Dynamics of extracellular release of *Aspergillus fumigatus* DNA and galactomannan during growth in blood and serum

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Aspergillus fumigatus is the major cause of invasive aspergillosis (IA), a disease associated with high rates of morbidity and mortality in patients undergoing treatment for haematological malignancies. This study investigated *A. fumigatus* growth *in vitro* and in a murine model of IA in order to provide insights into the dynamics of extracellular DNA and galactomannan (GM) release and their relevance to early diagnosis of IA. Following inoculation of whole blood with 20 *A. fumigatus* conidia ml⁻¹, DNA that corresponded to the inoculum could be detected by PCR but GM was not detected in plasma separated from the blood sample, indicating that the fungus did not grow in whole blood. The quantities of DNA detected by PCR, and GM, were proportional to the amount of fungal biomass present *in vitro*. Fungal DNA could be detected in the sera of mice experimentally infected with *A. fumigatus* with maximum detection in cyclophosphamide-treated mice.

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INTRODUCTION

Invasive aspergillosis (IA) is the leading cause of death due to fungal disease in patients undergoing intensive treatments for haematological malignancies, such as remission-induction chemotherapy and haematopoietic stem cell transplantation (Neofytos *et al.*, 2009). *Aspergillus fumigatus* is the species that most commonly causes IA (Alexander, 2002). A major factor in the poor outcome of this disease is its delayed diagnosis and consequent suboptimal therapy (Alexander, 2002; Perlin & Zhao, 2009).

A. fumigatus is ubiquitous in the environment and enters the respiratory system by inhalation of airborne conidia. In susceptible individuals, the fungus can become established in the lung, leading to pulmonary IA, and may progress to disseminated infection. Ideally, IA could be diagnosed using a non-invasive test but the almost invariable inability to grow the fungus from blood cultures during the course of the infection has hindered diagnosis (White *et al.*, 2009). During the course of the growth cycle, the fungus secretes a wide variety of molecules from enzymes to carbohydrates. Detection of circulating fungal antigens in serum using commercially available kits, e.g. for *Aspergillus* galactomannan (GM) or β -D-glucan, has become an accepted diagnostic

strategy (Perlin & Zhao, 2009) and these tests have been incorporated into the consensus definitions of invasive fungal disease of the European Organization for Research and Treatment of Cancer and Mycoses Study Group (EORTC/MSG) (de Pauw *et al.*, 2008). Despite there being a substantial literature on PCR for diagnosis of IA (Mengoli *et al.*, 2009), it has not been included in the EORTC/MSG definitions, as the ideal diagnostic blood sample to test and the extraction and amplification protocols to employ continue to be unclear. Whole blood or serum are the samples most commonly tested in studies evaluating *Aspergillus* PCR (White & Barnes, 2009). One potential advantage of testing serum is that no cellular DNA extraction steps are needed, and testing for GM and DNA can be achieved on the same sample (Botterel *et al.*, 2008; Suarez *et al.*, 2008).

Mennink-Kersten *et al.* (2006) found that the release of surrogate markers for IA *in vitro* was correlated with the growth phase of the fungus and was dependent on nutrient availability. A greater understanding of the dynamics of extracellular DNA release from *A. fumigatus* could assist the development of PCR diagnostics in serum.

METHODS

Fungal strain and culture conditions. A clinical isolate of *A. fumigatus* designated Sp101985 that had been recovered from a case

Abbreviations: CGD, chronic granulomatous disorder; GM, galactomannan; IA, invasive aspergillosis.

of IA was used in this study. The fungus was maintained on malt agar (Sigma-Aldrich) slopes from which conidia were harvested by inversion; concentrations of conidia were determined using a haemocytometer. Experimental cultures were grown in yeast nitrogen base medium (YNB) with amino acids, supplemented with 5 mM glucose (Sigma-Aldrich), pH 7.4 (buffered with 100 mM MOPS), in pooled human serum (Sigma-Aldrich) or in healthy volunteer donor blood. Donor blood was collected in 3 ml EDTA vacutainer tubes (Becton Dickinson) and used immediately after collection in each experiment. Prior approval was obtained from the St James's Hospital Research Ethics committee to collect and process volunteer donor blood for this research.

