

## ***Aspergillus fumigatus* Allergen I, a Major IgE-binding Protein, Is a Member of the Mitogillin Family of Cytotoxins**

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### **Summary**

A major 18-kD IgE-binding protein from *Aspergillus fumigatus* (*Asp f* I) has been purified. Partial amino acid sequencing of *Asp f* I showed extensive sequence homology (95%) between *Asp f* I and a cytotoxin (mitogillin) produced by *A. restrictus*. Crossinhibition radioimmunoassay using murine monoclonal antibody and human IgG and IgE antibodies showed that *Asp f* I and mitogillin were antigenically indistinguishable. Furthermore, both proteins inhibited protein synthesis in vitro by >90%. *Asp f* I was expressed in *A. fumigatus* but not in seven other *Aspergillus* species. The results suggest that *Asp f* I could play a dual role in the pathogenesis of *A. fumigatus*-related diseases by promoting colonization through cytotoxic activity and by causing inflammatory reactions involving IgE antibodies.

*A*spergillus fumigatus causes a diverse spectrum of human diseases, including allergic bronchopulmonary aspergillosis (ABPA), asthma, aspergilloma, and invasive infection in immunocompromised hosts (1). The respiratory tract of patients with chronic pulmonary diseases, particularly cystic fibrosis (2, 3), is frequently colonized by *A. fumigatus*. However, despite the ability of the fungus to cause tissue invasion, respiratory colonization by *A. fumigatus* is often regarded as saprophytic or benign. Serologic studies have shown that colonization can induce IgG and/or IgE antibodies to several *A. fumigatus* antigens (4–7), however, the structure and function of these antigens are largely unknown. In this report, we describe the purification of an 18-kD IgE-binding protein from *A. fumigatus* (*Asp f* I) that is selectively expressed in *A. fumigatus*. Structural, functional, and antigenic analyses show that *Asp f* I is a member of the mitogillin family of cytotoxins (8, 9).

### **Materials and Methods**

**Purification of *Asp f* I.** *A. fumigatus* culture (30 g, strain no. 5167; Greer Laboratories, Lenoir, NC) was extracted overnight in 300 ml borate-buffered saline, pH 8.0 (BBS), and centrifuged (20,200 g, 30 min). *A. fumigatus* antigens were partially purified from the supernate by gel filtration and phenyl-sepharose chromatography. A panel of six murine IgG1 mAbs was raised against an ~20-kD, 2 M NaCl phenyl-sepharose fraction. mAb 4A6 showed the highest binding to the  $^{125}\text{I}$  2 M fraction in RIA and recognized an 18-kD protein. This protein, *Asp f* I, was purified by affinity chromatography over mAb 4A6 immunosorbent followed by size

exclusion HPLC using a Sepharose 12 HR 10/30 column (Pharmacia Fine Chemicals, Piscataway, NJ). The yield was 16-mg *Asp f* I. Purity was assessed by SDS-PAGE using an 8–25% gel on a PhastSystem (Pharmacia Fine Chemicals).

**Amino Acid Sequencing.** 100 µg *Asp f* I was reduced and alkylated using 0.5% (vol/vol) 2-ME and 4% (vol/vol) vinyl pyridine and injected onto a reverse-phase HPLC column (C8; Brownlee Labs, Santa Clara, CA). The NH<sub>2</sub>-terminal amino acid sequence (27 residues) of the HPLC peak was determined using a gas phase sequencer (477-A; Applied Biosystems, Inc., Foster City, CA). Four tryptic peptides (T<sub>1</sub>, residues 22–28; T<sub>2</sub>, 29–38; T<sub>3</sub>, 43–60; and T<sub>4</sub>, 121–128) were sequenced as single sharp peaks from a reverse-phase HPLC column (C18; Applied Biosystems, Inc.) A CNBr fragment was also sequenced (residues 87–107).

