Amphotericin B enhances the synthesis and release of the immunosuppressive agent gliotoxin from the pulmonary pathogen Aspergillus fumigatus

Emer P. Reeves, Thomas Murphy, Paul Daly and Kevin Kavanagh

Medical Mycology Unit, National Institute for Cellular Biotechnology, Department of Biology, National University of Ireland Maynooth, Co. Kildare, Ireland

Exposure of the pulmonary pathogen Aspergillus fumigatus to amphotericin B alters membrane permeability as indicated by the escape of amino acids and protein from the mycelium. Amphotericin B exposure for periods of 2–4 h also leads to increased release of the immunosuppressive agent gliotoxin into the surrounding culture medium. Examination of the intracellular gliotoxin concentration following exposure to amphotericin B indicated elevated levels within the hyphae as well as in the culture medium – an effect which was also evident upon exposure of A. fumigatus to DMSO. These results indicate that in parallel with the ability of amphotericin B to act as a fungistatic agent it can also induce the synthesis of gliotoxin and facilitate its release by increasing the permeability of the fungal cell membrane. Increased synthesis of gliotoxin may result from the commencement of secondary metabolism in the presence of amphotericin B. The ability of amphotericin B to enhance the synthesis and release of gliotoxin may exacerbate the effects of the toxin and facilitate fungal invasion of pulmonary tissue.

INTRODUCTION

The fungus Aspergillus fumigatus is a pulmonary pathogen capable of inducing disease in those with pre-existing pulmonary malfunction (e.g. asthma, cystic fibrosis), disease (e.g. tuberculosis, lung cancer) or undergoing immunosuppressive therapy prior to organ transplantation (Denning, 1996a; Fraser, 1993; Daly & Kavanagh, 2001). Three forms of aspergillosis are recognized clinically: saprophytic, allergic and invasive. Invasive aspergillosis (IA) is the most serious form of disease as it involves the invasion of viable tissue and may produce a mortality rate of 80–95% (Denning, 1996b, 1998). IA has emerged as an important disease in recent decades due to the use of aggressive immunosuppressive therapy causing prolonged neutropenia in the treatment of cancer and leukaemia (Daly & Kavanagh, 2001). Despite aggressive anti-fungal chemotherapy, death due to IA usually results 7–14 days post-diagnosis (Denning, 1996b).

A. fumigatus produces a range of secondary metabolites such as gliotoxin, helvolic acid and fumagillin which may facilitate its growth and persistence in the lung (Amitani et al., 1995a; Hogan et al., 1996). Extracts obtained from sputum sols of patients with aspergillosis damage human respiratory epithelial cells (Amitani et al., 1995a). Subsequent analysis confirmed that gliotoxin derived from A. fumigatus was the toxic agent and that helvolic acid was also capable of complete ciliostasis and epithelial cell disruption (Amitani et al., 1995b).

Gliotoxin (C_{13}H_{14}N_{2}O_{4}S_{2}, molecular mass 326.4) is an epipolythiodioxopiperazine (Waring & Beaver, 1996) which displays immunosuppressive properties in vivo (Sutton et al., 1994). Gliotoxin is capable of inhibiting macrophage function and adherence to plastic surfaces (Bertout et al., 2002; Eichner et al., 1986) and may alter the immune response to Aspergillus as it can induce apoptotic cell death in macrophages (Waring, 1990), cells of the spleen (Braithwaite et al., 1987) and the immune system (Sutton et al., 1994). The immunosuppressive properties of gliotoxin have been evaluated for suppressing the whole body immune response prior to organ transplantation (Sutton et al., 1995). Gliotoxin is also capable of inducing cell death in a murine fibroblastic cell line (Piva, 1994). Gliotoxin has been detected in samples from animals (Richard & DeBey, 1995; Richard et al., 1996) and humans (Shah et al., 1995) where it may facilitate fungal persistence and colonization of tissue. In addition, gliotoxin has been implicated in the destruction of lung parenchyma in IA (Sutton et al., 1996) and the penetration of blood vessels in angio-invasive aspergillosis (Fraser, 1993).

