Flow cytometry light scattering was used to monitor size increase of Candida albicans (isolate ATCC 10231) cells in the presence or absence of the antifungal drug amphotericin B (AmB). This non-invasive and descriptive method allowed for the differentiation of dead and dormant sub-populations of cells. When inoculated into a growth medium without AmB, a progressive increase in light scattering was observed over a period of approximately 4 h, but without proliferation of the yeast. After this period, the light scattering distribution regressed to baseline level, whereas cell proliferation started. In the presence of AmB, all the cells shrank in size within approximately 4 h and proliferation was temporarily halted. However, in the presence of 0.4 μM AmB, a progressive increase of light scattering occurred after 21 h which was similar to that observed within the first 4 h in the absence of the antifungal. After approximately 24 h of incubation at this concentration of AmB, proliferation resumed.

These observations indicate that this renewed cell proliferation was due to the reawakening of dormant cells in the presence of AmB (45% in the presence of 0.4 μM AmB) rather than the result of the development of viable cells that had escaped detection. This simple descriptive approach could be extended to other fungal strains or species, to other antifungal drugs and possibly to bacteria.

Keywords Candida albicans, dormancy, amphotericin B, flow cytometry

Introduction

Antifungal susceptibility testing remains dependent on colony counts of replication-competent yeast cells which requires extended incubation and provides semi-quantitative and subjective endpoints. Minimum inhibitory concentration (MIC) is generally determined by using broth microdilution methods described in the Clinical and Laboratory Standards Institute guidelines (M27-A) [1]. The endpoint is defined as the complete inhibition of visible growth after 48 h of incubation in the presence of an antibiotic. In contrast, the minimum fungicidal concentration (MFC) is a >99.9% decrease in the log_{10} level of CFU/ml as compared with starting inoculum concentration. MIC and MFC represent only average measurements of the antifungal effects on organisms which may have potentially heterogeneous responses to these agents. Therefore, studies that quantify the potential variability of fungal populations after exposure to antibiotics are warranted. Flow cytometry is a good tool to quantify such microbial heterogeneity [2,3] and has been used with fluorescent probes to qualitatively and quantitatively evaluate the characteristics essential to fungal cell viability [4–9]. However, these approaches have not yet enabled discrimination between the cellular subpopulations which can be expected to be encountered in the presence of the polyene antifungal amphotericin B.
Recently, Liao et al. [10] have used a combination of vitality- and mortality-specific dyes that monitor membrane integrity, intracellular activity and alterations in membrane potential to assess the effect of AmB on Candida albicans. It appeared that cells exposed to AmB at concentrations between 0.5 and 1 μM did not take up the stains and while they did not replicate they might have been dormant rather than non-viable. More recently [11], these authors were able to demonstrate that fungal cells treated with AmB at a concentration of 0.5 μM were resuscitated by further incubation at 22°C for 15 h.

In the present study we have used light scattering and flow cytometry (without fluorescent probe) to quantify the cellular distribution of cultured fungal cells (C. albicans isolate ATCC 10231) as a function of size and aggregation. It appeared that it was possible to demonstrate cell viability under these conditions. In fact, in the absence of AmB, subcultured stationary phase cells grew in size and aggregated before any proliferation or budding. Simultaneously, a light scattering ‘burst’ was observed over a period of 4 or 5 h after cell inoculation. The same phenomenon was observed in the presence of AmB (0.4 to 0.8 μM), but after a time lag of approximately 24 h. This was interpreted as indicating that the cells treated with AmB had been in a dormant state rather than the result of the growth of a few cells that had escaped the effects of AmB.

Materials and methods

C. albicans yeast isolate ATCC 10231 was obtained from the American Type Culture Collection (Rockville, MD., USA). Amphotericin B was a kind gift of Bristol-Myers-Squibb France (Rueil-Malmaison, France). The drug was first diluted in DMSO at a concentration of approximately 10−3 M and then spectrophotometrically adjusted to 10−4 M in DMSO (ε416 = 121 400) and used immediately. Ten mM stock-solutions of 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) purchased from Molecular Probes (Eugene, OR, USA) were prepared in DMSO.

