisense)) and 162 (5' GAT-GAC-CAC-TAC-CTG-CT 3') were used in inverse PCR (15) to amplify circularized Eco RI and *Sau* 3a digested genomic spore DNA, to obtain the 5'- and 3'-ends of the sequence. To confirm the 3'-end sequence, primer M (5' TCA-AAT-

AAT-GAT-AAA-CA 3') was used in standard PCR, under conditions described above, using Pfu DNA polymerase. This primer sequence was obtained from a DNA sequence of the *A. restrictus* genome, adjacent to the open reading frame coding for the restrictocin gene (16). Amplification of mycelial DNA was carried out using the following primers, derived from the nucleotide sequence of Asp f I: 5' ACC-TGG-ACA-TGC-ATC-AA 3' and 5' CAC-AGT-CTC-AAG-TCT-CC 3' (antisense).

Southern blot analysis. PCR products were electrophoresed in 1.2% agarose gel, and DNA was transferred to a nylon membrane (Zetabind, Cuno, Meriden, CT) using 25 mM phosphate buffer, pH 6.7. Southern blots were hybridized with a ³²P end-labeled Asp f I 17-mer internal oligonucleotide primer, at 37°C. Blots were washed with 2 $\times$ SSC (0.9 M NaCl/0.09 M citrate) at room temperature, followed by 2 $\times$ SSC/0.1% SDS at 50°C, twice each for 20 min.

DNA sequence. PCR-derived DNA fragments were subcloned into plasmid vector pCR (TA Cloning System; Invitrogen, San Diego, CA), and sequencing was carried out by the dideoxy-chain-termination method described by Sanger et al. (17), using Sequenase (United States Biochemical, Cleveland, OH).

RNA blotting. Total RNA was extracted from *Aspergillus* sp. mycelia harvested at day 3, using the method of Chirgwin et al. (18). *A. fumigatus* mRNA was obtained using the PolyA Tract mRNA isolation system (Promega, Madison, WI). Samples containing 20 μ g total RNA or 4 μ g *A. fumigatus* mRNA were electrophoresed in formaldehyde denaturing 1% agarose gels (19), and RNA was transferred to a nylon membrane (Zetabind, Cuno, Meriden, CT) with 25 mM phosphate buffer, pH 6.7. A 380-bp PCR amplified product of Asp f I DNA and an 8.6-kb *Neurospora crassa* ribosomal DNA (pRW528) (20) were labeled with [α -³²P]dCTP by random priming (Boehringer Mannheim, Indianapolis, IN), and were used to probe the gel blots. Hybridization was performed at 37°C or 60°C in 6 $\times$ SSC, 5 $\times$ Denhardt's, 0.1% SDS, sonicated salmon sperm DNA (100 μ g/ml), 50 mM sodium phosphate, pH 6.7. When using Asp f I 380-bp probe, 10% dextran sulfate and 7 μ g cold ribosomal probe were added to hybridization mixture to decrease background hybridization. Washes were performed as described above for Southern blots.

CD spectroscopy. The CD spectra of Asp f I (270 μ g/ml) and mitogillin (200 μ g/ml) were obtained with a JASCO J600C spectropolarimeter (Jasco, Easton, MD) as described previously (21). Estimates of secondary structure were based on the method of Chen et al. (22).

RESULTS

Nucleotide and deduced amino acid sequence of Asp f I. Previously we reported 54% of the Asp f I amino acid sequence, obtained by Edman degradation (8). The complete nucleotide sequence of Asp f I was determined by analysis of PCR products. Initially, two degenerate oligonucleotide primers were designed to code for amino acid residues 3 to 9 and 123 to 128 and used for PCR amplification of genomic spore DNA. A 380-bp PCR fragment was generated, which coded for amino acids 3 to 128. "Inverse" PCR was used to determine the adjacent 5' and 3' coding sequences (15). Two internal primers of the 380-bp fragment, complementary to opposite strands and oriented outward, were used to amplify circularized Eco RI or *Sau* 3a digested spore DNA fragments. PCR amplified 183-, 302-, and 445-bp DNA fragments were obtained, which overlapped the 380-bp fragment (Fig. 1A). The sequence 5' to the 380-bp fragment revealed a 52-bp intron immediately after the GCG codon for the N-terminal alanine residue. The sequence lacked the stop codon at the 3' end, therefore an oligonucleotide primer was generated based on the recently published sequence of the restrictocin gene (*res*) of *A. restrictus* (16), downstream from the 3' end of the coding region, and was used in standard PCR. This approach enabled us to obtain the stop codon, TAG, at the 3' end of Asp f I sequence. In addition, we were able to determine the sequence of a putative signal peptide, starting with ATG, 81 nucleotides from the N-terminus of the mature protein.