Extraction of extracellular DNA from samples. DNA extraction was performed using a phenol:chloroform method adapted from Schmidt *et al.* (2005). The modified protocol consisted of proteinase K (Roche) digestion at 65 °C for 45 min, followed by protein precipitation using 4.5 M NaCl. The supernatant was extracted with 1 vol. phenol:chloroform:isoamyl alcohol (25:24:1) followed by a single chloroform:isoamyl alcohol wash. DNA was precipitated by addition of 0.7 vol. cold 2-propanol and 0.1 vol. sodium acetate (3.5 M, pH 5.2) to the sample and incubated on ice for 45 min. After washing with 70 % ethanol, the pellet was air-dried and resuspended in 50 µl sterile distilled water (Sigma).

DNA clean-up of the samples extracted from serum and blood plasma was necessary using the JetQuick DNA Clean-Up Spin kit (Genomed).

Stability of free DNA in serum. Five hundred microlitre aliquots of pooled human serum (Sigma-Aldrich) and donor blood (dispensed from 3 ml EDTA vacutainer tubes) were spiked with *A. fumigatus* genomic DNA at concentrations of 300 fg ml⁻¹, 3 pg ml⁻¹ and 30 pg ml⁻¹ and incubated at 37 °C for up to 144 h. Samples for DNA detection were collected at times 0, 24, 48, 96 and 144 h. There were three replicates per sample. DNA was extracted by the phenol:chloroform method and PCR was performed with primers targeting the ITS ribosomal region.

Fungal morphotype development in different media. Conidia were inoculated into 1 ml of either serum or YNB at a final concentration of 5×10^5 ml⁻¹ and incubated at 37 °C with shaking at 200 r.p.m. Samples were taken at hourly time points over a 1-day period, fixed with 2 % paraformaldehyde (Sigma-Aldrich) and examined by light microscopy at $\times 400$ magnification. The fungal morphotype development at each time point was noted and growth in each medium was compared. This was done to see whether DNA or GM release was associated with a particular growth phase and to see whether the culture medium affected this.

Time-course experiments to measure extracellular DNA and GM release *in vitro*. Conidia were inoculated into 3 ml YNB, donor serum (Sigma-Aldrich) or donor blood (collected in 3 ml EDTA tubes) in 15 ml Falcon tubes at a final concentration of 2×10^1 ml⁻¹. The inoculum was 20 µl of a stock solution of 10^3 conidia ml⁻¹; quantitative plating confirmed a mean inoculum of $17 (\pm 2)$ conidia. The cultures were incubated at 37 °C with shaking at 200 r.p.m. for a maximum of 9 days for YNB and serum and 4 days for blood. At each time point (0, 3, 6, 14, 24, 48, 120, 168 and 216 h or 0, 3, 6, 9, 14, 24, 48, 72 or 96 h for blood), 2 ml culture medium was taken and separated into two 1.5 ml microcentrifuge tubes. Four replicate experiments were performed. The samples were centrifuged at 15 000 g for 2 min to pellet any cellular debris; the supernatant was carefully transferred to new 1.5 ml microcentrifuge tubes and stored at -20 °C for DNA extraction or GM measurement. Therefore, the extraction from the blood samples was from plasma but for the purposes of DNA extraction there was no effective difference observed between

plasma and serum. There were four replicate time-course experiments with each medium for dry weight measurement and DNA detection.

Measurement of GM release and dry weight measurement. The release of fungal GM into the medium was measured using the Platelia *Aspergillus* EIA kit (Bio-Rad) in accordance with the manufacturer's instructions. There were three replicate experiments with time points and conditions as previously described. A GM index value greater than 0.5 was considered positive.

Fungal dry weight was measured by collecting samples onto pre-weighed Whatman number 1 filter paper. The samples were dried at 65 °C for 72 h and weights were measured in triplicate on a four decimal point balance.

Quantification by real-time PCR of DNA released by *A. fumigatus*. Real-time PCR was performed in an Applied Biosystems 7000 sequence detection system. Detection of target genes in samples from blood or serum was done using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) with 2 mM MgCl₂ and cycling as described in the manufacturer's instructions. Real-time PCR was done using ITS primers (Asp_fum_F: 5'-GCAGTCTGAGTTGATTATCGTAATC-3' and Fungi_5.8_R: 5'-CAGGGGGCGCAATGTGC-3') provided by J. Loeffler; ITS was chosen because of its high copy number and specificity (Perlin & Zhao, 2009). In preliminary studies, this ITS assay had a sensitivity of 94 % and a specificity of 100 % in donor blood samples spiked with either *A. fumigatus* or other clinically relevant fungi, with cross-reactions observed with *Aspergillus terreus* and *Aspergillus flavus*, but not with *Candida* species (J. Loeffler, unpublished data). ITS primers were used at a final concentration of 1 µM in 20 µl reactions and were synthesized by Eurofins MWG Operon. There were four replicate experiments and three PCR replicates per sample.