Microscopy

Microscopic observations were carried out with a Nikon Optiphot-2 epifluorescence microscope and cells were observed directly in the growth media using a Zeiss Ultrafluar ×32 objective. Images were collected by a cooled CCD camera (Micromax; Princeton Instruments, Evry, France) with a 12-bit detector (RTEA-1317 K-1; Kodak). After storage, display and analysis were performed with IPLab software (Scanalytics, Fairfax, VA). The number and shape of cells were determined using a haemocytometer slide. Cell size (diameter) was measured directly on images by comparison with an image of a Zeiss standard (5 mm grid) slide.

Acquisition of flow cytometry data

Yeast cells grown on Sabouraud dextrose agar (Institut Pasteur, France) plates were transferred into 15 ml of yeast broth medium (YM; Difco) with a final pH of 6.2. Once a growth plateau had been reached (approximately 9 h, as determined by absorption at 595 nm, see below), the cells were incubated on a shaker at 30°C for a supplementary 15 h. Thereafter the cell concentration was adjusted spectrophotometrically to approximately 2 × 10^6 cells/mL in 10 ml of fresh YM and incubated again on a shaker at 30°C. Two hundred μl samples were taken hourly and their scattering measured with a Beckton Dickinson FACSCalibur 3C (argon laser wavelength at 488 nm) flow cytometer. Ten thousand cells per sample were analysed for forward-angle light scattering (FSC).

Susceptibility tests – Broth macrodilution method

The absorbance of samples obtained under the same conditions as those used in flow cytometry measurements was determined at 595 nm with a Cary 1E UV-visible spectrophotometer in one centimeter path cells, after dilution (1/20) in YM if necessary.

Time-kill

Every hour, 100 μl samples were removed from the YM cultures and serially diluted in cold sterile water. One hundred μl of the diluted samples were plated onto agar plates and incubated for 48 h at 30°C. Experiments were done in triplicate.

Live-dead-cell determination, using H2DCFDA and epifluorescence microscopy

Samples (in the presence or absence of AmB and after the appropriate incubation period) were directly incubated for 2 h in the presence of 250 μM H2DCFDA. Samples were viewed and photographed in a manner similar to the direct observation described above. Optical filters were used to select the excitation wavelength at 492 nm, and the emission was measured at 530 nm.

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Results

Microscope and haemocytometer studies

Samples of cell suspensions (2 × 10^6 cells/ml, initially) incubated under the same conditions as those used for flow cytometry measurements were taken hourly and microscopically examined in a haematocytometer for the first 5 h of incubation in the absence of amphotericin B. After a time lag of 1 h, the cells began to increase in size and buds began to appear. After 3 h, almost all cells had buds (some aggregation occurred to a low extent) but the number of cells per ml remained approximately constant (Fig. 1A, 1B). After 4 h of incubation, small cells began to be seen near the large ones and as a consequence, the number of cells started to increase. This process continued for 10 h of incubation, after which the appearance of the cells was the same as noted at the beginning of incubation and remained constant for the remaining 24 h of incubation (Fig. 1A). After one day of incubation, the distribution and appearance of cells changed (see below and Fig. 3, 32h), possibly due to the exhaustion of nutrients within the medium. In the presence of 0.4 μM AmB, the size of the cells decreased (Fig. 1C) and their number remained constant until 25 h of incubation. Size and number began to increase thereafter. After 27 h of incubation, the culture presented a mixture of large and small cells (Fig. 1D). For longer incubation times (32 h for instance), large cells progressively became the major constituents of the population, with an appearance similar to that observed at 0 or 21 h in the absence of AmB (Fig. 1A).

