

Secretion of a potential virulence factor, a fungal ribonucleotoxin, during human aspergillosis infections

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Summary

We show by cloning and nucleotide sequence analysis that the 18kDa antigen found in the urine of patients suffering from aspergillosis is related to the fungal protein toxins restrictocin and mitogillin. These are inhibitors of translation which act by catalytic inactivation of eukaryotic ribosomes; they may be implicated in the pathogenesis of the disease.

Introduction

Fungi are ubiquitous in nature and numerous fungal diseases in humans, cattle, poultry, etc. have been described. In humans, fungal infections are found predominantly in immunocompromised patients, especially following major surgery, for which the prognosis is generally pessimistic. There has been a marked increase in opportunistic secondary fungal infections in recent years because of acquired immune deficiency syndrome (AIDS) infections, the resurgence of diseases such as tuberculosis (TB), and following drug treatment of leukaemic patients (Musial *et al.*, 1988).

The major pathogenic species of *Aspergillus* for humans is *Aspergillus fumigatus*, which is responsible for a variety of allergic and invasive diseases (Vanden Bossche *et al.*, 1988); the most severe form is invasive aspergillosis which has been reported as an increasingly important cause of mortality in severely immunocompromised patients, especially those with acute leukaemia. *A. fumigatus* is also known to produce a number of interesting physiologically active metabolites (Ingber *et al.*, 1990). During human fungal infections a variety of complex circulating antigens are found in the urine and serum of patients; these antigens are mostly polysaccharides and proteins, and their detection has been used for the diagnosis of the disease (Vanden Bossche, 1988). It has not been con-

sidered previously whether any of these antigens might influence the course of infection, i.e. whether they were virulence factors.

Results and Discussion

The major circulating antigen found in the urine of patients with invasive aspergillosis is a protein of 18kDa, which we have recently purified to homogeneity by cation-exchange chromatography (Haynes *et al.*, 1990; Latge *et al.*, in press). This is the first of the disease-associated *Aspergillus* antigens to be purified in this way. The N-terminal amino acid sequence (10 residues) of this protein was determined by gas-phase analysis and was found to be identical to that of the ribonucleolytic protein toxins restrictocin and mitogillin (Lopez-Otin *et al.*, 1984), produced by two strains of *Aspergillus restrictus* (Table 1). In addition, the amino acid compositions of the three proteins were in agreement.

These cytotoxins were discovered by Olson and collaborators (Olson and Goerner, 1965) and, with the close analogue alpha-sarcin, were studied extensively as potential antitumour agents (Goldin *et al.*, 1966). Apparently the toxicity of these molecules did not encourage their pharmaceutical development. The ribotoxins were subsequently found to be among the most potent and specific inhibitors of eukaryotic translation (Fando *et al.*, 1985; Fernandez-Puentes and Vazquez, 1977; Jimenez and Vazquez, 1985), acting to cleave 28S rRNA enzymatically at a single site (Schindler and Davies, 1977), thereby rapidly and irreversibly inactivating all ribosomes in the cell. The mode of action and site specificity of the ribotoxins have been reviewed extensively by Wool (Wool, 1984; Wool *et al.*, 1990).

Since we had recently cloned and sequenced the gene for restrictocin (16.85kDa) (Lamy and Davies, 1991), we carried out a detailed comparison of the 18kDa antigen (isolated from *A. fumigatus* culture medium or from human urine) with restrictocin. They had the same apparent molecular weight in sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE), were immunologically cross-reactive (Fig. 1), and both inhibited protein synthesis on eukaryotic ribosomes *in vitro* at low concentrations (results not shown). In particular, the 18kDa antigen, like restrictocin and the related ribotoxin alpha-sarcin, releases a c. 400-base fragment from the 3' end of

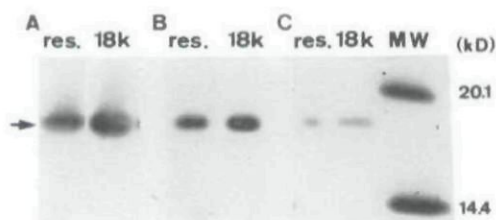


Fig. 1. Comparison of restrictocin and the 18kDa protein. The purified proteins were compared on 15% SDS-PAGE (C); and immunoblots revealed by anti-alpha-sarcin rabbit antiserum (A) or monospecific anti-18kDa rabbit antiserum (B). Restrictocin (res.) and 18kDa protein (18k) had the same apparent molecular weight and cross-reacted strongly.