Real-time PCR data were expressed as *A. fumigatus* genomes ml⁻¹ (one genome being equivalent to 30 fg DNA) based on comparison of samples to a standard curve of *A. fumigatus* genomic DNA that was included on each PCR plate. The standard curve was a dilution series over eight logs and the typical slope of the linear regression of the data points was -3.4 (96 % PCR efficiency).

Detection of *A. fumigatus* DNA in sera from infected mice. Mice were used as an *in vivo* model of IA to compare the findings with the *in vitro* experiments using serum. In this model, it was possible to compare sera from infected mice with cyclophosphamide-induced neutropenia and sera from infected chronic granulomatous disorder (CGD) mice. Eight- to ten-week-old C57BL/6 wild-type mice were purchased from Charles River or Jackson Laboratories. Breeding pairs of homozygous *p47phox*^{-/-} mice raised on a C57BL/6 background (Romani *et al.*, 2008) were bred under specific pathogen-free conditions at the breeding facilities of the University of Perugia, Perugia, Italy. Experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143-01. The infecting *A. fumigatus* strain was obtained from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of the University of Perugia (Romani *et al.*, 2008). For infection, mice were anaesthetized by i.p. injection of 2.5 % avertin (Sigma-Aldrich) before intranasal instillation of a suspension of 2×10^7 resting conidia in 20 µl saline for 3 consecutive days; mice were sacrificed at 24, 72 and 168 h after inoculation. Cyclophosphamide 150 mg kg⁻¹ i.p. was given a day before the infection to the C57BL/6 group. No immunosuppression was given to the CGD mice. Blood was collected from anaesthetized mice by cardiac puncture; the blood was allowed to clot for 1 h at room temperature and then stored overnight at 4 °C. The serum was then pipetted off the clot, placed into a clean test tube and clarified by centrifugation at 3000 g for 10 min.

DNA was extracted from serum samples (starting volume varied between 100 and 200 µl) by the phenol:chloroform method having

first adjusted them to 200 μl with sterile water (Sigma-Aldrich). DNA was quantified by real-time PCR using ITS primers as previously described; there were three samples per treatment. There was insufficient material to measure GM.

Statistical analysis. Quantitative data for the *in vitro* and *in vivo* experiments were compared by one-way ANOVA for each dataset followed by Dunnett's test using Graphpad Prism Version 5.02 for Windows.

RESULTS AND DISCUSSION

PCR performance and stability of *A. fumigatus* DNA in serum

The standard curve for real-time PCR was reproducible to a detection limit of 0.3 *A. fumigatus* genomes ml^{-1} , equivalent to approximately 10 fg genomic DNA ml^{-1} .

A. fumigatus genomic DNA that was spiked into serum and blood could be detected at all time points up to 144 h by PCR, with PCR product C_t values increasing with incubation time. There was greater degradation of DNA in blood, with only 30 pg *A. fumigatus* DNA ml^{-1} detectable at 144 h, whereas the DNA spiked at concentrations of 300 fg ml^{-1} , 3 pg ml^{-1} or 30 pg ml^{-1} was detectable in serum at 144 h. *Candida* DNA has also been shown to be stable in plasma for up to 72 h (Kasai *et al.*, 2006). This demonstrates the potential stability of extracellular fungal DNA in serum and blood.

In vitro growth and development of *A. fumigatus*

The rate of fungal development did not vary between the media tested. The fungus developed from resting conidia to germ tubes within 6 h and to mycelium within 24 h of inoculation (data not shown). The growth dynamics of *A. fumigatus* in serum were similar to those in YNB (Fig. 1), with the exponential growth phase from 48 h to 120 h for both, but more biomass was produced in serum over 216 h than in YNB, indicating a greater nutrient availability in serum, which is consistent with previous findings (Gifford *et al.*, 2002). The earlier peak in the growth cycle in YNB (120 h) compared to serum (168 h) may have been due to nutrient exhaustion. There was no evidence of fungal growth in blood and this was confirmed by PCR (Fig. 2).

