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Increased resistance to antifungal antibiotics of *Candida* spp. adhered to silicone

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SUMMARY

The minimal inhibitory concentrations (MICs) and minimal fungicidal concentrations (MFCs) of amphotericin B, miconazole, ketoconazole, fluconazole, and itraconazole were determined for non-adhered cells and cells adhered to sections of a silicone urinary catheter. The densities of adhered cells were established with cells radiolabeled with tritiated leucine. Well defined MICs and MFCs were established for amphotericin B for representative adhered strains. In contrast, the azoles, especially fluconazole, did not give clear end points and the MICs and MFCs were arbitrarily determined. MFCs for the adhered cells generally were 2- to 5-fold higher than those of non-adhered cells. Techniques that include adhered-cell susceptibilities may be necessary before antifungal regimens for prosthetic device-associated yeast infections are appropriately defined.

INTRODUCTION

Systemic infections by species of *Candida* are associated frequently with colonization of prosthetic devices or various types of catheters [5,24]. These infections are frequently difficult to diagnose early and are often fatal [1,24]. The grave outcome for invasive yeast infections persists for strains that are shown to be susceptible to chemotherapy in vitro [2,4,18].

Colonization of inorganic surfaces by bacteria to produce a biofilm is a recognized factor in the recalcitrance of bacterial infections [3]. Various investigators have reported that bacteria adhere to and grow on polymer surfaces and produce a biofilm or an extracellular slime substance [8,17]. There is strong evidence that such biofilms are responsible for inhibiting the activity of antibacterial antibiotics [23]. It has been predicated that bacterial adherence induces slime formation that serves as a physical barrier to antibiotics or as a matrix that binds antibiotics before their contact with the cell wall of the organism can occur [23]. Also the metabolic state or growth of the adherent bacteria may be altered (reduced), rendering them less susceptible to an antibiotic [11]. Biofilm bacteria can also be induced to form enzymes that can inactivate antibiotics. It has been demonstrated that the presence of β-lactam antibiotics can induce biofilm bacteria to produce β-lactamase enzymes which can degrade the antibiotics [10].

In contrast to bacteria, there is relatively little information available on attachment of yeasts to alloplastic materials. Changes in the cell-surface hydrophobic and hydrophilic proteins alter the adherence capabilities of *Candida* spp. Cells that express greater cell surface hydrophobicity adhere better to cell cultures, polyvinyl chloride, and other materials [14]. Apparently, increased hydrophobicity enhances the pathogenesis of candidiasis, especially at the early phase of adhesion [13]. Weakly virulent *C. tropicalis* has been reported to demonstrate a stronger degree of adherence to bio-materials than more virulent *C. albicans* [9]. The external cell wall of *C. albicans* contains a fibrillar layer that is composed of high molecular weight mannoproteins. Critchley and Doughlas [9] have shown that cells extracted for their mannoproteins are less capable of adherence to buccal epithelial cells and to acrylics.

There is little information available on the susceptibilities of *Candida* biofilms to antifungals. This study compares the relative resistance to selected antifungals of adhered and non-adhered cells of *Candida* spp.

MATERIALS AND METHODS

Antifungal agents

Susceptibility tests were performed with the following antifungal drugs: amphotericin B (AMB) (Fungizone, E.R. Squibb & Sons, Princeton, NJ, USA), ketoconazole (KCZ) and miconazole (MCZ) (Sigma Chemical Company, St Louis, MO, USA), fluconazole (FCZ) (Diflucan, Roerig/Pfizer, New York, NY, USA), and itraconazole (ICZ) (Janssen, Beerse, Belgium). Amphotericin B and miconazole were dissolved in dimethylsulfoxide (DMSO); the former was held in an amber bottle at room temperature for 30 min prior to cold storage. Fluconazole was dissolved in sterile distilled water. Ketoconazole was dissolved in 0.4 N HCl. Itraconazole was dissolved in polyethylene glycol. All stock drug solutions were stored at 4 °C and used for only one week.

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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Cultures

Seven isolates of clinical origin representing three species of *Candida* were obtained from the culture collection at Georgia State University (Table 1). All cultures were maintained on Sabouraud dextrose agar and transferred monthly.

Assay media

Antibiotic medium (Difco laboratories, Detroit, MI, USA) was employed with amphotericin B. Yeast nitrogen base (Difco) fortified with 0.5% glucose and adjusted to a pH of 7.0 with 0.165 M morpholinopropane sulfonic acid (MOPS) was employed for all other antifungal assay methods.

