Correlation between in vitro growth rate and in vivo virulence in Aspergillus fumigatus

DEREK PAISLEY*, GEOFFREY D. ROBSON† & DAVID W. DENNING**

*Clinical Sciences Building, Hope Hospital, Salford, †School of Biological Sciences, University of Manchester and ‡Education and Research Centre, Wythenshawe Hospital, Manchester, UK

We describe a kinetic microbroth method of measuring the growth rate of Aspergillus fumigatus spectrophotometrically. Using this method, growth rates were determined for nine A. fumigatus isolates for which an LD₉₀ value in immunosuppressed CD-1 mice had previously been obtained. Comparison of the growth rates and LD₉₀ values of these isolates suggests that a correlation exists between the two parameters.

Keywords fitness, amphotericin, resistance, genome

Introduction

Previous studies have suggested that reduced virulence in Aspergillus fumigatus is associated with reduced growth rate. For example, it was found that double mutation of the chsC and chsG chitin synthase genes in A. fumigatus led to a reduced colony radial growth rate as well as a decrease in mortality and a delay in onset of illness in a murine model of invasive pulmonary aspergillosis [1]. In separate studies, A. fumigatus mutants deficient in either chitin synthase gene chsD or chsE were generated. Despite having morphological abnormalities that included hyphal swelling, both mutant strains showed no changes in colonial growth rate accompanied with normal virulence in a similar murine model of invasive pulmonary aspergillosis [2,3]. A tentative correlation between growth rate and virulence in A. fumigatus has therefore previously been demonstrated.

Numerous direct and indirect methods have been employed to measure the growth rate of filamentous fungi including determination of hyphal length and branching [4,5] dry weight, chitin, ergosterol, protein and ATP determination [6,7].

The spectrophotometric measurement of turbidity has also been employed as it allows the non-destructive direct estimation of biomass levels to be made repeatedly over time in the same sample [8]. However, the application of spectrophotometry to the measurement of growth of filamentous fungi has been regarded as unsatisfactory because the morphology of these species provides no identifiable growth unit. Advances in spectrophotometry that enable small changes in absorbance of microcultures in a 96-well plate format to be detected have, however, facilitated the improvement of this methodology. After demonstrating a correlation between absorbance of microbroth suspensions and mycelial weight for a variety of filamentous fungi, Granade et al. described a method of spectrophotometric measurement of filamentous fungal growth in a 96-well plate microbroth format [9]. More recently, Meletiadis et al. used a similar method to analyse the growth characteristics of three species of filamentous fungi with the intention of optimising methodologies for antifungal susceptibility testing [10,11].

Here we describe a modified microbroth method for kinetic measurement of A. fumigatus growth. We selected nine A. fumigatus isolates for which virulence data, in the form of the LD₉₀ dose in a CD1 mouse model of invasive aspergillosis, was already known. Our microbroth method was used to measure the growth rates of these isolates allowing us to demonstrate a correlation between A. fumigatus growth rate and virulence.
Materials and methods

Isolates

Nine clinical isolates of the filamentous fungus *A. fumigatus* were selected, Af10, Af65, Af71, Af72, Af90, Af91, Af210, Af293 and Af294. Isolates were revivied from liquid nitrogen storage by subculturing on Sabouraud dextrose agar (Sab) (Oxoid, Basingstoke, UK) for 7 days at 37°C. Conidia and spores were then collected into PBS/0.05% Tween-20 using a cotton swab. Concentrations of the suspensions were determined using a haemocytometer and viable counts confirmed by plating serial dilutions for each suspension.

Measurement of growth rates

Spore suspensions were inoculated into Sab liquid medium (Oxoid), RPMI-1640 medium (RPMI) (Sigma, Poole, UK) or yeast peptone dextrose (YPD) broth (Becton Dickinson, Oxford, UK) to produce the spore concentration required. The spore suspension in broth was pipetted into 96-well microtitre plates in quadruplicate. Plates were then sealed using Breathe Easy gas-permeable sealing membranes (Diversified Biotech, Boston, MA). The optical density (OD) at 405 nm was determined for each well using a haemocytometer and viable counts confirmed by plating serial dilutions for each suspension.

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Results

Development of microbroth method for growth-rate measurement

We wanted to assess the growth characteristics of a variety of *A. fumigatus* isolates by using a microbroth kinetic method based upon absorbance measurement at 405 nm over a 24-h period. To determine the most suitable microbroth conditions for this, growth curves were obtained for the isolate Af293 under a variety of different conditions (Inoculum: 10^4, 10^5 or 10^6 total spores per well; final volume per well: 50, 75, 100 or 125 μl; media: RPMI, Sab or YPD) and compared.