Molecular weight markers (MW) are indicated in the right-hand margin. Restrictocin and 18kDa protein were purified as described in Table 1. Rabbit antiserum to alpha-sarcin was kindly provided by Drs Y. Endo and G. Stoeffler. Monospecific anti-18kDa rabbit antiserum was experimentally obtained after immunization of New Zealand rabbits with 0.5 mg of the 18kDa protein. Detection was with alkaline-phosphatase-conjugated anti-rabbit IgG (Promega).

the 28S rRNA (the 'alpha-fragment') upon incubation of rabbit reticulocyte lysates with a low concentration of the antigen (Wool *et al.*, 1990) (Fig. 2). Southern blotting and analysis of polymerase chain reaction (PCR)-amplified fragments of DNA from *A. fumigatus* indicate that the gene for the 18kDa protein is similar in size and organization to that of restrictocin (results not shown). Independently isolated PCR fragments from *A. fumigatus* were sequenced and confirm that the two genes are almost identical (Fig. 4). Two differences were detected: the first

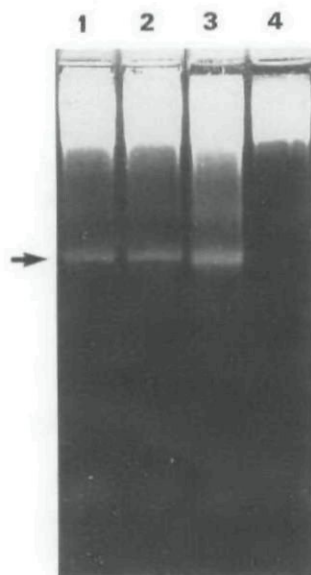


Fig. 2. Specific ribonuclease activity of restrictocin and the 18kDa protein shown by release of the 400-base 'alpha-fragment' by cleavage of the 28S rRNA of eukaryote ribosomes. Rabbit reticulocyte lysates (Promega) were incubated with 100 ng of the toxins (1, alpha-sarcin; 2, restrictocin; 3, 18kDa protein; 4, without toxin, for 5 min and rRNAs extracted and analysed by electrophoresis on agarose as described by Endo (Endo and Wool, 1982). The arrow shows the position of the 'alpha-fragment' (c. 400 nucleotides) cleaved from the 28S rRNA.

results in the substitution of a serine residue by asparagine (position 52) as in mitogillin, which differs from restrictocin by a single amino acid (Fernandez-Luna *et al.*, 1985); the second at nucleotide no. 439 is silent. Thus, there is no doubt that the 18kDa antigen found in the urine of humans with invasive aspergillosis is a member of the family of fungal ribotoxins, which includes the cytotoxic agents alpha-sarcin, mitogillin, and restrictocin. The strain *Aspergillus restrictus* (MDH 13462L, ATCC 34475) producing restrictocin has been reclassified as a member of the *A. fumigatus* family and phenotypic comparisons confirm that the *A. restrictus* strain does in fact belong to the *A. fumigatus* species.

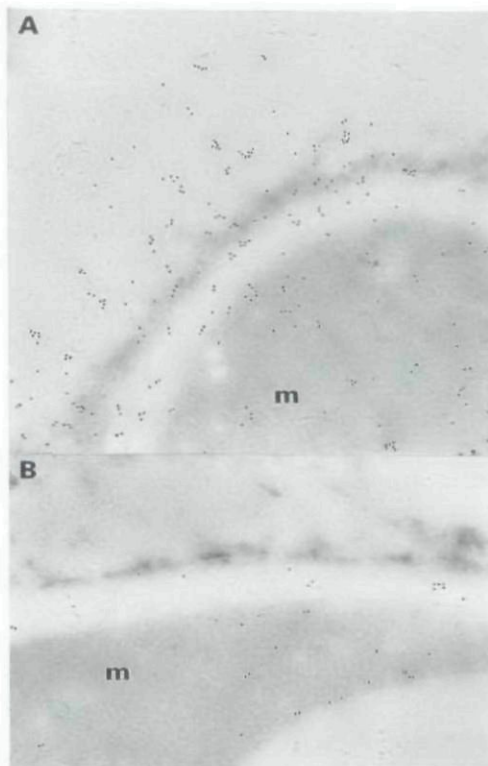


Fig. 3. Immunolabelling of mycelium (m) of *Aspergillus fumigatus* growing in kidneys of CD1 mice infected experimentally by intravenous injection. Ultrathin sections of material embedded in Lowicryl K4M were sequentially incubated with an anti-18kDa protein rabbit antiserum (A) or a pre-immune rabbit antiserum (B) diluted 1:300 and with a 10 nm gold-labelled goat anti-rabbit IgG (H+L) conjugate. Gold particles demonstrate the secretion of the 18kDa antigen in tissue ($\times 34\,000$).