**IgE Antibody Assays.** mAb 4A6 was used in a solid-phase RIA to present *Asp f* I to IgE antibodies. Bound IgE antibodies were detected by the addition of 2 ng affinity-purified,  $^{125}\text{I}$ -labeled goat anti-human IgE. Specific IgE antibody to *Asp f* I or to mitogillin was also determined by double antibody antigen-binding RIA (10). IgE antibody to *A. fumigatus* was measured by quantitative radioallergosorbent test (RAST) (11).

**Crossinhibition RIA.** Serial fourfold or 10-fold dilutions of cold *Asp f* I or mitogillin were incubated for 2 h with 60 ng mAb 4A6 or with 0.1 ml 1/4 or 1/25 dilution of serum from a patient with ABPA (J.H.) for the IgE and IgG assays, respectively. Subsequently, 6 ng of  $^{125}\text{I}$  *Asp f* I or 4 ng  $^{125}\text{I}$  mitogillin was added for 2 h, and antigen-antibody complexes were precipitated overnight using monospecific goat or sheep antisera (10). Precipitates were washed with BBS and counted in a gamma counter.

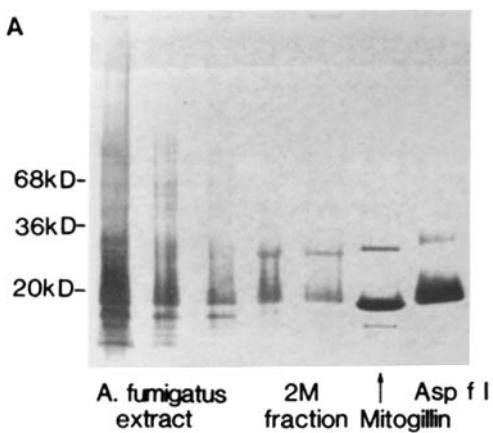
**Inhibition of Protein Synthesis.** *Asp f* I and mitogillin were used to inhibit protein synthesis in a rabbit reticulocyte lysate in vitro translation system for Brome Mosaic Virus (Promega Biotech,

Madison, WI). *Asp f I* or mitogillin was added at 0.3–80 ng/ml in 7  $\mu$ l RNAase free distilled water. Total  $^{35}$ S-methionine incorporation (TCA precipitable counts) was determined by adding 7  $\mu$ l distilled water only, and a reaction mixture containing no BMV-mRNA was used to determine background counts.

**Human Sera and Skin Testing.** Sera were obtained from six nonallergic control subjects and from 46 patients who had either positive immediate skin tests or serum IgE antibodies to *A. fumigatus* and clinical symptoms of ABPA ( $n = 14$ ), cystic fibrosis and ABPA ( $n = 21$ ), or asthma ( $n = 11$ ). Intradermal skin tests were carried out using serial 10-fold dilutions of *Asp f I*, from  $10^{-6}$   $\mu$ g/ml (10).

## Results and Discussion

We initially raised a mAb (clone 4A6) against partially purified *A. fumigatus* antigen and found that it recognized a single 18-kD band on immunoprecipitation. The mAb was used in a solid-phase RIA to present the antigen to human IgE antibodies. Of 13 patients with ABPA or asthma and positive serum IgE antibodies to *A. fumigatus* (RAST > 200 U/ml), 11 (85%) had IgE antibodies to the 18-kD antigen.

