

Identification and evaluation of a major cytotoxin of *A. fumigatus*

Taruna Madan, Naveen Arora and P. Usha Sarma

Centre for Biochemical Technology, Mall Road, Delhi-110007, India

Received 11 March 1996; accepted 2 July 1996

Abstract

Aspergillus fumigatus is a highly pathogenic fungus causing a wide spectrum of diseases in immunocompromised as well as immunocompetent hosts. The present work was undertaken to evaluate the cytotoxic nature of fractionated antigens of *A. fumigatus* against the mammalian cell lines (J774, RAW, CHO and L929). An enriched protein antigenic fraction of *A. fumigatus* was subjected to con A Sepharose and phenyl Sepharose chromatography. Antigenic fractions, ConAub (conA unbound) and PSC III (fraction III of phenyl Sepharose column) containing low mw antigens showed higher cytotoxicity as compared to other antigenic fractions. PSC III was further purified on HPLC resulting in an 18 kDa homogeneous protein. The purified protein showed high ELISA absorbance values for specific IgG and IgE antibodies in sera of ABPA patients. Monoclonal antibody raised against Asp fl, a major allergen/antigen of *A. fumigatus* recognised the purified 18 kDa by ELISA and western blot. The 18 kDa allergen/ antigen or Asp fl showed similar toxicity towards all the four cell lines (macrophage and fibroblast) with an IC_{50} of 75 ng/ml or 4.16 nM. Reduction in toxicity of 18 kDa at low temperatures and potentiation in presence of ammonium chloride and monensin indicates mechanism of internalisation of 18 kDa in eukaryotic cells is similar to α -sarcin. The present work shows that the 18 kDa allergen/ antigen (Asp fl) is a major cytotoxin secreted by *A. fumigatus* which may play multiple roles in the pathogenesis of Aspergillosis through allergenicity, antigenicity and cytotoxicity. (Mol Cell Biochem **167**: 89–97, 1997)

Key words: *Aspergillus fumigatus*, Asp fl, major allergen, cytotoxicity, eukaryotic cell lines, internalisation

Introduction

Aspergillus fumigatus is the principal fungal species causing allergic and invasive infections in humans and animals [1]. Virulence of *A. fumigatus* is attributed to secretion of various immunologically and biologically active macromolecules such as toxins, elastases, proteases and ribonucleases [2]. However, direct evidence is lacking for their involvement in the disease as only a few of them have been well characterised [3]. Identification of virulent factors and their complete immunochemical and biological characterisation not only leads to a better understanding of the mechanism of pathogenesis but also provides development of standard diagnostic reagents.

Extensive tissue damage such as granuloma formation, interstitial fibrosis, tissue necrosis has been observed in *Aspergillus* induced disorders which results in irreversible lung damage [2]. Tissue damage observed may be attributed to inflammatory response to the allergens/antigens and biological

activity of some of these allergens/antigens. Many species of *Aspergillus* such as *A. oryzae*, *A. restrictus* and *A. giganteus* produce cytotoxins but they are not reported to be pathogenic. The first cytotoxin to be isolated was alpha-sarcin from *Aspergillus giganteus* followed by restrictocin and mitogillin from *Aspergillus restrictus* [4, 5, 6]. Interestingly, Asp fl a major allergen/antigen secreted by *A. fumigatus*, a pathogenic fungus, showed more than 95% sequence homology to mitogillin [7]. The above fact emphasised the role of cytotoxins in the pathogenesis of *A. fumigatus* related disorders. However, absence of any data on the cytotoxicity of *A. fumigatus* antigens towards eukaryotic cell lines necessitated the present study. Evaluation of cytotoxicity of fractionated antigens of *A. fumigatus* against the mammalian cell lines RAW, CHO, J774 and L929 was carried out to identify the major cytotoxin secreted by *A. fumigatus*. Further studies were carried out to investigate the uptake mechanism of the major cytotoxin by the eukaryotic cells.

Address for offprints: P. Usha Sarma, Diagnostics Laboratory, Centre for Biochemical Technology, Mall Road, Delhi 110007, India

Materials and methods

Human sera

Sera of patients of ABPA (based on Rosenberg criteria) were received from the VP Chest Institute, Delhi. Sera from healthy donors without any indication of pulmonary disease were taken as controls.

Monoclonal antibodies raised against Asp fl were a kind gift from Dr. L. Karla Arruda, Division of Allergy and Clinical Immunology, University of Virginia.

Enriched protein antigenic fraction (crude antigen)

An enriched protein antigenic fraction of *A. fumigatus* was prepared as reported previously [8]. Briefly, a pathogenic strain of *A. fumigatus* (285) isolated from an ABPA patient, was grown for 3 weeks in asparagine synthetic medium at 37°C. Culture filtrate proteins were concentrated by ammonium sulfate precipitation, dialysed, lyophilised and used for further studies. Fractionation of enriched protein antigenic fraction was carried out by conA Sepharose as well as phenyl Sepharose as they fractionate the antigens based on the extent of glycosylation and hydrophilicity of the antigens respectively.

