Human albumin promotes germination, hyphal growth and antifungal resistance by *Aspergillus fumigatus*

ACACIO GONÇALVES RODRIGUES†, RICARDO ARAÚJO† & CIDALIA PINA-VAZ‡

†Department of Microbiology, Faculty of Medicine, University of Porto, Alameda Prof. Hernani Monteiro, 4200-319, Porto, ‡Department of Anesthesiology and Intensive Care, Faculty of Medicine and Hospital S. João, Alameda Prof. Hernani Monteiro, 4200-319, Porto, and †IPATIMUP – Institute of Pathology and Molecular Immunology of University of Porto, Rua Roberto Frías, 4200-319, Porto, Portugal

Invasive aspergillosis is one of the most common deep-seated fungal infections among patients with an impaired immune system. Albumin is a serum protein commonly administered to critical patients. Our objective was to evaluate the *in vitro* effect of human albumin upon germination and hyphal growth of *Aspergillus* species, especially the most pathogenic, *Aspergillus fumigatus*, as well as its influence on antifungal drug activity. Human albumin induced, at normal serum concentrations (2–4%), a significant promotion of conidial germination by *A. fumigatus*, but not by *Aspergillus flavus* and *Aspergillus niger*. However, mycelium growth following germination was enhanced in all *Aspergillus* species. Minimal inhibitory concentrations (MIC) of all strains tested to amphotericin B and itraconazole increased in the presence of physiological concentrations of human albumin. Voriconazole activity was not, however, significantly affected by the presence of the protein. Conidial germination represents a crucial initial step in the progression to invasive disease, involving metabolic pathways that may differ considerably among *Aspergillus* species. Our results support the concept that human albumin may promote a faster onset and enhanced dissemination of invasive aspergillosis.

**Keywords** *Aspergillus*, albumin, germination, mycelium formation, antifungal activity

---

**Introduction**

*Aspergillus fumigatus* is among the fungal species responsible for the significant increase of fungal infections registered in the last decades. *A. fumigatus* is at present responsible for over 90% of all human *Aspergillus* infections [1]. Its medical relevance is justified by the high mortality of invasive aspergillosis (estimated between 13% and 95%, despite treatment), particularly in patients with an impaired immune system [2]. Neutropenic, bone marrow and other solid organ transplant patients are the most common targets of invasion by this organism [2–4]. Therapeutical approaches usually involve the use of amphotericin B or alternatively the use of itraconazole and more recently voriconazole and caspofungin [5–7].

Virulence factors and mechanisms responsible for *A. fumigatus* infection have not been yet fully characterized, although progressive advances have been registered in recent years [8,9]. D’Enfert et al. [10] referred a *pyr*G mutant unable to germinate without uridine and uracil and Brown et al. [11] described an auxotroph unable to germinate in the absence of p-aminobenzoic acid. Both situations affected the morphogenesis of strains and germination was considered the crucial step reducing virulence of strains. Differences regarding the germinative potential among *Aspergillus* species have been noted...
been described but not yet related to pathogenicity [12,13]. Moreover, individual risk factors, such as neutropenia and corticosteroid therapy, may be considered to act synergistically with fungal virulence traits [8,9,14–16].

A wide array of factors may thus influence the germination and growth of Aspergillus. Therefore, it is of relevance to evaluate carefully specific factors affecting germination of fungal conidia, other than the use of corticosteroids. Albumin, a human plasma protein, accounts for nearly 50% of plasma proteins and plays multiple physiological roles. Human albumin solutions are used clinically in a variety of procedures and disease states, namely as a volume expander for hypovolemia, in cirrhotic patients, in various oedematous states, acute management of burns and as an adjunct during cardiopulmonary surgery [17,18]. This array of indications justifies the widespread use of albumin solutions in patients admitted in intensive care units and others with critical clinical conditions. In patients with acute or chronic illness, serum albumin concentration is inversely related to risk of death [19]. However, increased permeability of the vessels due to direct cellular damage and inflammatory mediators raised questions about the rationale of albumin administration [20,21]. It has been recently postulated that albumin might be responsible for six extra deaths per 100 patients treated [22]. Previous reports suggested that albumin may enhance the pathogenesis of Candida albicans by promoting germ tube formation of this fungal agent [23], but did not affect its susceptibility to itraconazole and ketoconazole [24]. Additionally, bovine albumin stimulated the in vitro growth of A. fumigatus [25].