Detection of extracellular DNA and GM from *A. fumigatus* grown *in vitro*

Cultures of *A. fumigatus* released genomic DNA and GM during the exponential growth phase. DNA was detected after 48 h ($P < 0.05$, Dunnett's test comparing all time points to T0 h) in culture supernatants of *A. fumigatus* grown in YNB and serum, and after 72 h ($P < 0.05$, Dunnett's test comparing all time points to T0 h) in whole blood (Fig. 2).

The data for real-time PCR from cultures grown in YNB medium and serum (Fig. 2) closely resembled the dry

Fig. 1. Dry weight measurements of *A. fumigatus* grown in YNB medium (pH 7.2) (●) or human serum (○) over 9 days. The inoculum density was 2×10^1 conidia ml^{-1} . Means $\pm$ SEM are shown on the chart for each data point; $n=4$. There was more biomass produced in serum than in YNB medium ($P < 0.05$ by *t*-test).

weight data (Fig. 1). Maximum DNA release was at 120 and 168 h, which were the time points at which the maximum dry weight was measured. The GM index (Fig. 3) reached a maximum intensity at 48 h in YNB and serum but the absence of a significant GM signal from the conidia inoculated into whole blood indicated that there was no or very limited fungal growth in the blood samples. The detection of GM can be impaired/inhibited by antifungal treatment in patients, which can also result in the detection of DNA in the absence of GM detection (Cuenca-Estrella *et al.*, 2009). This indicates an association between viable *A. fumigatus* and the production of GM.

The release of DNA from conidia incubated in whole blood was maximum between 72 and 96 h but the amounts were low, approximately 500 fg ml^{-1} (approximately 17 genomes ml^{-1}), and corresponded to the initial inoculum of 20 conidia ml^{-1} (Fig. 2) confirming the GM data (Fig. 3). This demonstrates that DNA from the fungus could be detected in the absence of any apparent growth. Growth inhibition in blood may have been due to the low number of inoculated conidia being processed by surviving immune cells since fungal DNA was only detected after the blood cells underwent autolysis; alternatively *A. fumigatus* may not be viable in whole blood as suggested by the infrequency of its recovery from blood culture samples (White *et al.*, 2009). There is no evidence that conidia circulate in the bloodstream but this experiment demonstrates that fungal elements present in blood could yield detectable amounts of extracellular DNA.

Our results for the detection of extracellular *A. fumigatus* antigens *in vitro* are consistent with the findings of Mennink-Kersten *et al.* (2006), who found that maximum release of GM and DNA was correlated with increased biomass, with GM being detectable earlier than DNA. In

Fig. 2. Quantification of DNA release from *A. fumigatus* grown in YNB medium (pH 7.2) (shaded bars), human serum (black bars) and donor human blood (dotted bars) by real-time PCR targeting the ITS ribosomal region. Data values were derived by extrapolating C_t values from a standard curve of quantified *A. fumigatus* genomic DNA (1 genome ≈ 30 fg genomic DNA). No samples from serum or YNB were processed at 72 and 96 h. Data shown are means $\pm$ SEM for each sample; $n=12$. Asterisks over a bar indicate $P < 0.05$ by Dunnett's test; all time points compared to T0 h for each treatment.

contrast to their study, we showed that DNA could be detected in the exponential phase of the growth cycle as early as 2 days into the 9-day growth period. This difference was probably attributable to our significantly lower inoculum, 20 conidia ml^{-1} compared to 10^6 conidia ml^{-1} , and our use of real-time PCR, which is more sensitive than conventional PCR. The kinetics of DNA release from *Candida* (Kasai *et al.*, 2006), where it could be detected at 24 h in minimal medium, is also consistent with our findings. The differences in the timing of DNA release between the two fungi were probably due to the differences in the growth of the organisms and the inoculum size: 1×10^7 c.f.u. *Candida* ml^{-1} compared to 20 *A. fumigatus* conidia ml^{-1} .

