Effect of *Candida* Morphology on Amphotericin B Susceptibility

KENNETH M. NUGENT,¹* KENDRA R. COUCHOT,¹ AND LYNN D. GRAY²

Departments of Medicine¹ and Cell Biology², The University of Texas Health Center at Tyler, Tyler, Texas 75710

Received 16 June 1986/Accepted 3 November 1986

We showed that brief exposures to amphotericin B (AmB) inhibited the induction of new *Candida* germ tubes and the lengthening of partially induced germ tubes. Blastoconidia with germ tubes were more susceptible to AmB killing, and this varied directly with the induction period and the AmB exposure period. AmB did not preferentially affect germ tube adherence to fibrin matrices.

We recently showed that sublethal concentrations of amphotericin B (AmB) inhibited germ tube formation in *Candida albicans* cultures (7). Niimi et al. reported that germ tube-forming cells of *C. albicans* are more susceptible to clotrimazole-induced killing than are yeast cells (6), and Borgers and co-workers found that pseudomycelium formation was very sensitive to dilute concentrations of imidazoles (1). These studies suggest that the biochemical and morphological changes associated with germ tube formation produce increased susceptibility to antifungal agents.

Germ tube formation. In earlier studies with a clinical isolate of *C. albicans* (11), we observed that AmB inhibited the induction of germ tubes in serum (7). In the experiments described here, we measured the effects of brief exposure to low concentrations of AmB (0.1 µg/ml) on germ tube development and lengthening in partially induced cultures. Blastoconidia were incubated in 10% newborn calf serum in phosphate-buffered saline (NBCS; GIBCO Laboratories, Grand Island, N.Y.) for 45 min to induce germ tubes, incubated with AmB for 15 min, washed free of AmB, and then reincubated in 10% NBCS for an additional 60 min. After 45 min, 34.2 ± 9.9% (standard error) of blastoconidia had germ tubes identified by light microscope criteria (9).

blastoconidia had more germ tubes per cell than did control blastoconidia (Table 1).

Germ tube killing. In pilot experiments, we tested various AmB concentrations on *C. albicans* cultures which had been induced for 60 min at either 37 or 0°C in 10% NBCS. The results demonstrated that *C. albicans* with germ tubes (i.e., 37°C cultures) were more susceptible to AmB than control cultures incubated at 0°C (data not shown). On the basis of these results, we used AmB at concentrations of 0.25 µg/ml in the next three series of experiments. Blastoconidial suspensions were incubated in 10% NBCS for 0 to 120 min and then exposed to AmB for 60 min. Longer induction periods increased susceptibility to AmB killing, and the percent survival decreased from 97 ± 3% after 0 min of induction to 84 ± 7% after 30 min, 57 ± 13% after 60 min, and 13 ± 4% after 120 min (*n* = 6 for each time point). In a second series of experiments, germ tubes induced for 60 min were incubated with AmB for 0 to 120 min. Longer exposures produced greater killing, and AmB had a more pronounced effect on *Candida* cells with germ tubes (Fig. 1). Finally, germ tubes were induced in 10% NBCS for 60 min and then incubated with AmB for 60 min. Samples of these mixtures were supplemented with either KCl (85 mM) or MgCl₂ (45

TABLE 1. Germ tube length and number^a

Culture	No. of yeasts measured	Length (µm; mean ± SE) of:		No. of tubes/yeast (mean ± SE)	% Yeasts with 2 or more tubes
		1° Tubes ^b	All tubes		
Control	164	15.44 ± 0.79	12.50 ± 0.83	1.41 ± 0.05	34
AmB treated	155	3.60 ± 0.33 ^c	3.33 ± 0.31 ^c	1.71 ± 0.09 ^c	59

^a Cover slips with adherent *C. albicans* cells were fixed with 0.1 M phosphate-buffered 3% glutaraldehyde, postfixed with 0.1 M phosphate-buffered 2% OsO₄, dehydrated in ethanol, mounted in toto on aluminum planchets, and sputtered with approximately 400 Å (40 nm) of gold/palladium. Specimens were examined at 20 kV, 0° tilt, and at ×600 magnification in an AMR 1000A scanning electron microscope with 1-µm-diameter latex reference beads. Germ tube lengths were measured with a cursor interfaced to a Zeiss Videoplan image analysis system.