Conventional therapy for the control of aspergillosis relies upon the use of the polyene, amphotericin B (Ellis, 2002) and the azoles, itraconazole and fluconazole (Canuto & Rodero, 2002). Amphotericin B displays fungistatic activity and functions by forming apertures in the cell membrane, by
complexing the membrane sterol, ergosterol (Abu-Salah, 1996). Each pore consists of an annulus of eight amphotericin B molecules linked hydrophobically to ergosterol. The hydroxyl residues of the polyene drug face inwards to give an effective pore diameter of 0.4–1.0 nm. Amphotericin B–ergosterol pores allow the exit of intracellular constituents (e.g. ions and small molecular mass compounds) and the entry of hydrogen ions leading to increased cytoplasmic acidity (Cohen, 1998). While amphotericin B can induce the formation of apertures in the fungal cell membrane, the effect may not be fatal to the cell and ‘recovery’ may be possible (Liao et al., 1999). It has been established that while a specific concentration of amphotericin B may inhibit replication, as indicated by lack of fungal growth, specific cellular functions (e.g. trans-membrane potential, intracellular enzyme activity and membrane integrity) still operate and, over time, the fungus may resume the ability to grow and divide (Liao et al., 1999).

An alternative explanation for the mode of action of amphotericin B has recently been proposed and suggests that, in addition to the formation of pores in the fungal cell membrane, amphotericin B also alters the permeability of the phospholipid bilayer by changing the membrane phase, i.e. increasing the fluidity of the bilayer (Venegas et al. 2003). However, the dominant effect of amphotericin B appears to be the formation of pores in the membrane by complexing ergosterol.

The ability of amphotericin B to induce the release of intracellular constituents in Candida albicans via membrane apertures has been established previously (Ghosh & Ghosh, 1963) and raises the possibility of the release of low molecular mass toxins from pathogenic fungi. Consequently, the aim of this study was to establish whether exposure of A. fumigatus to amphotericin B could lead to the release of a low molecular mass toxin such as gliotoxin; a process which could have the potential to exacerbate the immunosuppressive and invasive abilities of A. fumigatus.

MATERIAL AND METHODS

A. fumigatus culture conditions. A. fumigatus ATCC 26933 (obtained from the American Type Culture Collection) was used in this study. Aspergillus cultures were grown in minimal essential medium Eagle (MEM) (Sigma Aldrich) or RPMI medium supplemented with 5 % (v/v) fetal calf serum (Sigma Aldrich) at 37 °C and 200 r.p.m., for up to 4 days. Stocks were maintained on malt extract agar (MEA) (Oxoid).

Effect of amphotericin B on growth of A. fumigatus. MEA plates containing sporulating Aspergillus colonies were washed with 10 ml 0-1 % (v/v) Tween 80 (Merck) in PBS (pH 7.2) (Sigma Aldrich) to isolate conidia. Conidia were washed twice in sterile PBS, centrifuged (1500 g, 5 min in a Beckman GS-6 centrifuge) and counted using an haemocytometer. Flasks containing MEM (25 ml) were inoculated with 1 × 10^5 Aspergillus conidia to give a density of 4 × 10^3 ml^(-1) and incubated at 37 °C and 200 r.p.m. Cultures were supplemented with amphotericin B at final concentrations of 0-04, 0-08, 0-16 and 0-32 µg ml^(-1). Flasks were removed at each time point and the contents filtered through a Whatman No. 1 filter in a Büchner funnel and air-dried. A growth curve was constructed of dry fungal biomass versus incubation time.

Evaluation of membrane leakage. Seventy-two-hour-old cultures (25 ml) of A. fumigatus were harvested by centrifugation (2056 g, 20 min), washed three times in PBS and the hyphal mass resuspended in 25 ml PBS. Amphotericin B (0-04, 0-08, 0-16 or 0-32 µg ml^(-1)) or DMSO (Sigma Aldrich) (0-5 % v/v) was added and the cultures incubated at 37 °C and 200 r.p.m. for a further 1, 2 or 4 h. At each time point a culture was removed and the contents filtered through a Whatman No. 1 filter. The filtrate was passed through a 0-45 µm syringe filter (Sartorius) and free amino acids or protein were measured as described below.