The objective of the present study was to evaluate the effect of human albumin upon conidial germination and hyphal development by A. fumigatus, A. flavus and A. niger, the three most important pathogenic species of Aspergillus. Furthermore, the influence of human albumin upon the in vitro activity of antifungal agents commonly used in the treatment of invasive aspergillosis was elucidated.

**Materials and methods**

**Organisms and growth conditions**

Clinical isolates of A. fumigatus (5 strains), A. flavus (3 strains) and A. niger (3 strains), from the fungal collection of the Department of Microbiology of the Faculty of Medicine, University of Porto, were used in these studies. Fungal conidia were kept frozen at −70°C, in Brain-Heart infusion (Difco, Detroit, MI, USA) broth with 5% glycerol, before testing. After thawing and assuring the purity of the cultivated strains, organisms were grown on Sabouraud agar slants (Difco) at room temperature (20°C), for 5 and 11 days. Conidia were harvested by flooding the agar surface with a phosphate buffered saline solution (PBS; Sigma, St Louis, USA), filtered and resuspended in PBS. The density of conidia suspensions was evaluated by haemocytometer counts and adjusted to a concentration of 2.5 × 10⁶ conidia/ml with sterile PBS.

**Chemicals**

Human albumin 20% (Albuman®) was purchased from Octapharma (Octapharma Pharmazeutika, Vienna, Austria). Bovine albumin, RPMI 1640 culture medium and all other chemicals (analytical grade) were purchased from Sigma. Amphotericin B was obtained from Pharmatek (Huntington, NY), itraconazole was a kind gift by Janssen-Cilag (Sanderton, UK) and voriconazole was graciously donated by Pfizer, Inc. (New York, NY).

**Germination assay**

Following a previously standardized procedure [12], Aspergillus conidia (final concentration 5 × 10⁵ conidia/ml) were suspended in RPMI 1640 medium, containing 2% and 4% human or bovine albumin. Controls were prepared in RPMI 1640 medium without albumin. All samples were incubated at 37°C for up to 12 h. The percentage of germination was determined hourly by evaluating the formation of germ tubes, i.e. a projection that was two times longer than the conidial diameter, of 200 conidia as evaluated under phase contrast microscopy (400 ×). All tests and determinations were run in triplicate.

**Hyphal growth assessment**

Suspensions of 1 × 10⁶ and 5 × 10⁴ conidia/ml were prepared in RPMI 1640 medium from which 100 μl aliquots of each suspension were inoculated in wells of a 96 well microplate. Serial dilutions of human albumin were prepared in sterile saline solution and 100 μl aliquots of each were added to the conidial suspensions, giving a final concentration of human albumin ranging from 0.02 to 10%. A positive control (conidia suspension plus saline solution) and a negative control (plain RPMI 1640 medium plus human albumin) were included in each experiment. Microplates were incubated at 37°C for up to 72 h. Absorbance was evaluated at 450 nm (differential filter of 630nm), in a microplate reader (Awareness Technology, Palm City), with all tests run in duplicate.

© 2005 ISHAM, Medical Mycology 43, 711–717
Antifungal activity assay

The MIC of all fungal strains to amphotericin B, itraconazole and voriconazole were determined in RPMI 1640 medium, following the NCCLS M38-A protocol [26]. Similar studies were simultaneously conducted with RPMI 1640 containing 2 and 4% of human albumin. All MIC were read following incubation for 48 and 72 h at 37°C and tests were run in duplicate.