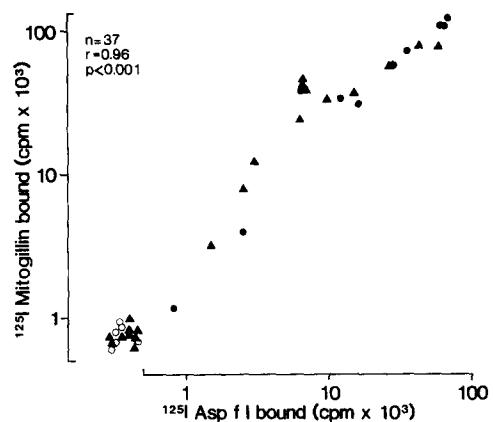


Mitogillin:	A T W T C I N Q Q L N P K T N K W E D K <sup>20</sup>
<i>Asp f I</i> :	A T W T C I N Q Q L N P K T N K - E - -
	*
Mitogillin:	R L L Y N Q A K A E S N S H H A P L S D <sup>40</sup>
<i>Asp f I</i> :	G L L Y N Q A K A E S T S H H A P L - -
	*
Mitogillin:	G K T G S S Y P H W F T N G Y D G N G K <sup>60</sup>
<i>Asp f I</i> :	- - T G S S Y P H - F T D G Y D G N F K
	*
Mitogillin:	<sup>87</sup> G K D D H Y L L E F P T F P D G H D Y K F <sup>107</sup>
<i>Asp f I</i> :	G K D D H Y L L E F P T F P D G H D Y K F
	*
Mitogillin:	<sup>121</sup> V I Y T Y P N K <sup>128</sup>
<i>Asp f I</i> :	V I Y T Y P N K

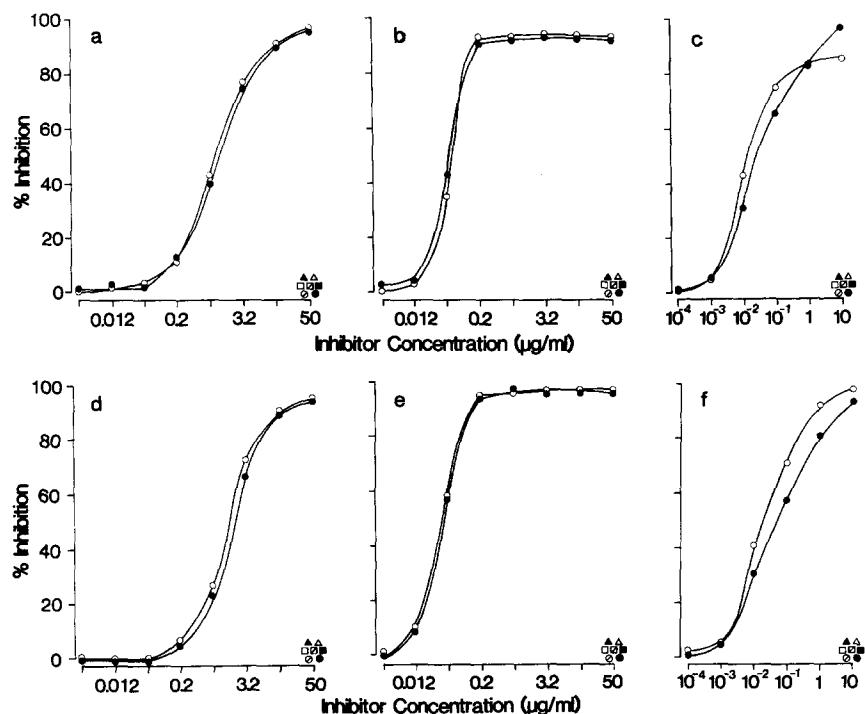
**Figure 1.** SDS-PAGE and amino acid sequence comparisons of *Asp f I* (18 kD) and mitogillin (16.2 kD). (A) Silver-stained SDS-PAGE analysis of *A. fumigatus* extract (lanes 1–3), 2 M phenyl-sepharose fraction (lanes 4 and 5), mitogillin (lane 6), and *Asp f I* (lane 7). *Asp f I* shows a high molecular weight band similar to that observed in mitogillin preparations and previously interpreted as a dimer (9). (B) Amino acid sequence homology between *Asp f I* and mitogillin: 77/81 (95%) residues of *Asp f I* were identical to the published mitogillin sequence (8). *Asp f I* residues that differ from mitogillin are indicated (\*). Mitogillin was kindly provided by Dr. Ted Watson (Michigan Department of Public Health, Lansing, MI).