ConA Sepharose 4B chromatography

The enriched protein antigenic fraction was subjected to conA Sepharose 4B chromatography according to the procedure reported earlier [9]. A column (0.9 × 30 cm) containing conA Sepharose was equilibrated with 10 mM tris-HCl pH 7.4 buffer containing 5 mM MgCl₂ and 5 mM CaCl₂. Twenty mg of enriched protein antigenic fraction was applied to the column. The unbound fraction (ConA ub) was collected with equilibration buffer. Column was washed with 50 ml of equilibration buffer and conA bound proteins were eluted using 2 M alpha-D-methyl mannoside in equilibration buffer (ConA b).

Phenyl Sepharose CL4B (PSC) chromatography

The enriched protein antigenic fraction was subjected to PSC (hydrophobic) chromatography as reported earlier [10]. A PSC column (0.9 × 30 cm) was equilibrated with 25% saturated ammonium sulfate solution in 10 mM phosphate buffer, pH 7.4. Sixty mg of enriched protein antigenic fraction was applied to the column. Fractions were obtained on successive elution with a gradient of decreasing ammonium sulfate concentration (25, 20, 15, 10, 5, 0%) and simultaneously increasing ethylene glycol concentration (0, 10, 20, 30, 40, 50%).

High pressure liquid chromatography

The low mw protein rich fraction III of PSC (15% ammonium sulfate + 20% ethylene glycol) was subjected to reverse phase HPLC on C-18 column using a 10–70% gradient of acetonitrile in water containing 0.1% v/v trifluoroacetic acid.

SDS-PAGE analysis of various fractions

The enriched protein antigenic fraction, conA Sepharose fractions and phenyl Sepharose fractions were electrophoretically analysed on 10% SDS-polyacrylamide gels [11]. Purified protein was analysed on a 15% SDS-polyacrylamide gel.

Immunological characterisation of various fractions

Indirect ELISA (Enzyme linked immunosorbent assay) for specific IgG and IgE antibodies was carried out using various fractions and purified antigen with sera of ABPA patients by the method reported earlier [9]. The ELISA and immunoblot analysis of HPLC purified antigen was carried out with monoclonal antibody against Asp fl [9, 12].

Cell lines

L929 (fibroblast), J774 (macrophage) and RAW (macrophage) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 2 mM L-glutamine, 25 mM HEPES buffer, 10% FCS and 10 µg/ml gentamicin. Chinese hamster ovary (CHO) (fibroblast) was grown in alpha-MEM, 2 mM glutamine, 10% FCS and 50 µg/ml gentamicin. All cell lines were maintained at 37°C in 5% CO₂ in a humidified atmosphere.

Cytotoxicity assay

Cytotoxicity assays were carried out on above mentioned cell lines using a rapid colorimetric assay [13]. L929, RAW and J774 cells were scraped in fresh media and CHO cells were trypsinized. Cells were centrifuged, resuspended in the fresh medium and dispensed into 96 well plates at a density of 10⁴ cells/100 µl/well. On the following day cells were incubated with various dilutions of antigen for 8 h. In control wells no antigen was added. Cell viability was measured by incubating the cells with 0.5 mg/ml MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) in DMEM for 30 min. Medium was aspirated and cells were lysed using 100 µl of acidified isopropanol (40 mM HCl in isopropanol). Absorbance was measured at 570 nm in NUNC ELISA reader. Cytotoxicity of HPLC purified protein on RAW cells was studied in presence

of 10 mM ammonium chloride, 1 μ M monensin and at different temperatures ranging from 4–37°C. Cells were plated (1×10^4 /well) and pre-incubated for 40 min with assay medium containing ammonium chloride or monensin. Fresh medium containing various concentrations of purified 18 kDa was added to the pre-incubated cells followed by the similar steps as described above. All cytotoxicity assays were performed 3 times. Values given represent the average of three independent readings.

Results

Electrophoretic analysis of fractions

SDS-PAGE profile of enriched protein antigenic fraction (crude antigen; CrAg) shows the proteins in the mw range of 14–70 kDa (Fig. 1). ConA ub fraction was rich in proteins in the mw range of 14–50 kDa (Fig. 1). ConA b fraction contained both high and low mw proteins (Fig. 1).

Six fractions were obtained from phenyl Sepharose chromatography. Fraction I (25% ammonium sulfate) and fraction III (15% ammonium sulfate and 10% ethylene glycol) each showed two unresolved peaks on the chromatogram and were collected separately. However, the SDS-PAGE analysis of fraction Ia and Ib showed similar proteins in both the lanes (Fig. 2). Fraction I was rich in low mw proteins ranging from 14–43 kDa with a doublet in 14–20 kDa region (Fig. 2; lanes PSC Ia, PSC Ib). Fraction III was also rich in low mw proteins in the range of 14–30 kDa. Fraction IIIa and IIIb both showed a strong band at 18 kDa with PSC IIIb containing less number of protein bands (Fig. 2; lanes PSC IIIa and PSC IIIb). Fraction IV, V and VI contained proteins in the whole range of 14–80 kDa (Fig. 2. lanes PSC IV, PSC V and PSC VI).