Statistical analysis

Excel 2000 (Microsoft Corp., NY) and SPSS 11.5 (SPSS Inc., Chicago, IL) applications were used for data elaboration and analysis. The Wilcoxon signed rank test and Student’s t-test for paired samples were used for statistical analysis. Data were compared at significance level of 0.05.

Results

Germination of conidia and hyphal growth

Human albumin significantly promoted the germination rate of all tested strains of *A. fumigatus*, particularly following 6–8 h of incubation, a time interval corresponding to the critical period of exponential germination of its conidia. Germination of 5-day-old conidia of *A. fumigatus*, following 6 h of incubation, increased 30 and 50% in presence of 2 and 4% of human albumin, respectively (Fig. 1). Figure 2 depicts enhanced conidial germination by *A. fumigatus* in presence of human albumin (2 and 4%). Less significant enhanced germination was found with 11-day-old conidia of *A. fumigatus* (data not shown).

Conversely, the germination of *A. flavus* and *A. niger* was reduced in presence of human albumin. Following 11 h of incubation, the germination of 5-day-old conidia of *A. flavus* decreased by 20 and 25% in presence of 2 and 4% of human albumin, respectively (Fig. 1), with considerably shorter germ tubes in comparison to control (Fig. 2). Five-day-old conidia of *A. niger* experienced a reduction in germination of around 25% in presence of 2% and 4% of human albumin (Fig. 1). No differences were found in the germination of 5- and 11-day-old conidia of *A. flavus* and *A. niger* in the presence of human albumin.

Similar to the effect of human albumin, bovine albumin induced a significant promotion of germination by *A. fumigatus* after 5 h incubation. Reduced germination of around 15% of all tested strains of *A. flavus* and no significant interference with germination of *A. niger* (data not shown) were found with bovine albumin.

Increases in the concentrations of human albumin correlated with enhanced absorbance readings in microplate cultures of *A. fumigatus* (A), *A. flavus* (B) and *A. niger* (C), at 37°C. (black bars-control; grey bars–2% human albumin; white bars–4% human albumin)

Fig. 1 Effect of human albumin on conidial germination (mean percentage ± standard error of mean) of 5-day-old conidia of *Aspergillus fumigatus* (A), *Aspergillus flavus* (B) and *Aspergillus niger* (C), at 37°C. (black bars-control; grey bars–2% human albumin; white bars–4% human albumin)
16 h incubation. Dose-dependent hyphal growth was also found after 24 h in all tested strains of *A. flavus* and *A. niger* (Fig. 4).

The formation of conidiophores and maturation of conidia by *A. fumigatus* was also faster in the presence of human albumin. Thus, it was possible to visualize macroscopically mycelium after 20 h, while conidia, which provide the characteristic blue colour to culture, could be observed soon after 48 h incubation. Conversely, 36 and 72 h were needed to detect similar features in positive controls. *A. flavus* and *A. niger* presented a similar behaviour to *A. fumigatus* regarding these characteristics (Fig. 5).

The described effects of albumin were independent of conidial inoculum concentrations of $2.5 \times 10^4$ and $5 \times 10^5$ conidia/ml (data not shown).

**Antifungal activity**

The effect of human albumin upon the *in vitro* activity of antifungals with fungicidal activity against *Aspergillus* species, e.g. amphotericin B, itraconazole and voriconazole, is shown in Table 1. MIC values, evaluated after 72 h of incubation, matched or differed in just one single dilution from the MIC values evaluated after 48 h, in the control and experimental wells containing human albumin. No trailing behaviour was detected among the tested strains. Albumin was associated with a systematic increase of one to three dilutions of the MIC to amphotericin B and itraconazole in all tested strains of *Aspergillus*. Such differences were significant for both antifungal agents. However, MIC values of all strains to voriconazole did not change significantly, after 48 h, in the presence of the tested concentrations of human albumin ($P > 0.05$).