Inoculum preparation

Yeasts were streaked on Sabouraud's agar (Difco) and incubated for 48 h. Five colonies of at least 1 mm diameter were picked with an inoculating loop and the cells were suspended in 5 ml of 0.9% NaCl to an absorbancy of 0.05 at 530 nm. Final cell densities determined with a standard pour plate method were $2–5 \times 10^5$ colony forming units (CFU) ml^{-1}.

Antifungal suspectibilities

A broth dilution procedure that involved four rows each of twelve dilutions of the antifungals was adapted from the methods of Shadomy and Pfaller [22]. Starting with twelve sterile tubes (16 × 125 mm), 9.0 ml of assay broth was added to the first tube and 5.0 ml to all others. One milliliter of antifungal stock solution was added to the first tube containing the 9.0 ml of assay broth. The tube was vortexed; 5.0 ml was withdrawn and added to the next tube and this same procedure was repeated with the remaining tubes. The 5 ml of broth withdrawn from the eleventh tube was discarded. The twelfth tube containing only the assay medium was used as a control. From each of the above test tubes, 1.0 ml of the antifungal dilution was transferred to each of the smaller tubes (12 × 75 mm). The first 11 small tubes of the second and third rows were inoculated with 0.05 ml of test organism (final concentration 5×10^5 cells ml^{-1}). The fourth row, the sterility control for each dilution, was not inoculated. All tubes were incubated on a shaker at 35 °C for at least 72 h. All tests were run in duplicate. The lowest concentration of antifungal in the first row of tubes that inhibited visually detectable growth was considered the MIC. During incubation, 0.01 ml of suspension from each of the dilution tubes at 24, 48 and 72 h was subcultured into 5.0 ml of Sabouraud dextrose broth. These subcultures were incubated on a shaker at 35 °C for at least 72 h. The MFC for planktonic cells was defined arbitrarily as the lowest concentration of the antifungal drug that did not show visibly detectable growth in the Sabouraud recovery medium after 48–72 h of incubation. Concentrations of each antifungal ranged to 100 $\mu g\ ml^{-1}$.

Substrate for adherence

Commercially available, sterile silicone, single-use urethral catheters were sectioned above the balloon in 0.5-cm lengths. In certain experiments, these sections were sliced longitudinally so that the inner lumen was opened.

Quantitation of adhered cells

The procedure described by Sawant et al. [21] for quantitation of adhered cells on catheter sections was followed. Cells were grown in Sabouraud dextrose broth for 12–18 h, harvested and washed twice in 0.9% saline, and suspended in minimal broth at 25 °C for 1 h. Radiolabeled L-[3,4,5-^{3}H] leucine (1–3 $\mu Ci\ ml^{-1}$; NEN Research Products, DuPont Company, Wilmington, DE, USA) was added to the cell suspension and the system was incubated for 20 min. The cell suspension was harvested and washed four times in saline. Catheter sections were added to 5.0 ml of radiolabeled cell suspension (5.0×10^5 cells ml^{-1}) in scintillation vials and incubated at 30 °C for 2 h; separate experiments involved 6 and 24 h of incubation. The sections were removed from the vials after incubation, washed in three changes of 100 ml of saline, and transferred to 20-ml glass scintillation vials containing 10 ml of OptiFluor scintillation cocktail (Packard Instrument C., Downers Grove, IL, USA). The vials were vortexed and coun-

TABLE 1

MICs of selected antifungals for *Candida* spp.[a]

Species	Source	Antifungals[b]				
		AmB	Ket	Mic	Flu	Itr
C. albicans 30	vagina	1.6[c]	1.6	0.8	50	3.1
C. albicans SM44	oropharynx	1.6	1.6	0.8	50	3.1
C. albicans 9	feces	1.6	1.6	0.8	50	3.1
C. parapsilosis 39	blood	0.8	0.8	0.8	12.5	1.6
C. parapsilosis 32	skin	1.6	1.6	0.8	25	3.1
C. tropicalis 772	urinary	3.1	1.6	0.4	25	6.3
C. tropicalis 224	maxillary sinus	1.6	0.8	0.4	12.5	6.3

[a] Inocula about $10^5–10^6$ cells ml^{-1} in antibiotic medium (AmB, only) and yeast nitrogen base.