Immunoreactivity of fractions

ELISA absorbance values of various fractions for specific IgG and IgE binding with sera of ABPA patients and normals is shown in Table 1. Fraction PSC III shows high ELISA values for both specific IgG and IgE (1.192 and 0.602). (For ELISA analysis Fraction PSC IIIa and PSC IIIb were pooled and Fraction PSC Ia and Ib were pooled).

Cytotoxicity of antigenic fractions

Potent antigenic mixture (Crude antigen) was found to be toxic to RAW, L929, CHO and J774 with similar values of IC_{50} (43 ng/ml) (Table 2, Fig. 3). Therefore, further experiments were done on RAW cell line. Both the glycosylated (ConA b) and nonglycosylated (ConA ub) proteins were found to be toxic

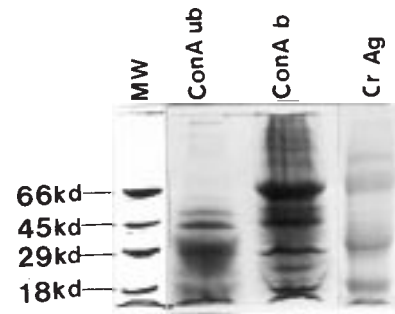


Fig. 1. SDS-PAGE analysis of antigenic fractions of *A. fumigatus* on a 10% gel.

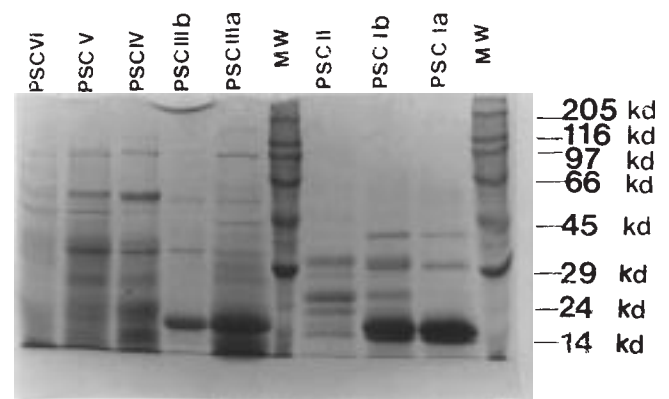


Fig. 2. SDS-PAGE analysis of phenyl sepharose fractionated antigens on a 10% gel.

Table 1. Specific IgG and IgE for various antigenic fractions in sera of ABPA patients

Fraction	Patient sera		Normal sera	
	Sp.IgG	Sp.IgE	Sp.IgG	Sp.IgE
Crude Ag	1.432	0.689	0.067	0.053
ConA b	0.842	0.427	0.042	0.057
ConA ub	0.870	0.515	0.070	0.062
PSC I	0.926	0.432	0.035	0.026
PSC II	0.632	0.243	0.028	0.022
PSC III	1.192	0.602	0.043	0.055
PSC IV	0.739	0.308	0.025	0.080
PSC V	0.626	0.218	0.036	0.040
PSC VI	0.438	0.127	0.028	0.025
18 kd	1.205	0.632	0.028	0.036

Antigenic fraction coated: 1 μ g protein/well; Patient sera: Pooled sera of 10 ABPA patients; Dilution of sera: 1:100; Normal Sera: Sera from healthy without any indication of pulmonary was taken as normals; OD was measured at 490 nm.

to RAW cells in a dose dependent manner (Fig. 3). ConA ub fraction containing nonglycosylated low mw proteins was found to be more toxic than ConA b fraction (Table 2). Cytotoxicity experiments with PSC fractions on RAW cells (Table 2, Fig. 4) shows that fraction I and III are more toxic in comparison with other fractions.

Table 2. Toxicity of various antigenic fractions on RAW cells

S.No.	Antigenic fractions	IC ₅₀ (ng/ml)
1	Enriched protein antigenic fraction (Cr. Ag)	43
2	ConAub	55
3	ConAb	240
4	Fraction I of PSC	90
5	Fraction II of PSC	175
6	Fraction III of PSC	60
7	Fraction IV of PSC	260
8	Fraction V of PSC	660
9	Fraction VI of PSC	1500

Cells were incubated with various antigenic fractions for 8 h and viability was measured by MTT assay as described in Materials and methods. IC₅₀: Concentration causing 50% inhibition; PSC: Phenyl Sepharose Chromatography.

Identification of the major cytotoxin

High toxicity shown by low mw rich protein fractions ConAub, PSC fraction III and PSC fraction I indicated that a low mw protein is probably the major cytotoxin secreted by *A. fumigatus*. Hence, fraction IIIb of PSC, containing significant amount of low mw proteins with minor contaminants, was subjected to reverse phase HPLC. It resulted in five peaks. The major peak at retention time 5.94 min at 22% acetonitrile gradient resulted in a homogeneous protein (Fig. 5) with an apparent mw of 18 kDa on SDS-PAGE (Fig. 6, lane B). Purified 18 kDa reacted with monoclonal antibody raised against Asp fl by ELISA (OD at 490 nm; 18 kDa + MoAb = 1.389; Control = 0.067). Recognition of 18 kDa by monoclonal antibody by immunoblot established the similarity of the purified protein to Asp fl (Fig. 6, lane C).