**Discussion**

In a previous work, we reported that *A. fumigatus* presented faster conidial germination in comparison to other *Aspergillus* species under human internal milieu conditions [12]. Thus, the promotion of germination by *A. fumigatus* may lead to a faster formation of

![Fig. 2 Conidial germination by 5-day-old conidia of *Aspergillus fumigatus* strain F16 (left column) and *Aspergillus flavus* strain F02 (right column), following 6 and 11 h, respectively, of incubation in presence of 2% (A, D) and 4% (B, E) human albumin; (C, F) control.](image)

![Fig. 3 Absorbance readings taken from culture of *Aspergillus fumigatus* (all strains) in presence of serial dilutions of human albumin, after incubation for 16, 24 and 36 h at 37 °C. (neg – negative control; pos – positive control.)](image)
mycelium and possibly to more severe invasive disease, at least to a faster onset of tissue invasion. However, the link between conidial germination, hyphal growth and pathogenicity of *A. fumigatus* in comparison with other *Aspergillus* species still remains unclear. There is no doubt that germination represents a crucial step in the development of mycelium and expression of virulence [10,11,13]. Natural differences in germination characteristics described among *Aspergillus* species [12] were stressed in the present study of human albumin. This protein, at concentrations of 2 and 4%, which corresponds to the physiological serum content and other human fluids, promoted significantly the germination of *A. fumigatus*, but delayed considerably the germination of *A. flavus* and *A. niger*. Several distinct mechanisms are involved in the germination process [13,27]. Such pathways seem to be considerably distinct among *Aspergillus* species, therefore supporting not only a native faster germination in the case of *A. fumigatus*, but also a distinct behaviour in the presence of specific factors.

Following initial germination, human albumin promoted hyphal development of all the three *Aspergillus* species along a wide range of concentrations, e.g. supporting a faster growth and maturation of mycelium. In fact, hyphae developed significantly faster, forming both microscopic and macroscopic mycelium sooner, as well as conidiophores and mature conidia. In accordance with our results, Gifford et al. stated that bovine serum albumin stimulated both growth and proteinase secretion by *A. fumigatus* [24].

The nutrient medium is a major factor influencing fungal growth and consequently the result of susceptibility tests. All our assays were conducted in RPMI 1640 medium, as it provides adequate germination and growth of the organisms [28] and is the recommended medium by NCCLS M38-A protocol [25]. Comparing the inhibitory effect of amphotericin B and itraconazole upon pathogenic *Aspergillus* species, we verified that susceptible strains become increasingly resistant in vitro in the presence of human albumin. Thus, it seems plausible that clinical or environmental strains, already presenting high MIC values can potentially become more dangerous, as a result of further increases of MIC values of antifungals. Voriconazole activity remained, however, relatively stable. This fact adds against the presumable assumption that albumin may act as an unspecific stimulator of fungal growth, that is the higher the growth, the higher the amount of antifungal drug needed to kill the mycelium. In fact, an active synthesis generally provides a larger number of targets to an active antifungal agent. A plausible hypothesis to
explain such discrepancy among activity of antifungals may result from protein binding, which is very high for amphotericin B and itraconazole, and considerably lower (around 50%) for voriconazole.

According to our results albumin can be involved in promotion of virulence by A. fumigatus, namely following conidia germination and hyphal development. Additionally, our study raises the issue of its impact upon antifungal activity of antifungal drugs. In this way, albumin may possibly be involved, if not in the genesis, in the spreading of invasive aspergillosis, among critical patients receiving intravenous infusions of such protein, as well as in neutropenic and other recently iatrogenical immunosuppressed patients, with normal serum content of this protein. While the clinical relevance of our results needs yet to be ascertained, our results ultimately support the concept that human serum may increase the pathogenic potential of specific fungal agents, namely promoting the A. fumigatus germination process, a crucial step in pathogenesis and progression to tissue invasion.

### References