Is the restrictocin-like 18kDa protein a factor that contributes to the pathogenesis of aspergillosis? This question cannot be answered definitively until the gene is inactivated in pathogenic *A. fumigatus* and studies of the infectivity of this mutant carried out in experimental animals. Alternatively, it might be possible to demonstrate that a fungal ribotoxin gene, when cloned into an innocuous fungal strain, changes the infectious character of the host (Lamy and Davies, 1991). Aspergillosis is characterized by

the presence of inflammatory granulomatous lesions showing necrosis of the tissue surrounding the fungus. Phagocytic cells (polymorphonuclear leucocytes (PMNs), macrophages) are the main immune defence mechanism against *Aspergillus* infections (Waldorf, 1989). It is conceivable that phagocytosis of the infecting *A. fumigatus* cells

with subsequent cytoplasmic release of one of the ribotoxins, such as restrictocin, is the cause of localized cell necrosis. In fact, it is possible to detect the ribotoxin within the surrounding host cells in infected mouse kidney (Fig. 3).

The clinical effects of the release of a toxin such as

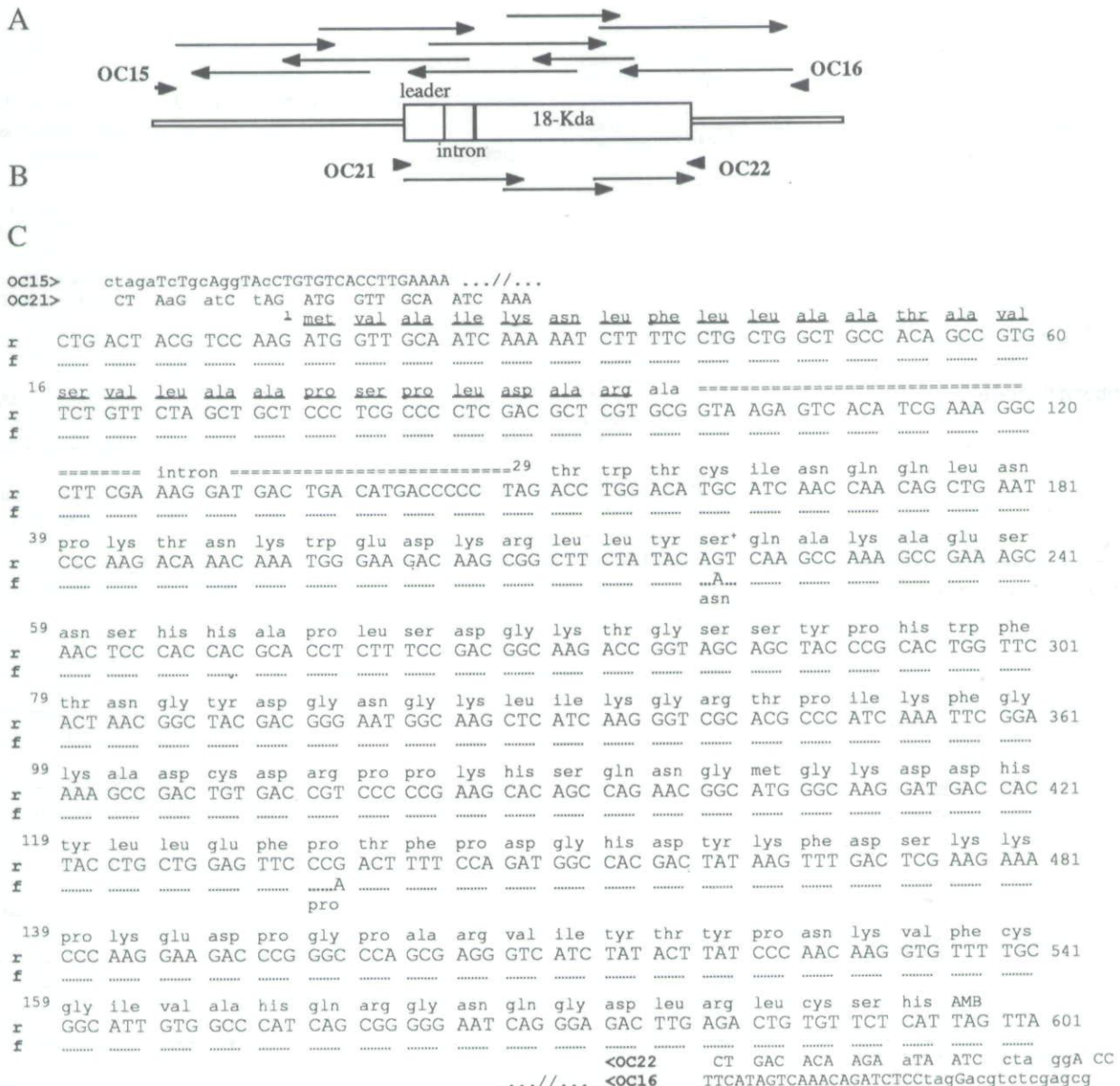


Fig. 4. Sequencing of the gene of 18kDa protein of *A. fumigatus* and comparison with the sequence of restrictocin. Two independent polymerase chain reactions were performed using the following: (A) the oligonucleotides OC15, OC16 and a preparation of *A. fumigatus* DNA (strain CBS 143.89), or (B) the oligonucleotides OC21, OC22 and a second preparation of DNA of the same strain. The four oligonucleotides are partially homologous with the restrictocin gene of *A. restrictus* (uppercase in C), and contain restriction sites useful for subcloning of the amplified fragments in M13mp19. The arrows indicate the direction and the relative sizes of the fragments sequenced; (A) the *Pst*I fragment of 1.3kb, and (B) the *Bam*HI-*Bgl*II fragment of 0.6kb.

C. The aligned nucleotide sequences of the *res* gene (r) (Lamy and Davies, 1991) and the 18kDa gene of *A. fumigatus* (f). Dots indicate identical nucleotides. The deduced amino acid sequence of restrictocin is shown above, and the amino acid differences in the 18kDa gene are shown below the nucleotide sequences. The predicted signal peptide is underlined. The serine residue (position 52) which differentiates restrictocin from mitogillin (asparagine in the latter) is indicated (+).

These sequence data will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Data Libraries under the accession numbers X56176 (*res*) and X58278 (18kDa).

Table 1. Comparison of the N-terminal amino acid sequences of the 18kDa protein from *A. fumigatus*, restrictocin, and alpha-sarcin.

Restrictocin:	A - T W T C I N Q Q L
18kDa:	A - T W T C I N Q Q L
Alpha-sarcin:	A V T W T C L N D Q K