In view of presence of cytotoxicity in fractions PSC III and PSC I, a dot-blot assay with monoclonal antibody was carried out using various fractions. The results indicated positive reaction with crude antigen (++), Con A ub (++), PSC III (++) and PSC I (+). The reaction was negative with the other fractions (Data not shown). These results suggest the presence of cytotoxicity in some of the antigens other than 18 kDa allergen. Further investigations are in progress to identify other cytotoxins present in other fractions.

Cytotoxicity of Asp fl

Analysis of cytotoxicity of HPLC purified protein (18 kDa protein or Asp fl) at various concentrations (1 pg–10 µg/ml) showed that 50% of the cells in all the four cell lines were killed at a concentration of 75 ng/ml or 4.16 nM (Fig. 7). Cytotoxicity assays of Asp fl for different incubation time showed that cells became sick after 1 h of incubation with the toxin and were killed by 8 h (data not shown).

Routing of Asp fl

All the cell lines having different origins gave similar IC₅₀ with crude and purified antigen which prompted us to investigate the internalisation mechanism of 18 kDa protein. Toxicity of Asp fl was evaluated at different temperatures. It was found to be maximum at 37°C and it reduced with decrease in temperature (Fig. 8). No toxicity was found at 4°C and it was minimal at 15°C indicating that uptake is blocked at low temperatures. The cytotoxic activity of Asp fl was potentiated by 2-fold in presence of 10 mM ammonium chloride (Fig. 9). Monensin, which inhibits the translocation of protein from endosomes, also potentiated the toxicity of Asp fl (Fig. 9).

Discussion

The present study shows that 18 kDa allergen/antigen is the major cytotoxin secreted by *A. fumigatus* with an IC₅₀ of 4.16 nM. Further, it has been established that 18 kDa protein purified in this study is similar to Asp fl, already reported as a major allergen/antigen secreted by *A. fumigatus*. IC₅₀ of Asp fl for protein synthesis inhibition in cell free lysates was reported to be 0.25 nM which is significantly lower than the concentration required by Asp fl (4.16 nM) to kill 50% of eukaryotic cells [7]. This difference may be due to the regulated uptake of Asp fl by eukaryotic cells. Further, IC₅₀ of the enriched protein antigenic fraction is lower than purified Asp fl indicating that more than one component may be contributing to the cytotoxicity of enriched protein antigenic fraction or during purification some biological activity may have been lost.

The 18 kDa antigen seems to be a potential virulence factor as it contributes to the type I and type III hypersensitivity reactions in allergic forms of aspergillosis. Its role in the invasive forms of disease is yet not clear although it has been found as a major antigen excreted in the urine of patients of invasive aspergillosis [14]. Further, it may be playing a significant role in a variety of clinical conditions by contributing to the necrosis and tissue damage. However, alkaline protease and Asp fl gene disrupted mutants of *A. fumigatus* still retained their pathogenicity [15]. Hence, it appears that multiple virulent factors with various biological activities probably may be contributing to varying host-pathogen interactions resulting in a plethora of clinical conditions such as allergic bronchopulmonary aspergillosis, aspergilloma, invasive aspergillosis and chronic necrotising aspergillosis.

Of particular interest, cytotoxicity of Asp fl and crude antigen was found to be similar on all the four cell lines used; two macrophage cell lines (RAW and J774) and two fibroblast cell lines (CHO and L929). Thus the mechanism of uptake of 18 kDa by different cells appears to be similar. Fusion of phos-

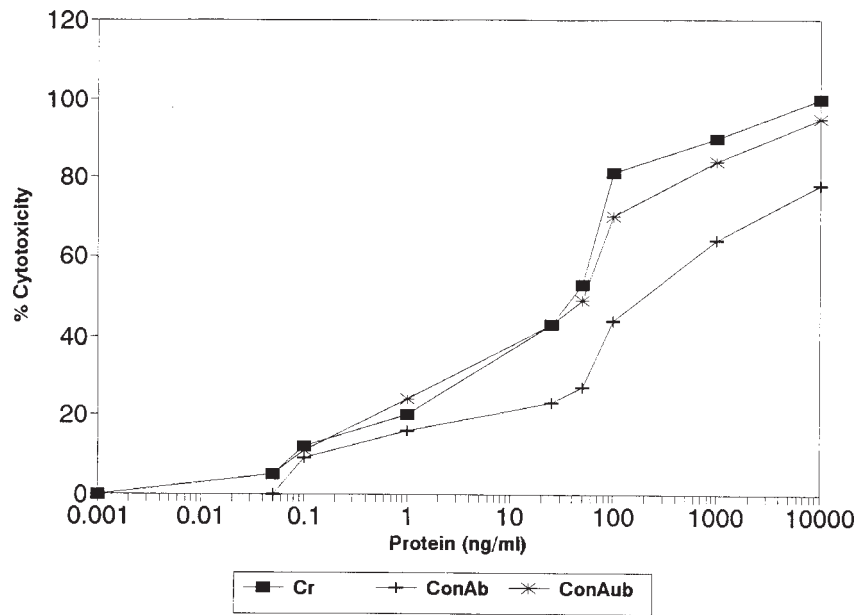


Fig. 3. Cytotoxicity analysis of fractionated antigens on RAW cells. RAW cells (10^4 cells/well) were incubated with the antigenic fractions for 8 h and viable cells were stained with MTT as described in Materials and methods.