Amino acid concentration was determined by the ninhydrin colorimetric method and is expressed in terms of aspartic acid and glutamic acid, which were used as standards. A ninhydrin (Sigma Aldrich) solution (200 µl; stock: 0-35 g in 100 ml ethanol) was added to each sample (1 ml) and heated to 95 °C for 4 min. After cooling to room temperature in an ice bath, the absorbance at 570 nm was recorded on a spectrophotometer (Beckman DU 640).

To determine the quantity of protein released from the hyphal mass, samples were assayed using the Bradford reagent (Bio-Rad), with BSA (Sigma Aldrich) as standard.

Extraction of gliotoxin from A. fumigatus culture filtrate. Hyphae of Aspergillus were removed from the MEM culture medium or RPMI medium by filtration and an equal volume (25 ml) of chloroform (Hyper Solv; BDH) was added to the filtrates. Following continual mixing for 30 min, the chloroform fraction was collected and evaporated to dryness in a Büchi (Brinkmann Instruments; Westbury, NY) rotor evaporator. Dried extracts were dissolved in 250 µl methanol (Hyper Solv, BDH) and stored at −70 °C until assayed.

Extraction of gliotoxin from hyphae of A. fumigatus. The extraction of intracellular gliotoxin from hyphae of A. fumigatus was performed as follows: hyphae were recovered from MEM culture medium (50 ml) or RPMI medium (50 ml) by filtration. Hyphae were washed in PBS and ground to a fine powder under liquid N2 using a pre-chilled pestle and mortar. The ground hyphae were resuspended in 10 ml 6 M HCl. Chloroform (30 ml) was added and the mixture stirred at room temperature for 30 min. The sample was poured into a separation funnel and the gliotoxin extracted in the lower chloroform layer. Following chloroform evaporation, the dried extracts were dissolved in 250 µl methanol and levels of gliotoxin quantified by Reversed Phase-HPLC.

Quantification of gliotoxin by HPLC. Gliotoxin was detected by Reversed Phase-HPLC (Spectra-Physics). The mobile phase was 34.9 % (v/v) acetonitrile (Hyper Solv, BDH), 0-1 % (v/v) trifluoroacetic acid (Sigma Aldrich) and 65 % (v/v) deionized-distilled water. Gliotoxin extract (20 µl) was injected onto a C18 Hewlett Packard column. A standard curve of peak area versus gliotoxin concentration was constructed using gliotoxin standards (50, 100 and 200 ng ml^(-1)) dissolved in methanol (Sigma Aldrich).

Statistical analysis. All assays were performed on three independent occasions. Results presented are the mean ± standard error. Statistical analysis were performed using Student’s two tailed t-test with values of P < 0.05 considered statistically significant.
RESULTS

The effect of amphotericin B on the growth of A. fumigatus

Culture medium was inoculated with conidia of A. fumigatus at an initial density of $4 \times 10^3$ ml$^{-1}$ and incubated as described. An untreated culture and four cultures supplemented with different concentrations of amphotericin B were used. The results (Fig. 1) indicate that while the concentrations of amphotericin B employed here act as fungistatic agents over the first 24 h, the rate of growth of the cultures supplemented with 0.04 and 0.08 g amphotericin B ml$^{-1}$ is approximately the same as the control over the 24–72 h period. Following 96 h incubation, the treated cultures reached a mass of approximately 65–70 % of that of the control. This experiment demonstrated that the concentrations of amphotericin B chosen for subsequent work were fungistatic rather than fungicidal, as used clinically (Liao et al., 1999).