Fig. 1  Microphotographs of Candida albicans cells: (A) in the absence of AmB, after a 30 min incubation in YM (mean round-shaped cell diameter = 4.4 ± 0.8 μm (n = 30)); similar to cells observed in the absence of AmB after 10 h up to 24 h, and in the presence of 0.4 μM AmB after 32 h; (B) in the absence of AmB, after a 180 min incubation in YM (mean round-shaped cell diameter = 7.4 ± 0.5 μm (n = 30)); (C) in the presence of 0.6 μM AmB, after a 300 min incubation (mean round-shaped cell diameter = 3.3 ± 0.5 μm (n = 30)). Bar = 5 μm; (D) fluorescence microscopy in the presence of 0.4 μM AmB, after a 27 h incubation followed by a 2 h incubation in the presence of 250 μM H2DCFDA. Both transmission and fluorescence images were recorded together.
Growth as monitored by absorbance and CFU

In the absence of AmB, sample absorbance was recorded at the same time as microscopy measurements were conducted. After only slight changes during the first 2 h of incubation, absorbance grew exponentially, i.e., 3.5 and 5.8 fold after 3 and 4 h, respectively (Fig. 2A). The CFUs were $2 \times 10^6$ per mL at the beginning of incubation and remained constant over 3 h, after which they increased up to $40 \times 10^6$ after 12 h incubation.

In the presence of AmB, the absorbance changes were determined (Fig. 2A) for concentrations of AmB ranging between 0.4 and 1.2 μM. No absorbance increase could be detected before 21 h of incubation, and then only in the presence of 0.4 μM AmB. At higher AmB concentrations, absorbance finally increased with extended incubation (25 h with 0.6 μM AmB). The CFUs were $2 \times 10^6$ per mL at the beginning of incubation and remained constant over 25 h before starting to increase.

AmB stability in YM was checked by incubating the antifungal in the medium for 24 h at 30°C prior to its use in the studies. Under these conditions, its activity appeared to be strongly reduced with no growth inhibition observed at 0.2 or 1.2 μM by absorbance measurement.

Flow cytometry light scattering measurements

In the absence of AmB, FSC was monitored by flow cytometry as a function of time after inoculation of stationary phase C. albicans cells into fresh YM.

At time $t=0$, two peaks were observed in forward light scattering at 98 and 193 AU (arbitrary units). Over time, a new peak appeared at higher scattering values, whereas the initial peaks disappeared (Fig. 3A). The new peaks were observed at 306 and 537 A.U. after 2 and 4 h, respectively. Beyond 4 h, the scattering progressively regressed to the initial profile. After 7 h of incubation (and until 24 h), the scattering distribution was similar to that observed at time $t=0$. Beyond 24 h, the medium was probably nutrient-exhausted and the distribution changed (see 32 h). Fig. 2B shows the time dependence of the median of the scattering histogram. Importantly, the same pattern was observed regardless of initial cell concentration ($2 \times 10^2$ to $2 \times 10^7$ cells/ml). The initial scattering profile could not be recorded for the lower cell concentrations (Fig. 2C).
AmB from the $10^{-4}$ M stock solution in DMSO was added at various concentrations (0.4–1.2 μM) immediately after inoculation of stationary phase C. albicans cells into fresh yeast medium. The initial scattering increase noted within four hours in the absence of AmB, was obliterated. Instead, changes in the scattering pattern of C. albicans cells occurred in three steps, as for example as shown in Fig. 3B in the presence of 0.4 μM AmB. First, scattering decreased with the peaks at 98 and 193 AU observed in the absence of AmB shifting to lower values and eventually being replaced by two peaks at 63 and 125 AU. The rate of this change was dependent upon the AmB concentration and the elapsed time with changes taking place more rapidly at higher concentrations of AmB. For all AmB concentrations above 0.4 μM, the change was radical and only peaks at 63 and 125 AU were observed. Then, in a second step, after a time lag of 21 h in the presence of 0.4 μM AmB (25 h in the presence of 0.6 μM AmB), the scattering profile started to shift to higher values and in approximately 4 h moved to a median of 276 AU (with a distinct peak at 426 A.U., see Fig. 2B). However, in contrast with what had been observed in the absence of AmB, this new distribution only partially replaced the initial distribution, which remained unchanged in position, although less significant in counts. As noted above, microscopic observations revealed no proliferation before 24/25 hr of incubation. Finally, after 27 h, the peak at 426 AU shifted back to lower values of scattering, whereas cell proliferation started, accompanied by an increase in light absorption. After 32 h, the distribution profile was similar to that observed in the absence of AmB at 21 h.