(up to 6 µg/ml), the ratio of Asp f I:protein was 100-fold lower in hyphae than in culture filtrate. Comparison of Asp f I:protein ratios suggested that by day 3, Asp f I accounted for ~30% of the filtrate protein, and this observation was confirmed by SDS-PAGE. The culture filtrate contained three predominant bands, including an 18-kDa band that co-migrated with Asp f I. This band was specifically absorbed from the filtrate by monoclonal anti-Asp f I antibody (Fig. 3).

Expression of Asp f I protein and mRNA, and detection of Asp f I gene in other *Aspergillus* species. Previous analysis of *Aspergillus* sp. extracts suggested that Asp f I was a specific marker for *A. fumigatus* and *A. restrictus*. However, those studies were performed using commercial *Aspergillus* extracts, and we could not exclude the possibility that, under different growth conditions, other *Aspergillus* species would also produce Asp f I. Therefore, we compared Asp f I protein production under the same conditions in a panel of nine *A. fumigatus* and two *A. restrictus* strains, and four other selected *Aspergillus* species. Asp f I was detected in filtrates of all *A. fumigatus* and *A. restrictus* cultures, harvested on days 2 or 3, at levels of 867 to 12,110 ng/ml. On the other hand, Asp f I was undetectable (<1.5 ng/ml) in filtrates from *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans* cultures harvested at either day 3 or day 8 (Table II).

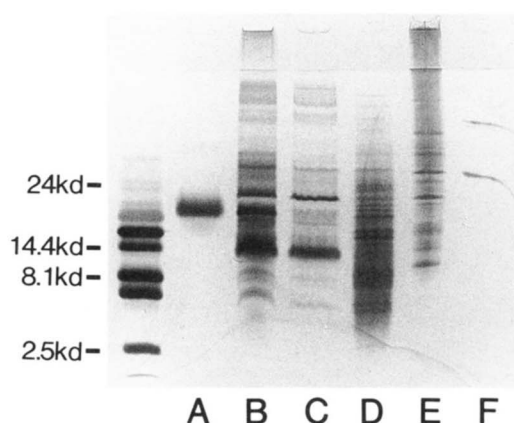


Figure 3. SDS-PAGE analysis of *A. fumigatus* extracts. Samples are as follows: A, Asp f I; B, *A. fumigatus* culture filtrate (day 3); C, filtrate absorbed with monoclonal anti-Asp f I antibody, 4A6; D, disrupted hyphae; E, disrupted spores; F, intact spore extract. Left lane, Low molecular weight protein standards.

TABLE II
Levels of Asp f I in *Aspergillus* sp. culture filtrates^a

<i>Aspergillus</i>		Day 2 or 3 (ng/ml)	Day 8 (ng/ml)
Species	Strain		
<i>A. fumigatus</i>	O.S.	7,620	1,580
<i>A. fumigatus</i>	17.65	1,146 ^b	ND
<i>A. fumigatus</i>	17.66	1,055	ND
<i>A. fumigatus</i>	17.68	10,842	ND
<i>A. fumigatus</i>	17.69	3,540	ND
<i>A. fumigatus</i>	17.71	12,110	ND
<i>A. fumigatus</i>	17.74	867	ND
<i>A. fumigatus</i>	ATCC 34475	10,520 ^b	ND
<i>A. fumigatus</i>	ATCC 36607	2,120 ^b	ND
<i>A. restrictus</i>	ATCC 34506	5,950	9,500
<i>A. restrictus</i>	17070	6,592 ^b	ND
<i>A. flavus</i>		<1.5	<1.5
<i>A. niger</i>		<1.5	<1.5
<i>A. terreus</i>		<1.5	<1.5
<i>A. nidulans</i>		<1.5	<1.5

^a Harvested at days 2 or 3, and day 8, and assayed unconcentrated for Asp f I by mAb-based ELISA.