To verify that the amphotericin B concentrations employed in subsequent experiments were fungistatic rather than fungicidal, 48 h cultures of A. fumigatus [hyphal mass 127.9 mg (dry weight)] grown in MEM culture medium were supplemented with amphotericin B at final concentrations of 0.16 or 0.32 g amphotericin B ml$^{-1}$ and grown for a further 24 h. Hyphae were harvested, washed with PBS and resuspended in fresh medium for 24 h in the absence of amphotericin B. The hyphal mass of the culture supplemented with 0.16 g amphotericin B ml$^{-1}$ had increased to 155.4 mg (dry weight) while that of the culture supplemented with amphotericin B at a concentration of 0.32 g ml$^{-1}$ was 111.6 mg (dry weight). This finding indicates that amphotericin B at 0.16 g ml$^{-1}$ does not prevent subsequent growth of the culture, and that the 0.32 g ml$^{-1}$ amphotericin B treatment does not cause wide-scale hyphal cell death, as the dry weight was still 87 % of the untreated control.

The effect of amphotericin B on release of intracellular constituents from A. fumigatus

It has been established previously that amphotericin B can induce the release of intracellular constituents from pathogenic fungi (Ghosh & Ghosh, 1963; Cohen, 1998). We sought to establish whether this occurred in A. fumigatus and to determine whether it contributed to the enhanced release of low molecular mass compounds. Seventy-two-hour-old cultures of A. fumigatus were exposed to amphotericin B at final concentrations of 0.04, 0.08, 0.16 or 0.32 g ml$^{-1}$ or 0.5 % (v/v) DMSO for 1, 2 or 4 h and the release of amino acids and protein was monitored. DMSO was employed here as a positive control since it is known to alter the permeability of the cell membrane (Yu & Quinn, 1998). The results show that exposure to 0.16 or 0.32 g amphotericin B ml$^{-1}$ or 0.5 % DMSO for periods of 2 or 4 h lead to the release of elevated levels of amino acids compared to the relevant controls (Fig. 2). In the case of exposure of A. fumigatus to 0.32 g amphotericin B ml$^{-1}$ for 4 h, 2.741 ± 0.069 g amino acid (mg hyphae)$^{-1}$ was released.
Enhanced protein release from *A. fumigatus* was also evident after exposure to amphotericin B for 2 or 4 h (Fig. 3). In this case the greatest release occurred when 0·32 µg amphotericin B ml\(^{-1}\) was employed for 4 h (35·57 ± 2·56).

**Release of gliotoxin from *A. fumigatus* exposed to amphotericin B**

Amphotericin B can induce the release of amino acids and protein from cultures of *A. fumigatus* over a relatively short time period (Figs 2 and 3). Since gliotoxin is a low molecular mass dipeptide it could escape from *A. fumigatus* in the same manner as amino acids and proteins. Cultures of *A. fumigatus* which had been grown for a period of 72 h to a hyphal mass of 29 mg were supplemented with amphotericin B at final concentrations of 0·04, 0·08, 0·16 or 0·32 µg ml\(^{-1}\) and the amount of gliotoxin in the culture filtrates was assessed at various time points after the addition. After 1 h, the control culture demonstrated a gliotoxin concentration of 210·3 ± 36·1 ng (mg hyphae)\(^{-1}\) (Fig. 4). Those cultures supplemented with 0·16 or 0·32 µg amphotericin B ml\(^{-1}\) showed elevated levels of gliotoxin in the culture supernatants, particularly after 2 and 4 h. The highest concentration of gliotoxin [506·6 ± 24·7 ng (mg hyphae)\(^{-1}\)] was detected in the culture supplemented with 0·32 µg amphotericin B ml\(^{-1}\) after 2 h incubation (Fig. 4).

**Effect of DMSO on gliotoxin release by *A. fumigatus***

Amphotericin B functions by binding ergosterol in the fungal cell membrane and creating pores through which intracellular constituents may escape (Abu-Salah, 1996). It was postulated that the elevated levels of gliotoxin observed in amphotericin-B-supplemented cultures could be due to the increased permeability of the fungal cell membrane. To test this hypothesis 72-h-old cultures of *A. fumigatus* were supplemented with DMSO, which also has the ability to alter the permeability of cell membranes, but in a different manner to amphotericin B (Yu & Quinn, 1998). The results demonstrated a significant increase in gliotoxin in the culture medium after 2 h in those cultures supplemented with 0·5 % DMSO (Fig. 5). Following 4 h exposure to DMSO, the gliotoxin concentration in the culture medium supplemented with 0·5 % DMSO reached a value of 431·14 ± 23·1 ng (mg hyphae)\(^{-1}\).