From the double distribution of the scattering profile, it is possible to determine the percentage of cells that restart (Fig. 4). In fact, the distribution profiles observed from 6 to 20 h of incubation present a distinct peak at 63 AU that is representative of shrunken cells and different from the distribution profile observed with cells in the terminal phase of scattering burst. It is therefore possible to subtract this shrunken cells related peak from the latter obtained at 20 h and normalized on the peak at 63 AU, to obtain the percentage of cells that renewed their proliferation. Considering that cell growth does not resume before 24 to 25 h (0.4 μM AmB) and 28 to 29 h (0.6 μM AmB), according to the resulting histogram profile, these percentages determined in the presence of 0.4 and 0.6 μM AmB were $45 \pm 5$ and $20 \pm 6\%$, respectively.

Fig. 3 Histograms showing the time dependence of the FSC of Candida albicans cells in the absence (A, vertical bar at 193 AU) or presence (B, vertical bar at 3 AU) of 0.4 μM AmB.

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Live-dead-cell determination, using H2DCFDA and epifluorescence microscopy

After 2 h incubation with H2DCFDA, in the absence of AmB, most of exponentially growing C. albicans cells (ATCC 10231) appeared fluorescently labelled under fluorescence microscopy. In contrast, cells incubated in the presence of 0.4 μM AmB for 5 to 15 h which were smaller than the preceding ones (see above), were not labelled. The same culture incubated for 27 h showed a mixture of large and small cells (see above) but only the large ones were labelled (Fig. 1D).

Discussion

In the absence of AmB

After the transfer of C. albicans cells (ATCC 10231) into fresh yeast medium, important changes were noted during the first 3 h in the scattering profile as determined by flow cytometry. At the same time, the number of cells, as determined by haemocytometer cell and CFU counts remained approximately constant. However, the appearance of the cells and the absorption at 595 nm revealed increase in the size and budding of the cells. After longer incubation, regression to the initial state occurred. It is important to note that the description of these events for an initial inoculum of 2 × 10^6 cells/mL also applies to inoculum concentrations varying from 10^5–10^7 cells/mL (Fig. 2C).

In flow cytometry, FSC is considered to be related to cell size. The continuous increase of FSC and therefore of size of the cells after a 4 h incubation, that is after the first emergence of newborn cells, can be explained by increases in volumes of the parental cells with each generation of buds, as observed in S. cerevisiae. Indeed, it has been shown that during each cell division cycle, C. albicans cell size increases slowly whether they are budding or not so that each generation is significantly larger than the preceding [12]. However, the relative number of daughter cells also gradually increases and becomes preponderant as regards the number of mother cells. As flow cytometry measurements are performed on a constant number of cells (10,000), the scattering signal of the mother cells eventually becomes negligible. Microscope examination and flow cytometry indicated that these changes were synchronized.

Bud formation as observed microscopically, started after approximately 1 h of growth in fresh medium following 15 h incubation in YM. This synchronization is consistent with a former observation [13] of C. albicans cells grown in stationary phase in Lee’s medium and maintained in stationary phase at least 24 h prior to subculturing in fresh medium at 37°C.

As concerns the volume changes, size distribution studies combined with microscopic analysis of C. utilis [14] showed that cells expanded only during the phase of growth in which budding was not occurring. Usually cells stopped increasing in size approximately 30 min before bud emergence. The growth process of S. cerevisiae cells appears to be much closer to that observed here than that of C. utilis. Woldringh et al. presented detailed results [15] on increase in absorbance, cell number and cell volume (as measured by image cytometry) as a function of time that are entirely similar to ours. Immediately after inoculation of S. cerevisiae cells into YM, the absorbance at 450 nm began to increase whereas the number of cells remained stationary for 100 min. Cell volume as measured by image cytometry increased continuously until 100 min had elapsed. Then, subpopulations of smaller volume cells appeared while the larger cells kept on growing. Similarly Johnston et al. have shown [16] that when abnormally small S. cerevisiae cells produced by nitrogen starvation were placed in fresh medium, they did not start budding until they had grown to a critical size. On the other hand, an exponential increase in the size of individual cells of S. cerevisiae during the cell cycle of asynchronous populations has been demonstrated by forward angle scattering measurements [17].

