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Effect of sub-inhibitory concentrations of amphotericin B on the yeast surface and phagocytic killing activity

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Abstract

Amphotericin B (AmB) is the major antifungal drug used for the treatment of opportunistic fungal pathogens. In this study, we investigate another more subtle effect of this antifungal than ergosterol interaction, the effect of AmB pre-treatment of several fungi on cell surface charge. Pre-treatment of the yeasts with sub-inhibitory concentrations (SIC) of AmB also affects the phagocytic killing of yeasts. Phagocytosis of yeast takes into account the cell surface properties and was greater in yeast treated by AmB than controls. In order to explain this phenomenon, the cell wall charged components were quantified and compared to the control. Results show an increase of Glc-N in cell wall treated cells and reduction of amino acids and phosphorus. In addition, the variation of cell wall components with cell surface properties and the consequences which can be generated on the phagocytosis of the yeasts have been correlated. Results show a significant difference of the zeta-potential, which is a measure of the net distribution of electrical charges of the fungal cell surface between the treated cells and the controls. Pre-treatment of the yeast by AmB produces variations of cell wall component. These events on cell wall composition are correlated to cell surface status.

Keywords: Phagocytic killing; Amphotericin B; Yeast

1. Introduction

The yeast cell surface plays a crucial role in the pathogenic yeasts. Candidiasis is an important life threatening fungal infection of immunocompromised patients and a common manifestation in patients with AIDS [1]. The *Candida albicans* cell wall is regarded as an important site that influences the virulence of this yeast [2]. The cell wall surface structure determines fungal adhesion to host substrata, presents immunomodulatory components to the host, and affects phagocytic events [3]. One of the antifungal agents used most frequently for treating candidiasis is Amphotericin B (AmB), a fungistatic drug which alters the plasma membrane by binding to ergosterol [4]. Nothing is published about the effect of AmB when its concentration is less than the concentration necessary for the fungistatic effect and it is now thought that AmB may have more than one mechanism of action on the cell walls of fungal cells [5]. There is also considerable evidence that AmB can play an immunomodulator role [6]. Morphological analyses of cells treated or not with AmB were achieved by fluorescence and scanning electron microscopy. In addition, the net distribution of electrical charges and biochemical cell wall components of cells treated or not with AmB were analyzed.

The objective of this study was to investigate the effect of AmB on yeast phagocytosis.

2. Materials and methods

2.1. Organisms and growth conditions

C. Albicans V.W 32 serotype A (Ca) was isolated from human renal candidiasis [7]. The yeast form (Ca y) was obtained after growth in Sabouraud medium (2% glucose and 1% peptone). The filamentous form (Ca f) was obtained after growth in medium 199 (Sigma) at pH 7.2 and at 37 °C for 15 h to obtain 5×10^8 to 10^9 cells ml^{-1} (Malassez counting cell) [8]. *Kluyveromyces lactis* (Kl, ATCC 96897) and *Kluyveromyces bulgaricus* (Kb, ATCC 96631), were isolated

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from dairy products. *K. lactis* mutant (Klm, ATCC 96896) [9] and *K. bulgaricus* mutant (Kbm) [10] were two amphotericin B resistant strains. Yeast cells were grown aerobically in a Sabouraud liquid medium (1% bactopeptone (Difco), 2% glucose) both in the absence (control) and in the presence of sub-inhibitory doses of Amphotericin B.

Sub-inhibitory concentration (SIC): is the concentration of AmB, that induces a lag phase of 40 h [9]. 10 mg of AmB (Squibb) were solubilized in a mixture of dimethylsulphoxide and ethanol (1:1, v/v). Various volumes from this solution were added to 20 ml of liquid media to have concentrations, which correspond to SICs of each yeast.