Patients with equivocal or weakly positive *A. fumigatus* RAST ( $n = 10$ ; geometric mean, 29.5 U/ml) had no detectable IgE antibody. These results showed that the 18-kD antigen defined by mAb 4A6 was an important allergen. The allergen was purified by affinity chromatography and size exclusion HPLC (Fig. 1A) and has provisionally been designated *Asp f I*. Partial amino acid sequencing of *Asp f I* showed extensive sequence homology (95%) between *Asp f I* and mitogillin, a cytotoxin produced by *A. restrictus* (Fig. 1B). Mitogillin is a member of a family of cytotoxins that includes  $\alpha$ -sarcin and restrictocin, produced by *A. giganteus* and *A. restrictus*, respectively. These cytotoxins are potent inhibitors of protein synthesis and act by cleaving the 3' end of the large RNA from the 60S ribosomal subunit (9). Mitogillin also causes regression of murine and canine tumors in vivo (12). Linear regression analysis showed an excellent quantitative correlation between IgE antibodies to *Asp f I* and to mitogillin (Fig. 2). On immediate skin testing, four of five patients with positive prick tests to *A. fumigatus* gave positive intradermal skin tests ( $> 6 \times 6$ -mm wheal) to *Asp f I* at  $10^{-2}$ – $10^{-6}$   $\mu$ g/ml. In contrast, two patients with positive skin tests to other allergens, but not to *A. fumigatus*, and four nonatopic controls, gave negative skin tests to 10  $\mu$ g/ml *Asp f I*.

We investigated the antigenic and functional relationships between *Asp f I* and mitogillin by crossinhibition RIA and by inhibition of cell-free protein synthesis in vitro. Superimposable, dose-dependent inhibition curves were obtained using cold *Asp f I* or mitogillin to inhibit the binding of either  $^{125}$ I-labeled proteins to mAb 4A6 or to human IgG or IgE antibody (Fig. 3). This effect was species specific in that extracts of seven other *Aspergillus* species, representative of distinct classification groups of the *Aspergilli* (13), caused <10% inhibition. Together, these results showed that *Asp f I* and mitogillin were antigenically indistinguishable. Using rabbit reticulocyte lysate to synthesize Brome Mosaic Virus protein, we observed >90% inhibition of protein synthesis by both mitogillin and *Asp f I* at concentrations of  $\leq 20$  ng/ml (Table



**Figure 2.** Correlation between the binding of  $^{125}$ I *Asp f I* or  $^{125}$ I mitogillin to IgE antibodies in sera from 10 patients with ABPA (●), 21 patients with cystic fibrosis and ABPA (▲), and six control subjects (○), determined by antigen-binding RIA.



**Figure 3.** Antigenic identity between *Asp f I* and mitogillin. Binding of  $^{125}\text{I}$  *Asp f I* to murine mAb 4A6 (a), human IgE (b), and human IgG (c), and of  $^{125}\text{I}$  mitogillin to mAb 4A6 (d), IgE (e), and IgG (f) was inhibited by cold *Asp f I* (●), cold mitogillin (○), or by extracts of *A. niger* (□), *A. nidulans* (△), *A. terreus* (■), *A. glaucus* (■), *A. clavatus* (○), *A. sydowi* (○), or *A. flavus* (▲). Results are expressed as the percent inhibition of  $^{125}\text{I}$  *Asp f I* or  $^{125}\text{I}$  mitogillin binding relative to the mean of four tubes containing 1% BSA-PBS-T (uninhibited controls). Using  $^{125}\text{I}$  *Asp f I*, uninhibited binding was 37,530 cpm (mAb 4A6), 5,903 cpm (IgE), and 40,973 cpm (IgG); and uninhibited binding using  $^{125}\text{I}$  mitogillin was 71,361 cpm (mAb 4A6), 21,045 cpm (IgE), and 67,854 cpm (IgG). Each point represents the mean of duplicate assays.