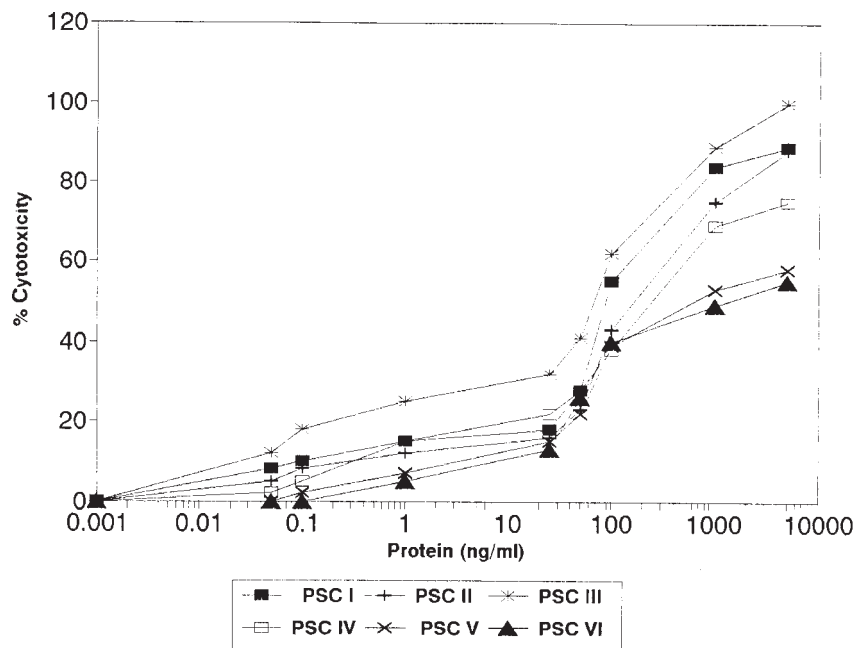


Fig. 4. Cytotoxic analysis of fractionated antigens of Phenyl Sepharose chromatography. RAW cells (10^4 cells/well) were incubated with the antigenic fractions for 8 h and viable cells were stained with MTT as described in Materials and methods.

pholipids, as shown by α -sarcin, may be one of the mechanisms of uptake for fungal ribotoxins [16].

Endocytosis is one of the most common ways of uptake of toxins. At temperatures less than 10°C endocytosis of various toxins is blocked as the lysosomal degradation is absent [17]. Inhibition of cytotoxicity of Asp fl at 4°C indicates en-

docytosis as probable mechanism of its uptake by the cells. Ammonium chloride and monensin raise endosomal pH in cells, reduce the proton gradient across the membrane and have powerful effects on the toxicity of protein toxins which have endocytosis as a step in their cytotoxicity. Uptake of α -sarcin is potentiated in presence of monensin [18].

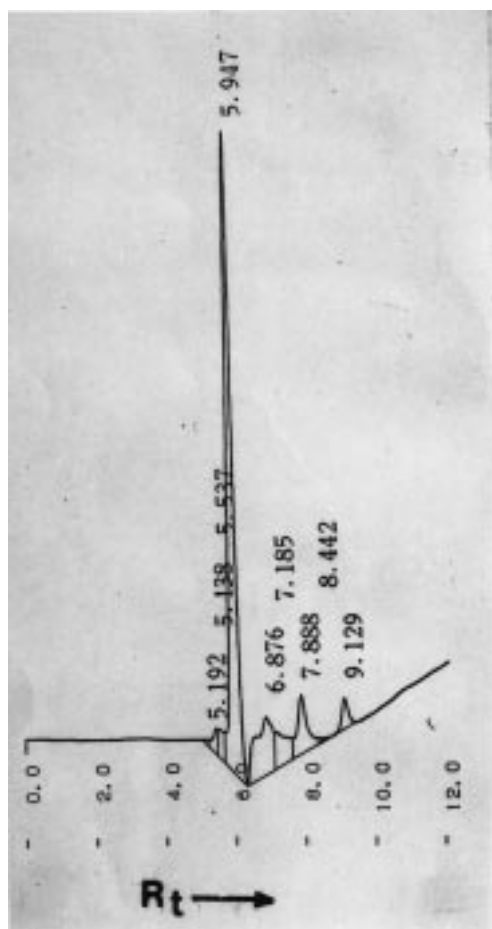


Fig. 5. HPLC profile of purified 18 kDa protein. Reverse phase HPLC was carried out on a CE-18 column using a 10–70% gradient of acetonitrile in water-trifluoroacetic acid. A major peak was identified at retention time 5.94 min. and 22% acetonitrile gradient.