Similar events were noted by Bedell et al. [18] and Soll et al. [13] with C. albicans at 100 min, in that buds appeared and dry weight and protein content increased.

It is interesting to note that our observations confirm that light absorption measurements should be cautiously considered as a method for counting cells as the...
initial absorption increase is related to cell size growth, not to cell number increase.

In the presence of AmB

The initial shrinkage of *C. albicans* cells in the presence of AmB, evidenced by microscopic examination and the decrease in light scattering, is a well-known phenomenon, and has frequently been demonstrated by flow cytometry [4,19–21]. This effect may possibly be related to the AmB-induced leakage of K⁺ ions [22].

In order to separately detect live and dead cells, we used the fluorescent marker H2DCFDA, a cell-permeant indicator that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Therefore, it is considered as specifically staining live cells as does carboxyfluorescein diacetate used in a study on *C. albicans*, which was similar to ours. The small cells observed after a 5–17 h incubation in the presence of 0.4 μM or more AmB were not stained and should therefore considered as non-metabolically active.

The original observation of this study is a transitory increase in light scattering (resulting in a new and transient flow cytometry histogram profile) after approximately one day (21 h in the presence of 0.4 μM AmB; 25 h in the presence of 0.6 μM AmB), without cell proliferation (as measured by CFU counts and absorbance measurements). However, in contrast with what was observed in the absence of AmB, part of the initial scattering distribution profile corresponding to the presence of shrunken cells remained unchanged. Microscopic examination revealed a portion of the cells remained small and were not stained by H2DCFDA, whereas another segment of cells grew in size and did stain. After a supplementary 3–4 h lag, (24–25 h and 28–29 h after seedling, respectively), proliferation restarts and the scattering distribution profile gradually comes back to that observed in the absence of AmB but with a time lag (compare 32 h of Fig. 3B to 21 h of Fig. 3A; note that at 32 h in the presence of AmB the medium is not nutrient-exhausted, in contrast with what is observed in its absence of the antifungal agent). The scattering contribution of cells with an unchanged profile is gradually diminished in comparison to the scattering caused by proliferating cells (most of them being H2DCFDA labelled).

The sequence of events that we observed has not been previously described as the result of the following possible factors. First, the purpose of earlier flow cytometry studies involved rapid susceptibility testing, leading to experimental conditions different to ours. For instance, shrinkage in the presence of AmB was only followed for 0.5–2 h after its addition. Second, most of these studies used viability dyes and did not take into account FSC or side light scattering (SSC) [6]. One study in which incubations were extended to 24 h represents an exception [23], finding that acridine orange fluorescence versus side scattering contour plot profiles suggested that SSC decreases in the presence of AmB concentrations above 0.4 μM AmB.

As concerns CFU counts, time-kill curves are generally not conducted beyond 24 h, at which time a general decrease of CFUs is observed for increasing AmB concentrations. However, Burgess *et al.* [24] extended their study to 48 h and obtained results similar to ours. Meunier *et al.* [25] and Klepser *et al.* [26] observed a temporary decrease of CFUs at 0.1 μM AmB at around 20 h of incubation, i.e., 25 fold and 10 fold, respectively. On the other hand, a recent physico-chemical study [10] carefully analyzed the different concentration-dependent steps of AmB activity. It was proposed that *C. albicans* cells exposed to AmB at concentrations between 0.5 and 1 μM (similar to those used in the present study) (1) did not take up vitality- or mortality-specific dyes, (2) were replication incompetent, but (3) may still be still capable of resuscitation. In a subsequent investigation, Liao *et al.* [11] observed that the CFUs decreased by 99.5% when exposed to 0.5 μM AmB for 10 h at 35°C. However, they did not consider that this reduction was due to cell death. Indeed, the cells did resume development through an additional 15 h incubation at 22°C and were 3 times more numerous than after initial inoculation. Their number would certainly have continued to increase with longer incubation times. While our results are in line with these observations, there was a major difference, i.e., we did not introduce a ‘resuscitation’ step (supplementary incubation at 22°C).