**Examination of intracellular and extracellular concentration of gliotoxin following exposure of *A. fumigatus* to amphotericin B or DMSO**

The elevated levels of gliotoxin in amphotericin-B- or DMSO-supplemented cultures may be due to increased membrane permeability. Alternatively, the two agents could exert a fungistatic effect and this might, in turn, lead to the increased synthesis of secondary metabolites such as gliotoxin. To ascertain whether this was the case the gliotoxin concentration in supplemented medium and in the hyphae of *A. fumigatus* was determined. In this case cultures were grown for 48 h and then supplemented with the relevant concentration of amphotericin B or DMSO for a further 24 h. The results revealed enhanced levels of gliotoxin in the hyphae as well as in the culture medium of those cultures supplemented with amphotericin B or DMSO (Fig. 6). The intra-hyphal gliotoxin concentration in the culture...
supplemented with 0.16 μg amphotericin B ml⁻¹ was 732.1 ± 37.9 ng mg⁻¹ compared to the control which showed a gliotoxin concentration of 88.6 ± 4.9 ng mg⁻¹. Similarly, the gliotoxin concentration in the MEM culture medium was 273.5 ± 11.9 ng ml⁻¹ compared to 146.1 ± 4.5 ng ml⁻¹ in the control. In the case of the cultures supplemented with 0.5% (v/v) DMSO, the gliotoxin concentration in the culture medium was 227 ± 12.1 ng ml⁻¹ (control: 146.1 ± 14.5 ng ml⁻¹) while that in the hyphae was found to be 405.5 ± 20.2 ng mg⁻¹ compared to 88.6 ± 4.9 ng mg⁻¹ in the relevant control. In the case of A. fumigatus grown in RPMI medium (Fig. 6) the extracellular gliotoxin concentration was 39.47 ± 1.2 ng ml⁻¹ in the control whereas it was 205.19 ± 8.2 ng ml⁻¹ when the culture was supplemented with amphotericin B at a final concentration of 0.16 μg ml⁻¹. The intracellular gliotoxin concentration increased from 3.63 ± 0.13 ng (mg hyphae)⁻¹ in the control to 41.4 ± 0.74 ng (mg hyphae)⁻¹ in the amphotericin-B-treated culture. Although different levels of gliotoxin were produced by A. fumigatus in MEM and RPMI culture media, the ability of amphotericin B to increase the intracellular and extracellular gliotoxin levels was observed in cultures grown in both media.

**DISCUSSION**

Infection by *A. fumigatus* is a serious threat to the health of patients immunocompromised as a result of disease, pulmonary malfunction or medical therapy (Daly & Kavanagh, 2001) and, in the case of IA, may have a mortality rate of 85–90% (Denning, 1996b). Part of the ability of *A. fumigatus* to colonize the lung is its capacity to suppress the local immune response (Hogan *et al.*, 1996). This can be achieved through the action of toxins and enzymes, and the ability of gliotoxin to facilitate colonization is well characterized (Sutton *et al.*, 1995 & 1996). Gliotoxin may suppress the function of pulmonary macrophages and also affect the general immune response to pathogens by destroying cells of the spleen and the immune system (Sutton *et al.*, 1994).

One of the principal drugs used to combat aspergillosis is the polyene amphotericin B, which creates pores in the fungal cell membrane through which intracellular constituents escape (Abu-Salah, 1996; Cohen, 1998). It was postulated that such pores might also serve to allow gliotoxin to escape from the hyphae of *A. fumigatus*, which could exacerbate gliotoxin-mediated damage to pulmonary tissue and the suppression of the immune response in the infected patient. As a consequence we sought to determine the response of hyphae of *A. fumigatus* to amphotericin B in terms of the synthesis and release of gliotoxin.