^b 1° Tubes, Primary tubes (>0.8 µm).

^c *P* < 0.05 by *t* tests.

This subsequently increased to 95.5 ± 1.1% in control suspensions and to 56.8 ± 8.6% in AmB-treated suspensions (*P* < 0.05 by analysis of variance; *n* = 6 experiments). We measured the length of germ tubes with scanning electron microscopy after *Candida* blastoconidia had completed the incubations described above. The primary germ tubes and all germ tubes were significantly longer in control cultures than in AmB-treated cultures (Table 1). In addition, AmB-treated

mM) during AmB exposure (3). Neither cation significantly prevented killing by AmB (data not shown; *n* = 6).

***Candida* adherence to fibrin matrices.** AmB strongly inhibits the adherence of *Candida* blastoconidia to fibrin matrices (7). In the present experiments, we compared the effect of AmB on germ tubes and on blastoconidial adherence to matrices prepared with thrombin-clotted fresh frozen plasma (4, 5). Control untreated blastoconidia with germ tubes tended to adhere to fibrin better than did control blastoconidia (56 ± 20 CFU/12 cm² at 10² CFU input versus 20 ± 4 CFU/12 cm² at 10² CFU input), but this difference did not reach statistical significance (by the paired *t* test). At low

* Corresponding author.

† Present address: Department of Internal Medicine, Texas Tech University Health Sciences Center, Lubbock, TX 79430.

FIG. 1. Amphotericin exposure time. *C. albicans* cultures were incubated in 10% NBCS for 60 min and then incubated with AmB (0.25 $\mu\text{g/ml}$) for 0 to 120 min (●). Control blastoconidial cultures were held at 0°C for the 60-min induction period in 10% NBCS (■). The results are presented as the percent surviving at various times after AmB exposure; the number of CFU per milliliter at zero time was assigned a value of 100%. There was no difference among AmB exposure periods in control experiments by analysis of variance ($n = 6$). In cultures with germ tubes, the differences between exposure periods were significant ($n = 6$; $P < 0.01$ by analysis of variance), and killing after 120 min of exposure was significantly greater than after 15 min ($P < 0.05$ by the Bonferroni t test).

concentrations of AmB, blastoconidial adherence was more sensitive to AmB than was germ tube adherence (Fig. 2).

In these experiments, low concentrations of AmB preferentially affected *Candida* germ tubes. The explanation(s) for this effect is uncertain but might include the following considerations. Firstly, cell wall synthesis and minor changes in membrane permeability during germ tube formation might allow increased AmB uptake, and this produces the increased killing observed in germ tube cultures. Secondly, during germ tube formation certain synthetic activities are concentrated at restricted regions of the cell (10). It

FIG. 2. Amphotericin effect on *Candida* adherence to fibrin matrices. Germ tubes were induced in 10% NBCS for 60 min. *Candida* suspensions were then incubated with AmB for 30 min, diluted 100-fold, and added to fibrin plates. The results were normalized to adherence values for untreated parallel cultures. Data represent the means for five experiments. Differences at 0.01- and 0.05- $\mu\text{g/ml}$ drug concentrations were significantly different by paired t tests. Symbols: ●, blastoconidia; ■, germ tubes.

is possible that AmB causes cell death by localizing to these regions and preferentially inhibiting the formation of new cell wall. Rast and Bartnicki-Garcia demonstrated that AmB inhibits chitin synthase at concentrations around 1 $\mu\text{g/ml}$ (8). This formulation would not require an increase in total drug uptake and would be supported by the observation that germ tube lengthening is inhibited by AmB but that new second germ tubes can develop. Finally, AmB may have unknown effects on critical membrane functions, and these effects may become physiologically important during periods of increased metabolic activity. The mechanism of action of AmB at low concentrations is usually attributed to changes in permeability which allow the release of critical ions and nutrients (3). However, we could not prevent the loss of viability by the addition of either Mg^{2+} or K^{+} to the germ tube cultures, and the AmB incubation was performed in fresh tryptic soy broth. At higher concentrations, AmB causes lipid peroxidation in erythrocytes (2). Oxidative injury probably occurs at lower concentrations and could damage critical enzymes. This formulation suggests that permeability measurements do not reflect AmB killing events.

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