The simplest interpretation of the burst of scattering we observed after approximately one day of incubation with 0.4–0.8 μM AmB is that a certain percentage of *C. albicans* cells (ATCC 10231) were still capable of development and proliferation, while others were not. In other words, a non-negligible number of cells had escaped the killing action of AmB and had become ‘dormant’. After one day, the cells broke their dormancy and were sufficiently numerous to be detected by means of the light scattering burst. The observed scattering burst could not have been the result of a few cells continuing to multiply from the time of initial incubation until reaching a detectable level. In support of this explanation, we have seen that in the absence of AmB, scattering and absorbance are practically constant from 20–30 h regardless of inoculum concentration (from 2 × 10⁵ to 2 × 10⁷ cells/mL). On the other hand, the increased cell size is not the result of swelling of dead cells as they
deteriorate since their budding clearly indicates their vitality, as well as their spherical morphology and white appearance. Furthermore, the fluorescent marker of cell viability specifically labeled the cells of increased size but not the smaller ones which were presumed to be dead. Therefore, these studies would appear to provide an answer to a controversy also encountered with bacteria, i.e., the in vitro observed the revival of viable cells rather than the growth of a few viable cells that had escaped detection.

Consequently, our results indicate that AmB activity develops in three steps. First all cells sustain strong perturbations that result in a marked shrinking. Then, for a long period that can extend over 24 h, no change appears in this state. Finally, a number of cells resume activity and growth, and may be assumed to have been in a ‘dormant’ state. The remainder, which will remain shrunk, may be assumed to be dead cells.

We think it preferable to use the term ‘dormancy’ rather than ‘time lag’ to describe the intervening period because shrinkage may be considered as implying more than a time lag. In fact, dormancy is defined as a reversible state of low activity, in which cells can persist for extended periods without division [27], which exactly corresponds to what we observed.

The existence of dormancy should be discussed in relationship to the phenomenon of persistence described with bacteria [28,29]. It has not been observed with C. albicans [30], although in this report the experimental conditions were different from ours (much higher AmB concentration). Furthermore, the high percentage of recovering cells (45% for 0.4 μM AmB) observed in our experiments seems to prevent them from being considered as ‘persister’ cells, the percentage of which are much lower (around 1% at maximum).

Questions arising in connection with the recovery of the cells from dormancy are whether it is (1) the result of the production by surviving cells of a factor that stimulates revival of dormant cells [31], (2) the consequence of recovery from AmB-induced membrane damage as has been shown for mammalian cells [32], or (3) caused by the possible dissociation of the channel-forming AmB-ergosterol complex [33]. In addition, it might be the result of possible AmB degradation during the 24 h of incubation as suggested by Liao et al. [11]. As a matter of fact, we observed a strong decrease of AmB activity following a 24 h incubation in YM. This degradation may explain why the recovery is concentration dependant, i.e., the higher the initial AmB concentration, the higher the AmB concentration remaining active after 24 h and the lower the percentage of recovering cells. It is certain that the new cells born after C. albicans cells revival are not subject to AmB antifungal action.

From a technical point of view, our approach (which could be applied to other yeasts and other antifungal drugs) presents the advantages of being time and material cost effective. In contrast, CFU enumeration is time consuming and not very practical if numerous assays are performed. As stated earlier, it would also appear that turbidimetry (as measured by light absorption) should be used with caution as a cell counting method as it is valid in the exponential phase of growth but not at the initial phase where it is not correlated to the number of cells.

A problem for which our approach could be of assistance is that of the post-antifungal effect (PAFE). PAFE is commonly defined as the time required for fungal cells to recover from the transient crippling injury sustained by the organism as a result of a brief exposure to the antifungal drug. Therefore, measurement of PAFE can be considered as revealing the physiological condition of the cells after exposure to given antibiotic concentrations and incubation times. PAFE in C. albicans in the presence of AmB has been examined using different approaches, such as absorbance [34–36], CFU counts [37–39], colorimetric detection of CO2 [40] and accumulation of 14C-labeled amino acids [41]. Flow cytometry was not applied to post-antifungal effect but its application to bacteria (FSC determination) appeared to be quite promising [42].

We are at present using our approach to assess the vitality of antisense oligonucleotide-loaded C. albicans cells in the presence of cationic derivatives of amphotericin B [43].

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References


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