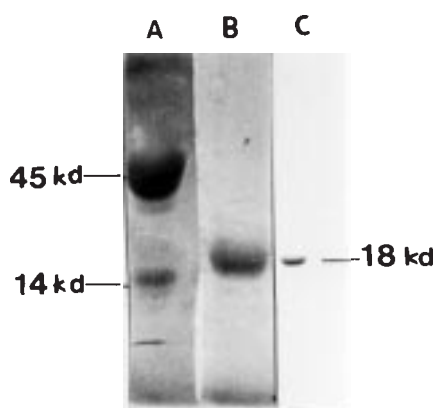


Fig. 6. SDS-PAGE analysis and immunoblot of purified antigen with monoclonal Ab raised against Asp fl on a 15% SDS-PAGE gel. A, MW markers; B, SDS-PAGE of purified 18 kDa; C, Immunoblot of purified 18 kDa with monoclonal Ab against Asp fl.

Cytotoxicity of Asp fl is also potentiated in presence of ammonium chloride and monensin which indicates that internalisation of Asp fl is probably similar to alpha-sarcin.

The partial amino acid sequence [7] and gene sequence [19] (Sarma *et al.* unpublished data) of Asp fl showed extensive homology (95%) with cytotoxins alpha-sarcin (*A. giganteus*), restrictocin and mitogillin (*A. restrictus*) known to inhibit protein synthesis by hydrolysing phosphodiester bond of 28S rRNA [20]. Asp fl has been shown to inhibit protein synthesis in cell free lysates [7]. Hence, the mechanism of cytotoxicity of Asp fl appears to be ribosome inactivation. However, precise mechanism of cytotoxicity of Asp fl is yet to be established.

Asp fl cytotoxicity against the eukaryotic cell lines is reported for the first time which is significantly higher than other fungal toxins (200 times restrictocin and 2 times Alpha sarcin) [18, 21]. Ribotoxins, when coupled with monoclonal antibodies against cell surface receptors become more toxic to eukaryotic cells. Alpha-sarcin conjugated immunotoxin was successfully targeted against the EJ human bladder carcinoma cell line and hepatoma cell line [18]. Mbr-1 restrictocin conjugates were 600–1500 times more efficient than the uncoupled restrictocin towards MCF-7 cells with no nonspecific cytotoxicity [21]. Asp fl may be conjugated with cell specific molecules to develop an immunotoxin for targeted cancer therapy. Immunological activity associated with toxic proteins limits their use in immunotherapy [22–24]. A similar problem of hypersensitivity hampered trials of mitogillin in cancer chemotherapy [25]. Since, B and T cell epitopes of Asp fl have already been identified (patent submitted, 1/NF/251/95-Pat), we suggest that elimination/modification of these immunologically active domains from the whole protein might result in a cytotoxic peptide devoid of immunogenicity.

In conclusion, Asp fl is the major cytotoxin secreted by *A. fumigatus* and its cytotoxicity is established towards four different eukaryotic cell lines. These studies were carried out with 18 kDa allergen/antigen purified from PSC IIIb, homogeneous by HPLC, SDS-PAGE and recognised by MoAb to Asp fl on western blot. The mechanism of internalisation of Asp fl is suggested to be similar to alpha-sarcin as shown by potentiation of Asp fl toxicity in presence of ammonium chloride and monensin. In view of pathogenic potential of fungal allergens, it would be important to examine the mechanism of cytotoxicity of Asp fl and the relationship between its allergenicity and cytotoxicity.

Acknowledgements

One of the authors, Taruna Madan is a recipient of a fellowship grant from Council of Scientific and Industrial Research, Government of India. We also express our gratitude to Dr. S.V. Gangal, Director CBT, for his encouragement and support.