The reasons for the extracellular release of DNA from *A. fumigatus* are varied. Stationary phase cultures undergo apoptosis (Mousavi & Robson, 2003) and cellular breakdown, which leads to the release of genomic DNA. DNA release prior to stationary phase cellular breakdown may be an active process related to biofilm formation; biofilm-forming microbes use DNA as a structural component of the extracellular matrix of the biofilm (Izano *et al.*, 2008). It has been demonstrated that *A. fumigatus* forms biofilms (Mowat *et al.*, 2009) and may secrete DNA when hyphal networks are forming. This suggests that circulating DNA may be a consequence of active fungal growth and not just a breakdown product. The *in vitro* data from YNB, serum and whole blood (Fig. 2) support the possibility that extracellular DNA can be generated by an active mycelium and by the action of immune cells.

In blood, apoptosis of macrophages can lead to the release of fungal DNA that dendritic cells can take up to initiate an immune response in a process known as cross-presentation. This has been demonstrated for both *Candida* (Miyazato *et al.*, 2009) and *Histoplasma* (Lin *et al.*, 2005) DNA.

Detection of *A. fumigatus* DNA in murine sera

The murine experiment was conducted to see whether the detection of extracellular DNA *in vitro* would be reproduced in an *in vivo* model of IA. *A. fumigatus* DNA could be detected in the sera of cyclophosphamide-treated mice at a level significantly greater than in the infected and uninfected control mice or infected CGD mice (Fig. 4). The amount of *A. fumigatus* DNA in sera from the infected controls and the CGD mice was not significantly different from that from the uninfected controls ($P > 0.05$, Dunnett's test comparison of all treatments to uninfected control treatment). This suggests that *Aspergillus* conidia were cleared efficiently by healthy mice while fungal growth was limited in CGD mice (Zarembek *et al.*, 2007). It has been proposed that the major

Fig. 3. Measurement of GM release from cultures of *A. fumigatus* grown in YNB medium (pH 7.2) (●), human serum (○) or human blood (▲). The inoculum density was 2×10^1 conidia ml^{-1} . Means $\pm$ SEM are shown on the chart for each data point; $n=4$. The amounts of GM detected in YNB medium and serum were significantly greater than in blood ($P < 0.05$ by one-way ANOVA).

Fig. 4. Quantification of *A. fumigatus* DNA detected in murine serum samples by real-time PCR targeting the ITS ribosomal region. Data values were derived by extrapolating C_t values from a standard curve of quantified *A. fumigatus* genomic DNA (1 genome=approximately 30 fg genomic DNA). Data are shown for each mouse category as mean $\pm$ SEM for each sample; $n=3$. The one-way ANOVA P -value for the dataset was 0.032; asterisks over horizontal bars indicate $P < 0.05$ by Dunnett's test. No samples were available at 168 h for cyclophosphamide-treated (Cy) mice and CGD mice due to mice dying and no sample material was available for Cy mice at 24 h.

cause of mortality in CGD mice is an excessive inflammatory response rather than an extensive invasive infection (Romani *et al.*, 2008) and this would explain the low levels of fungal DNA detected in samples from CGD mice. Cyclophosphamide-treated mice exhibit the typical pathological features of an invasive mycosis (Stephens-Romero *et al.*, 2005) and would be expected to have a relatively high fungal burden compared to the control and CGD mice. In a murine model of IA, real-time PCR data corresponded to the murine fungal burden determined by colony counts, with peak fungal burden coinciding with the highest mortality rate (Bowman *et al.*, 2001). This is supported by data from *Candida* infection of experimental rabbits, where extracellular fungal DNA could be detected in serum with the amount of fungal DNA increasing as the disease progressed (Kasai *et al.*, 2006). Extrapolating from this, the detection of fungal DNA in murine sera suggests a significant fungal burden in the host organism.

Conclusions

Extracellular *A. fumigatus* genomic DNA and GM were detected in the growth medium during the exponential and stationary phases of the *A. fumigatus* growth cycle, and *A. fumigatus* DNA could be detected in the sera of immunosuppressed mice with IA. The finding that *A. fumigatus* does not grow but can be detected in blood samples corroborates the observation that it is rarely recovered from blood cultures (White *et al.*, 2009). These observations support the diagnostic potential of PCR combined with GM detection for diagnosis of IA from serum, as recommended in several studies (Cuenca-Estrella *et al.*, 2009; Suarez *et al.*, 2008). Using the same sample for

both tests would simplify sample handling at the point of care and make serum a suitable sample to aid in diagnosis of IA (Suarez *et al.*, 2008).

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