^b Cultures harvested on day 2.

Northern analysis of *Aspergillus* mRNA, using the ³²P-labeled 380-bp fragment of Asp f I sequence as a probe, identified a 440-bp mRNA for Asp f I, which was present in *A. fumigatus* and *A. restrictus* but not in *A. flavus*, *A. niger*, *A. terreus*, or *A. nidulans* (Fig. 4). To test whether equivalent amounts of RNA were loaded for each of the *Aspergillus* species, a similar Northern blot was hybridized with a *N. crassa* ribosomal probe. The DNA probe strongly hybridized to two major bands in *A. flavus*, *A. niger*, *A. terreus*, *A. restrictus*, and *A. fumigatus* total RNA, and weakly to *A. nidulans* and *A. fumigatus* purified mRNA (data not shown). A PCR fragment of predicted size (437 bp) was generated after amplification of genomic mycelial DNA obtained from *A. fumigatus* and *A. restrictus*, but not from the other four *Aspergillus* species, using Asp f I specific primers. On Southern blot analysis, the 437-bp DNA fragment hybridized specifically to an internal Asp f I oligonucleotide probe (Fig. 5).

DISCUSSION

The results show almost complete homology between the deduced amino acid sequence of Asp f I and the published amino acid sequence of mitogillin, with only a single substitution at position 115 (9). The nucleotide sequencing confirms that Asp f I is a member of the mitogillin family of cytotoxic ribonucleases. The primary structure and nucleotide sequence of restrictocin (*A. restrictus*) and α-sarcin (*A. giganteus*), which are also members of this family of cytotoxins, has been reported and these proteins are 99% and 86% homologous to mitogillin, respectively (16, 26–28). Lamy et al. (29) also recently

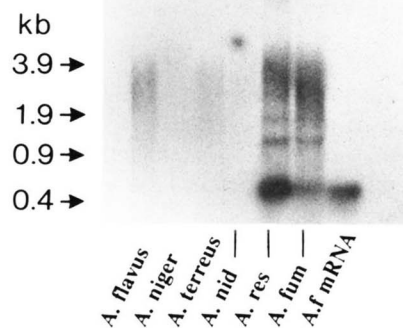


Figure 4. Northern blot analysis of *Aspergillus* sp. Twenty micrograms of total RNA from *A. flavus*, *A. niger*, *A. terreus*, *A. nidulans*, *A. restrictus*, and *A. fumigatus* and 4 µg *A. fumigatus* mRNA were electrophoresed in formaldehyde denaturing gel, blotted, and hybridized to a ³²P-labeled 380-bp Asp f I DNA probe.

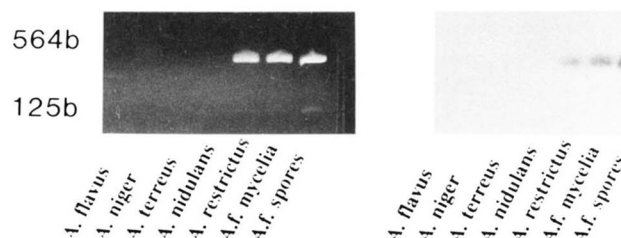


Figure 5. Analysis of PCR products amplified from *Aspergillus* sp. mycelial DNA and *A. fumigatus* spore DNA. Left panel, Ethidium bromide-stained 1.2% agarose gel; right panel, autoradiography of the Southern blot, hybridized with a ³²P-labeled Asp f I-specific internal oligonucleotide primer.

reported the nucleotide sequence of an 18-kDa *A. fumigatus* protein, which is identical to the sequence coding for Asp f I reported here, including the intron and the signal peptide sequences.