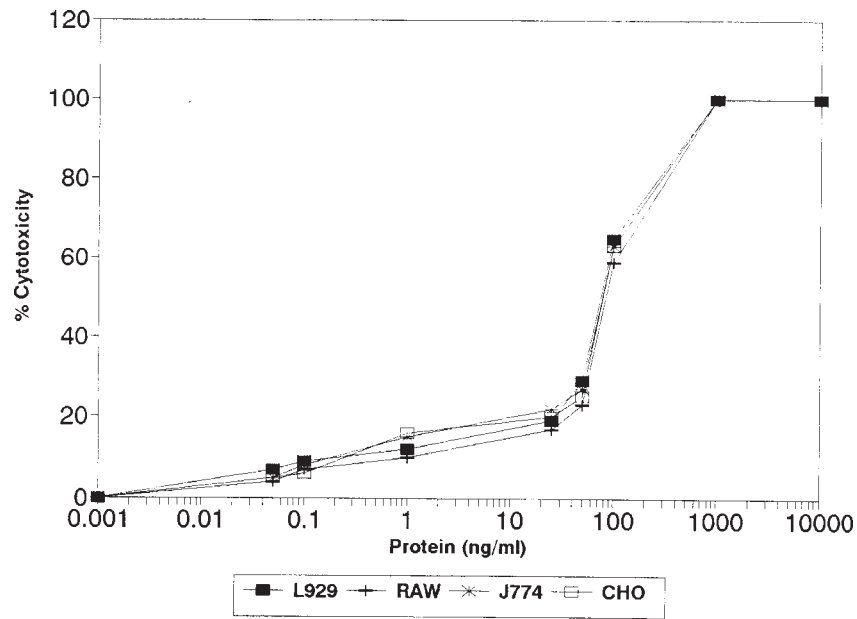


Fig. 7. Cytotoxicity of 18 kDa protein against different mammalian cell lines. Mammalian cells (10^4 cells/well) were incubated with the antigenic fractions for 8 h and viable cells were stained with MTT as described in Materials and methods.

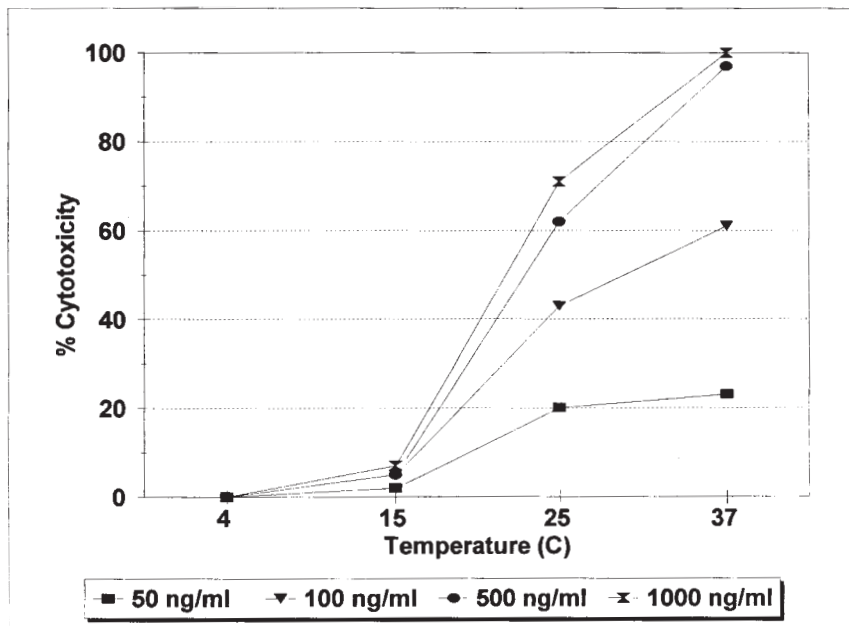


Fig. 8. Cytotoxicity of 18 kDa protein at different temperatures. RAW cells (10^4 cells/well) were incubated with the antigenic fractions for 8 h and viable cells were stained with MTT as described in Materials and methods.

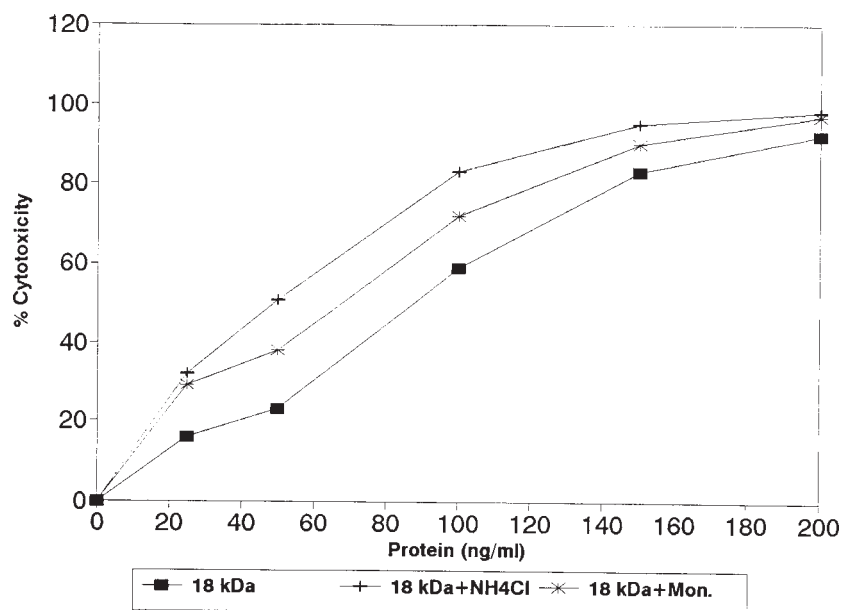


Fig. 9. Cytotoxicity of 18 kDa protein in presence of lysosomotropic agents. RAW cells were pre-incubated for 40 min in assay medium containing 10 mM ammonium chloride or 1 μ M monensin.