2.2. Fluorescence and electron microscopy

Fluorescence microscopy was performed as follows: aliquots of 10^7 cells counted in Malassez counting cell of overnight cultures in Sabouraud liquid medium were centrifuged and washed twice with deionised water. For the treated cells, yeasts were grown in the presence of the respective sub-minimal inhibitory concentration (sub-MIC) of AmB until the exponential growth phase. Aliquots of 10^7 cells of yeast grown or not in the presence of sub-MIC were suspended in 100 μl of 1 mg ml^{-1} of Calcofluor White (Sigma) then incubated for 10 min at room temperature. Aliquots were then applied on glass slides and observed with an UV filter in a fluorescence microscope (Zeiss Axioscope 50 microscope and a Zeiss MC80 camera) and counted by Malassez counting cell.

For electron microscopy, control and yeast grown in the presence of sub-MIC AmB were prepared for freeze-fracture electron microscopy as previously described [11].

2.3. Measurement of cellular charge (ζ)

The zeta-potential (ζ) is a measure of the cellular charge (in milli volts) defined as the potential gradient developed across the interface between a boundary liquid in contact with a solid and the mobile diffuse layer in the body of the liquid. It is derived from the equation $\zeta = 5(4phm)/D$, where D is the dielectric constant of the medium, h the viscosity, and m is the electrophoretic mobility of cells grown in Sabouraud medium [12]. The zeta-potential of suspensions of 10^6 cells ml^{-1} counted in (Malassez counting cell) [11], were measured as previously described [13] in *Cryptococcus neoformans* with slight modification. The size of particle (yeast) is fixed to 10 μm instead of 1 μm in the case of the bacteria.

2.4. Phagocytosis of yeast

Cells in exponential growth phase with or without AmB were harvested by centrifugation at 3000 × g at 4 °C for 5 min and washed twice at 3000 × g at 4 °C for 5 min with 5 ml PBS buffer. One milliliter of yeast grown in the presence of sub MIC AmB or not were incubated in the presence of polynuclear cells. Phagocytosis of yeasts was analysed using Phagotest Kit (ORPEGEN Pharma) with slight modification. Opsonized bacteria with fluorescein, were replaced by yeast marked by Calcofluor White. The phagocytosis of yeasts was analysed by flow cytometry in a FACS Calibur UV 295 nm using Calcofluor White as indicator. The signals of fluorescence were obtained by marking with Calcofluor White and collected on a photo multiplier FL1. Results are expressed in the form of cytogrammes with a fixed parameter: intensity of fluorescence (FL1 in ordinate) and a variable parameter: FSC or SSC.

Each yeast emits signals of light diffusion and signals of fluorescence. The signals of light diffusion forward scatter (FSC) of small angle are related to the size of the particles, while the refracted rays with 90° (great angle) constitute signal Side Scatter (SSC) correlated with the granulometry or the complexity of yeasts.

2.5. Cell wall preparations

Cells in exponential phase, grown in the presence or absence of AmB were harvested by centrifugation at 3000 × g at 4 °C at the end of exponential growth phase after 24 h culture (absorbance = 0.9 at 620 nm) and washed twice with deionized water. The cellular pellets were suspended in deionized water and crushed with glass beads (0.4 mm diameter) in a homogenizer (MSK Braun). Four successive disruptions during one minute were necessary to obtain more than 95% of disrupted cells. Cell walls were collected by centrifugation at 2000 × g for 5 min and washed at least five times with distilled water until the supernatant was clear and the broken cell walls appeared pure by optical microscopy observations (absence of whole cells). The final pellets corresponding to the isolated total cell walls (TCW) were then lyophilized. Different component were quantified [14].

2.6. Analytical procedures

Samples of 20 mg (TCW) were suspended in 2 ml of 6 M HCl and heated in sealed tubes at 100 °C for 12 h. After acid solution evaporation at very low pressure, the residues were washed in distilled water. A second evaporation led to a second residue that was dissolved in distilled water. The liberated glucosamine was then estimated following the technique described previously [15]. Free amino acids and phosphorus were measured according to the micro technique as described previously [16,17], respectively.

2.7. Poly nuclear fungicidal assay

Purified human poly nuclear macrophage cells were harvested and counted in Malassez counting cell and suspended in 10% fresh autologous serum. One milliliter of poly nuclear macrophage cells were supplemented with 100 μl of AmB treated or untreated yeast were added and then incubated at 27 °C or 37 °C with the *C. albicans* filamentous