The Asp f I sequence encodes a protein with a predicted m.w. of 16,889. On SDS-PAGE analysis, Asp f I migrates with a slightly higher m.w. than mitogillin, i.e., 18,000 (8, 29), which could represent differences in glycosylation, other post-translation modifications, or secondary structure. At present, the explanation is unclear, as there are no consensus sites for N-glycosylation in the sequences and the secondary structures, as determined by CD spectroscopy, are very similar. The CD results showing primarily random structure are in good agreement with previously reported spectra for mitogillin, restrictocin, and α -sarcin, though our estimate of α -helix (25%) is lower than that reported previously (38%) (30). Differences in the biologic activity of Asp f I and mitogillin have also been observed, both in relation to cytotoxic activity and ability to cause T cell proliferation. Asp f I is ~10-fold less active than mitogillin as an inhibitor of protein synthesis in vitro (8) (L. K. Arruda and S. Canevari, unpublished observations). It has also been reported to induce proliferative responses in T cells from patients with ABPA, whereas mitogillin failed to activate T cells and inhibited T cell responses to recall antigens (31). Our results suggest that these differences need to be re-evaluated in the light of the sequence data and that other possible causes of heterogeneity in Asp f I and mitogillin preparations need to be investigated.

Previous studies have suggested that germination or breakdown of spores might be necessary for sensitization to *A. fumigatus* (32). Our results clearly showed that Asp f I is secreted to the culture filtrate in the early phases of fungal growth. Asp f I levels in spores were negligible, even after disruption, and its concentration in hyphae was very low. Germination of spores and fungal growth is a prerequisite for Asp f I production and for the development of human antibody responses. Indeed, the highest levels of IgG and IgE antibodies to Asp f I are found in sera from patients with ABPA, aspergilloma, and cystic fibrosis, who have *A. fumigatus* growing in their lungs, presumably reflecting a higher degree of allergen exposure (8, 33, 34).

The predominance of *A. fumigatus* as the causative agent of *Aspergillus* diseases in humans and animals is intriguing, and has been attributed to factors such as ability to grow at human body temperature, small spore size, resistance to oxidative killing, along with production of small chemical metabolites and enzymes with immunosuppressive or proteolytic activity. However, the importance of these factors in the pathogenesis of disease is unclear. Unlike bacteria, few specific determinants of virulence have been proven in pathogenic fungi, and none have been described in *A. fumigatus*. In the present study, we provide strong evidence that Asp f I is a virulence factor for *A. fumigatus*. This protein belongs to a family of potent cytotoxins, and is secreted in abundance (up to 30% of total protein) after spore germination and during fungal growth. In addition, we have conclusively shown that Asp f I protein, mRNA, or DNA are not expressed in four other *Aspergillus* species, which are much less important in causing human disease. Asp f I could potentially cause extensive damage to lung tissue

through its cytotoxic activity, however, the production of IgG antibodies could have a neutralizing effect and serve to limit the growth of the fungus. Thus, patients with aspergilloma, ABPA, and cystic fibrosis, who have the fungus growing in the lumen of their bronchi or lung cavities, make strong anti-Asp f I antibody responses, but seldom develop invasive disease. The role of antibody responses in neutralizing pathogenicity is not well established, however it has been reported that immunosuppressed patients who develop invasive infection have undetectable or very low levels of specific anti-*A. fumigatus* antibodies (35, 36). Together, these observations strongly suggest that Asp f I constitutes a factor that favors colonization and infection by *A. fumigatus* over other *Aspergillus* species in humans and animals. *A. restrictus* also produces cytotoxins (mitogillin and restrictocin), but is considered to be nonpathogenic because of its inability to survive and replicate at 37°C (1, 8, 9, 16).

Molecular and genetic approaches have been instrumental in defining the role of virulence factors produced by other microbes. Our studies on Asp f I fulfill several of the criteria proposed by Falkow (37) and others (38–40) for defining microbial virulence factors. Formal proof would require the satisfaction of additional criteria, such as the demonstration that inactivation of the Asp f I gene would lead to loss of pathogenesis or virulence or, alternatively, reintroduction of the wild type gene would restore pathogenicity. Such studies are currently limited by the lack of established gene manipulation techniques for *A. fumigatus* and the absence of animal models for aspergillosis that are relevant to the different types of human disease. However, the present data provide the first step in establishing these techniques, which will be fundamental for the elucidation of pathogenetic mechanisms in aspergillosis. Also inherent from our results is the possibility that *A. fumigatus* produces other species-specific protein(s) that are important for colonization and that should be targeted for additional biochemical studies.

Note added in proof: The sequencing and expression of recombinant Asp f I has also recently been reported by Moser et al., *J. Immunol.* 149:454, 1992.

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