Growth in all volumes of RPMI with each concentration of inoculum was minimal. Both Sab and YPD gave more conventionally shaped growth curves (i.e. lag phase, first transition period, log phase, second transition period, stationary phase) but only when a volume of 50 μl per well was used. Growth curves in Sab appeared more uniform than those in YPD, with the second transition period in particular being more regular in shape (see Fig. 1). Specific growth rate (h⁻¹) was calculated for each of the growth curves obtained.

Values for Sab medium spore concentrations of 10^4 and 10^5 spores per well were greater than at a concentration of 10^6 spores per well. We thus decided to routinely measure growth using 10^5 spores in 50 μl Sab medium (see Fig. 1, expanded panel) as these conditions gave us uniformly shaped growth curves and allowed the measurement of specific growth rate values with a high level of sensitivity.

Correlation of *A. fumigatus* growth rates with LD90 in mice

Using the microbroth conditions described above we measured the growth characteristics of nine isolates of *Aspergillus fumigatus* for which the LD90 dose in CD1 mice had previously been determined. Growth was measured in quadruplicate in three separate experiments and the average specific growth rate across these experiments calculated (see Table 1). This enabled the isolates to be compared and ranked according to their growth rate (e.g. highest specific growth rate first, see Table 1). Isolates were also ranked according to their LD90 value (e.g. lowest LD90 ranked first, see Table 1).

The rank order of LD90 value was plotted against the rank order of average specific growth rate and regression analysis performed (Fig. 2). The correlation between these two parameters for the nine isolates was poor (r² = 0.1841). However, removal of Af65...
amphotericin B resistant) prior to ranking and regression analysis of the isolates (Table 1, Fig. 2) substantially improved the correlation between rank order of LD₉₀ and rank order of average specific growth rate ($r^2 = 0.6315$).

Discussion

We describe here a method for measuring the growth of A. fumigatus using spectrophotometric absorbance measurements of microbroth cultures in a 96-well plate format.
Indirectly measuring the growth of filamentous fungi by spectrophotometry has been regarded as unsatisfactory because the morphology of these species provides no identifiable growth unit. Studies by Meletiadis et al. have, however, described a correlation of a spectrophotometric method of growth measurement, with direct assessment of growth by measuring hyphal extension [10,11]. The method used in this instance is comparable to that described here.

Using this method, we have demonstrated a correlation between the specific growth rate (h⁻¹) and the virulence (as defined by the LD₉₀) of eight different A. fumigatus isolates. The correlation was substantially improved following the removal of Af65 prior to ranking and regression analysis of the isolates. Despite being amongst the least virulent of the isolates examined, Af65 was found to have the second highest average specific growth rate. It has been demonstrated that drug resistance in fungi can be associated with a reduction in fitness when compared with drug-sensitive organisms. In the yeast Candida albicans, populations evolved in the presence of fluconazole were found to diverge in fitness with a number of drug-resistant populations showing a significant reduction in fitness [18]. Furthermore, for a variety of a number of bacteria and viruses, drug resistance has been associated with a reduction in virulence in animal infection models as well as in other measures of fitness [19–21]. It is possible that the absence of a correlation between the growth rate and virulence of Af65 observed here could be related to a reduction in the ‘fitness’ of this isolate resulting from resistance to amphotericin B.

The finding that reduced virulence of A. fumigatus is associated with reduced growth rate means that genes involved in promoting the growth of this organism may be important virulence determinants. Some evidence for this exists. For example the rhbA, a gene implicated in nitrogen sensing, has been found to affect the growth rate of A. fumigatus and is also a virulence-determining factor [22]. Cyclic Amp signalling has also been shown to affect A. fumigatus growth rate and virulence [23,24].

Endogenous hypercortisolaemia or pharmacological doses of corticosteroid have been identified as a risk factor for disseminated aspergillosis [25,26]. This was thought to be due to inhibitory effects of corticosteroid upon the anti-fungal actions of monocytes [27] and
tissue macrophages [28]. However, Ng et al. have reported that corticosteroid promotes the growth of *A. fumigatus* in vitro [5]. The suggestion that increased growth rate is associated with increased virulence, could therefore provide an alternative hypothesis for the mechanism by which corticosteroids increase the risk of contracting disseminated aspergillosis. This in turn may present a novel target for the future development of anti-*Aspergillus* agents.

The growth rate of *A. fumigatus* thus appears to be an important parameter influencing the pathogenesis of what is now one of the most prevalent airborne fungal pathogens. The link between growth rate and virulence thus warrants further investigation.

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**References**