Restrictocin was extracted from the culture medium of *Aspergillus* strain ATCC 34475. Mycelium was grown for 50 h at 37°C in MOA4 medium (beef extract (Oxoid) 2% (w/v), Bactopeptone (Difco) 1% (w/v), corn starch (Prolabo) 2% (w/w), sodium chloride 0.5% (w/v), pH adjusted to 6.8). Purification of the protein was carried out on Mono S HR5/5 (Pharmacia LKB) with a NaCl gradient, after an initial batch extraction with SP-Sephadex C-25 (Pharmacia).

The 18kDa protein was extracted from culture medium of *A. fumigatus* (strain CBS 143.89, collection of Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) grown for 48 h at 25°C in Sabouraud medium. The culture filtrate was precipitated by ethanol and the 18kDa protein purified from the water-soluble material of this precipitate after dialysis and by following cation exchange (Mono S HR5/5, Pharmacia) and size exclusion (Superose 12, Pharmacia) chromatography.

The N-terminal sequence of these two proteins was determined by gas-phase sequencing (Applied Biosystems), and is identical to that described by Lopez-Otin (Lopez-Otin *et al.*, 1984). The alpha-sarcin sequence was determined by Sacco (Sacco *et al.*, 1983).

restrictocin into the bloodstream during systemic fungal infections cannot be assessed easily. Apparently, the single chain ribotoxins do not easily enter normal cells (this is distinct from the dimeric cytotoxins such as diphtheria toxin or ricin, in which one of the chains specifically mediates endocytotic entry), but virus infection of cultured cell lines makes them transiently permeable to the ribotoxins, and protein synthesis is rapidly inhibited when restrictocin or alpha-sarcin are added (Fernandez-Puente and Carrasco, 1980). The use of restrictocin as an immunotoxin is currently undergoing investigation (Conde *et al.*, 1989; Orlandi *et al.*, 1988); these studies have indicated that the presence of free restrictocin in the bloodstream of rabbits was not lethal. However, the situation is likely to be different when the infectious *A. fumigatus* is present, and the demonstration of the biological activity of the 18kDa antigen (restrictocin) will permit the study of a new aspect of human fungal infections.

During the preparation of this communication, Arruda *et al.* (1990) reported that an IgE-binding protein produced in cultures of another strain of *A. fumigatus* was similar to the ribotoxin mitogillin. Thus, it is probable that a family of allelic variant protein toxins that are potent inhibitors of eukaryotic protein synthesis are produced during *Aspergillus* infections in humans (and animals?). More extensive studies of the distribution of the ribotoxins and their potential role in disease are warranted, and oligonucleotide probes based on the restrictocin gene sequence (Fig. 4) are now available to permit rapid detection of the cytotoxin genes by *in situ* nucleic acid hybridization.

Acknowledgements

We are grateful to Ira Wool, Yaeta Endo, and Georg Stoeffler for providing samples of alpha-sarcin and antibodies, Robert Samson for confirmation of strain classification, and to Kenneth Haynes and Tom Rogers for providing a urine sample from a patient with invasive aspergillosis.

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