References

- Bardana EJ Jr.: The clinical spectrum of aspergillosis. Part 2: classification of saprophytic, allergic and invasive variants of *Aspergillus fumigatus*. *CRC Crit Rev Clin Lab Sci* 13: 85, 1980
- Kurup VP, Kumar A: Immunodiagnosis of aspergillosis. *Clin Microbiol Rev* 4: 439, 1991
- Kolattukudy PE, Lee JD, Rogers LM, Zimmerman P, Ceselski S, Fox B, Stein B, Copelan EA: Evidence for possible involvement of an elastinolytic serine protease in aspergillosis. *Infect Immun* 6: 2357, 1993
- Olson BJ, Jennings JC, Roga V, June AJ, Shuurmans DM: Alpha-sarcin a new antitumor agent. II. Fermentation and antitumor spectrum. *Appl Microbiol* 13: 314, 1965
- Conde FP, Orlandi R, Canevari S, Mezzanzanica D, Ripamonti M, Munoz SM, Jorge P, Colnaghi MI: The *Aspergillus* toxin restrictocin is a suitable cytotoxin agent for generation of immunoconjugates with monoclonal antibodies directed against human carcinoma cells. *Eur J Biochem* 178: 795, 1989
- Fernandez-Luna JL, Lopez-Otin C, Soriano F, Mendez E: Complete amino acid sequence of the *Aspergillus* cytotoxin mitogillin. *Biochemistry* 24: 861, 1985
- Arruda LK, Platts-Mills TAE, Fox JW, Chapman MD: *Aspergillus fumigatus* allergen 1, a major IgE binding protein, is a member of the mitogillin family of cytotoxins. *J Exp Med* 172: 1529–1532, 1992
- Banerjee B, Chetty A, Joshi AP, Sarma PU: Identification and partial characterisation of diagnostically relevant antigens of *A. fumigatus*. *Asian Pac J Allergy and Immunology* 18: 13–18, 1990
- Banerjee B, Madan T, Sharma GL, Prasad HK, Nath I, Sarma PU: Characterisation of 45 kD glycoprotein antigen of *A. fumigatus*. *Serodiag and Immunotherap* 7, 1996, In press
- Bir N: Immunochemical and biochemical characterisation of antigens of *A. fumigatus*. Thesis, 1996
- Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680, 1970
- Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets, procedure and some applications. *Proc Natl Acad Sci* 76: 4350, 1979
- Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55, 1983
- Latge JP, Moutaouakil M, Debeaupuis JP, Bouchara JP, Haynes K, Prevost MC: The 18 kilodalton antigen secreted by *Aspergillus fumigatus*. *Infect Immun* 59: 2586, 1991
- Smith JM, Tang CM, Noorden SV, Holden DW: Virulence of *Aspergillus fumigatus* double mutants lacking restrictocin and an alkaline protease in a low dose model of invasive aspergillosis. *Infect Immun* 62: 5247, 1994
- Gasset M, Onaderra M, delPozo AM, Schiavo G, Laynez J, Usobiaga P, Gavilanes JG: Effect of antitumor protein α -sarcin on the thermotropic behaviour of acid phospholipid vesicles. *Biochim Biophys Acta* 1068: 9, 1991
- Deurs BV, Petersen OW, Olsnes S, Sandvig K: The ways of endocytosis. *International Review of Cytology* 117: 131, 1989
- Youle RJ, Newton D, Wu Y, Gadina M, Rybak SM: Cytotoxic ribonucleases and chimeras in cancer therapy. *Crit Rev Therap Drug Carrier Sys* 10: 1, 1993
- Arruda LK, Mann BJ, Chapman MD: Selective expression of a major allergen and cytotoxin Asp fl in *A. fumigatus*. *J Immunol* 149: 3354–3359, 1992
- Endo Y, Huber PW, Wool IG: The ribonuclease activity of the cytotoxin alpha sarcin. *J Biol Chem* 258: 2662, 1982
- Orlandi R, Canevari S, Conde FP, Leoni F, Mezzanzanica D, Colnaghi MI: Immunoconjugate generation between the ribosome inactivating protein restrictocin and antihuman breast carcinoma MAB. *Cancer Immunol Immunother* 26: 114, 1988
- Pai LH, Pastan I: Immunotoxins and recombinant toxins for cancer treatment. Important advances in oncology. In: Vincent T DeVita, Samuel Hellman and Steven A Rosenberg (eds). *JB*

- Lippincott Company, Philadelphia 1994, p 3
23. Vitetta ES, Thorpe PE, Uhr JW: Immunotoxins: magic bullets or misguided missiles? *TIPS* 14: 148, 1993
24. Jin FS, Youle RJ, Johnson VG, Shiloach J, Fass R, Londo DL, Bridges SH: Suppression of the immune response to immunotoxins with anti-CD4 monoclonal antibodies. *J Immunol* 146: 1806, 1991
25. Roga V, Hedeman LP, Olson BH: Evaluation of mitogillin (NSC-69529) in the treatment of naturally occurring canine neoplasms. *Cancer Chemother Rep Part 1* 55: 101, 1971