1). Inhibition of protein synthesis by either molecule was not blocked by mAb 4A6, suggesting that the epitope recognized by mAb 4A6 is not located near the active site for ribosomal cleavage (Table 1).

*A. fumigatus* is the most common member of the genus *Aspergillus* that is associated with human disease, however,

the reasons why *A. fumigatus* frequently colonizes the lungs are poorly understood (14, 15). Cytotoxic metabolites of *A. fumigatus* (e.g., gliotoxin and fumagillin) are thought to facilitate fungal growth by inhibiting macrophage function and causing immunosuppression (15, 16). Unlike *Asp f I*, none of the low molecular weight metabolites induce IgE anti-

**Table 1.** *Asp f I* and Mitogillin Inhibit In Vitro Protein Synthesis

Inhibitor	$^{35}\text{S}$ -Met incorporation (percent inhibition)	
	<i>Asp f I</i>	Mitogillin
	$\text{cpm} \times 10^3$	
80.0 ng/ml	22.7 (96.0)	22.9 (96.3)
20.0 ng/ml	35.9 (92.2)	30.0 (94.6)
5.0 ng/ml	236.1 (64.9)	38.4 (92.8)
1.2 ng/ml	613.9 (-6.2)	42.9 (91.9)
0.3 ng/ml	417.3 (4.3)	175.0 (68.7)
20 $\mu\text{g}/\text{ml}$ inhibitor + 500 $\mu\text{g}/\text{ml}$ mAb 4A6	9.1 (97.7)	8.1 (98.2)
Background (no mRNA)		8.5
Uninhibited positive control		545.2

Serial fourfold dilutions of either *Asp f I* or mitogillin were used to inhibit incorporation of  $^{35}\text{S}$ -methionine in an in vitro translation system for BMV protein. Incorporation of  $^{35}\text{S}$ -methionine into protein was determined by TCA precipitation, and the results are expressed as cpm incorporated ( $\times 10^3$ ). In addition, for each reaction tube, the TCA precipitable counts were divided by the total counts added, and the percentage inhibition of the acid precipitable counts as compared with uninhibited controls is shown in parentheses. The estimated dose for 50% inhibition was 4 ng/ml for *Asp f I* and 0.25 ng/ml for mitogillin. In a parallel experiment, we included the major cat allergen, *Fel d 1*, at 20  $\mu\text{g}/\text{ml}$ , as a negative control and observed <2% inhibition of protein synthesis.

body formation. Mitogillin(s) were not previously known to occur in pathogenic *Aspergilli*, nor to cause IgE antibody responses, although, interestingly, hypersensitivity reactions (anaphylaxis and skin rashes) hampered U.S. trials of mitogillin in cancer chemotherapy (Dr. Ted Watson, personal communication; see also reference 12). Our results suggest that *Asp f I* could play a dual role in the pathogenesis of *A. fumigatus*-related diseases by promoting fungal colonization through cytotoxic activity and by causing inflammatory reactions involving IgE antibodies present on human lung mast cells. Cytotoxic damage to the respiratory mucosa would be

expected to be augmented by the production of IgE anti-*Asp f I* antibodies, which occur in most sera from patients with ABPA and cystic fibrosis. *Asp f I* was not detected in other pathogenic *Aspergillus* species. Thus, the selective expression of *Asp f I* in *A. fumigatus* could be a virulence factor that aids the growth of this fungus in man, in addition to its small spore size and ability to proliferate at human body temperature (13). It will now be possible to investigate this hypothesis and the immunobiologic role of *Asp f I* in *A. fumigatus*-related diseases.

We thank Dr. Robert Esch for providing *A. fumigatus* culture extract; Dr. Ted Watson for providing purified mitogillin; and Drs. Joan Longbottom, Jane El-Dahr, and George Ward for providing sera from patients with ABPA and cystic fibrosis. We are also grateful to Dr. Shu Man Fu for his critical review of the manuscript.

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