Using fungistatic concentrations of amphotericin B (Fig. 1) it was established that exposure of cultures of *A. fumigatus* to amphotericin B increased the release of amino acids (Fig. 2) and protein (Fig. 3) 2–4 h after addition. These findings suggested that amphotericin B was inducing the release of intracellular constituents from the fungal hyphae, possibly through the creation of pores in the cell membrane. Elevated
levels of gliotoxin were also detected in media of cultures supplemented with amphotericin B (Fig. 4) or DMSO (Fig. 5). DMSO is commonly used as a solvent in biological science and can alter cell permeability by affecting the structure and interaction of lipids in membranes (Yu & Quinn, 1998; Gordeliy et al., 1998) which may explain the release of amino acids, protein and gliotoxin observed here.

Given the previously established ability of amphotericin B to induce the release of intracellular constituents from C. albicans (Ghosh & Ghosh, 1963), it appeared that this mechanism might also be operating here to facilitate the release of gliotoxin. However, when the intracellular concentration of gliotoxin was ascertained it was found to be elevated, along with raised levels detected in the culture supernatant. In the case of MEM cultures supplemented with 0.08 or 0.16 μg amphotericin B ml⁻¹, the intracellular concentration of gliotoxin rose from 88.6 ± 4.9 ng mg⁻¹ to 217.38 ± 18.8 ng mg⁻¹ and 732.1 ± 37.9 ng mg⁻¹, respectively (Fig. 6). This effect was also apparent in RPMI-grown cultures of A. fumigatus.

The increased concentration of gliotoxin in culture supernatant may be explained by the well established ability of amphotericin B to increase the permeability of the fungal cell membrane via the creation of pores (Ghosh & Ghosh, 1963; Cohen, 1998). However, the elevated level of intracellular gliotoxin could also contribute to the higher levels detected in the culture. The ability of amphotericin B to increase the synthesis of gliotoxin may be mediated through its fungistatic abilities. When applied to cultures of C. albicans, amphotericin B halts replication but the cells remain viable (Liao et al., 1999) and the fungus may recover the ability to divide in the absence of the polyene. Halting the cell’s growth may force the production of secondary metabolites such as gliotoxin. In effect amphotericin B may have two distinct effects on the fungal cell: (i) The fungistatic effect may halt replication, leaving the cell alive but capable of synthesizing secondary metabolites such as gliotoxin; (ii) The ability of the polyene to create apertures in the fungal cell membrane allows the escape of small molecular mass compounds, including gliotoxin. The consequence of amphotericin B exposure at the cellular level would be elevated synthesis and release, via membrane apertures, of gliotoxin.

Although DMSO has a different primary effect on cell membranes (Yu & Quinn, 1998), it also induces elevated synthesis and release of gliotoxin. In the case of exposure of A. fumigatus to DMSO, the elevated levels of gliotoxin in the supernatant may be due to the increased permeability of the fungal cell membrane. The leakage of metabolites may hinder the cell’s ability to divide, temporarily allowing it to synthesize secondary metabolites such as gliotoxin.

The work presented here indicates that when exposed to amphotericin B in vitro A. fumigatus produces and secretes the immunosuppressive agent gliotoxin at an elevated rate. Amphotericin B therapy has many undesirable side effects (Cohen, 1998) and new formulations are designed to minimize the toxicity towards host tissue (Hartsel & Bolard, 1996). Apart from its direct effect on the host, amphotericin B may also induce the synthesis and release of secondary metabolites by A. fumigatus which may contribute to greater pulmonary damage and decreased immune function both locally and systemically. While the work presented here examined the response of A. fumigatus to amphotericin B in an in vitro model, in vivo evaluation of this phenomenon may be required to fully assess its clinical significance. Recent work has indicated that fungi can survive exposure to relatively high levels of amphotericin B, maintain certain cellular functions and potentially resume replication (Liao et al., 1999). This study has established that amphotericin B may induce the synthesis and release of gliotoxin from A. fumigatus. This phenomenon has the potential to contribute to elevated levels of pulmonary damage (Eichner et al., 1986; Sutton et al., 1996) and immunosuppression (Sutton et al., 1994, 1995) associated with this toxin.

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