[b] AmB, amphotericin B; Ket, ketoconazole; Mic, miconazole; Flu, fluconazole; Itr, itraconazole.

[c] $\mu g\ ml^{-1}$ at 48 h.

ted in a liquid scintillation counter (LS-7500, Beckman Instruments, Inc., Fullerton, CA, USA). The disintegrations per minute (DPMs) from the radiolabeled cells adhered to catheter sections were compared to DPM ranges established for samples of serially diluted radiolabeled cells. This procedure was employed for each species as a basis for estimating the number of cells adhered to a catheter section. The nonspecific background radiation of catheter sections was determined for each test series. Catheter sections without cells or with dead cells were exposed to radiolabeled leucine, and their radiation values were subtracted from the test values.

Antimicrobial activity of adhered cells

Cells were grown in Sabouraud dextrose broth, washed and harvested in saline and adjusted to a final concentration of about 5.0×10^5 cells ml^{-1}. Catheter sections were suspended in 5.0 ml of cell suspension and incubated at 30 °C for 24 h on a roller drum. The catheter sections were removed and washed five times in three successive changes of 100 ml of saline. The catheter sections were then transferred to tubes containing serial dilutions of antifungals in assay media (described above) and incubated for 6 and 24 h at 35 °C on a roller drum. The catheter sections were removed with forceps, washed in one change of 100 ml of saline, and transferred to 5.0 ml of Sabouraud dextrose broth. These cultures were read visually at 24, 48 and 72 h. A minimal fungicidal concentration (MFC) for adhered cells was defined as the minimal antifungal concentration from which the catheter sections when transferred to Sabouraud's dextrose broth did not produce visible growth at 48–72 h.

RESULTS

In preliminary susceptibility experiments, varied numbers (10^3–10^9 CFU ml^{-1}) of *C. albicans* and *C. tropicalis* were exposed to amphotericin B. The MICs and MFCs at 48 h for up to 10^7 CFU ml^{-1} were the same (1.56 μg ml^{-1}). Inocula above 10^8 CFU ml^{-1} had one- to two-fold greater values. Inocula of approximately 5×10^5 CFU ml^{-1} were maintained for subsequent tests.

All strains of *C. tropicalis* and *C. parapsilosis* agglutinated on exposure to azoles, especially itraconazole. On microscopic examination, the cells appeared to be enmeshed in the form of micelles of approximately 5–10 μm diameter. These clumps also contained numerous pseudohyphae. This phenomenon was not observed with any of the isolates of *C. albicans*.

Adherence of radiolabeled cells suspended in saline to silicone urinary catheter sections after 2, 6, and 24 h of exposure time is shown in Fig. 1. Overall there were no significant differences in the number of adhered cells for the varied times. Usually the densities of adhered cells were at least one to two logs less than the initial inoculum. The MICs or the antifungals for non-adhered cells of the species of *Candida* are given in Table 1. In duplicate experiments, results were usually identical and never differed by more than one dilution factor. Only amphotericin B produced for all isolates sharp end points that remained constant throughout an incubation period beyond 72 h. End points for the azoles, particularly fluconazole, were time-dependent, and after 48 h, usually showed an increase in titer. The MFCs for non-adhered and adhered cells, therefore are arbitrary except for amphotericin B. We have compared these MFCs for adhered cells exposed to the antifungals for 24 h to MFCs determined for non-adhered cells that had been in the presence of the antibiotics for 24 h (Table 2). The MFCs for adhered cells (based on growth after subculturing to Sabouraud's dextrose broth) were generally two- to five-fold greater than those for non-adhered cells. The end points were still clearly definable from the subcultures at 72 h with the exception of those for fluconazole for all species and for ketoconazole for *C. albicans* 30, *C. tropicalis* 224, and *C. parapsilosis* 32. Adhered cells of *C. albicans* 30 and SM44 showed clearly discernible end points (growth not visually observed) up to 72 h with all antifungals except fluconazole, whereas similar end points for *C. albicans* 9 could be determined only for amphotericin B, miconazole, and ketoconazole. Such sharply defined endpoints for adhered cells of all isolates of *C. tropicalis*, and *C. parapsilosis* were evident only with amphotericin B and itraconazole. Interestingly, MFCs for adhered cells exposed to antifungals for only 6 h were lower (only one dilution) or equivalent to those exposed for 24 h (data not shown).

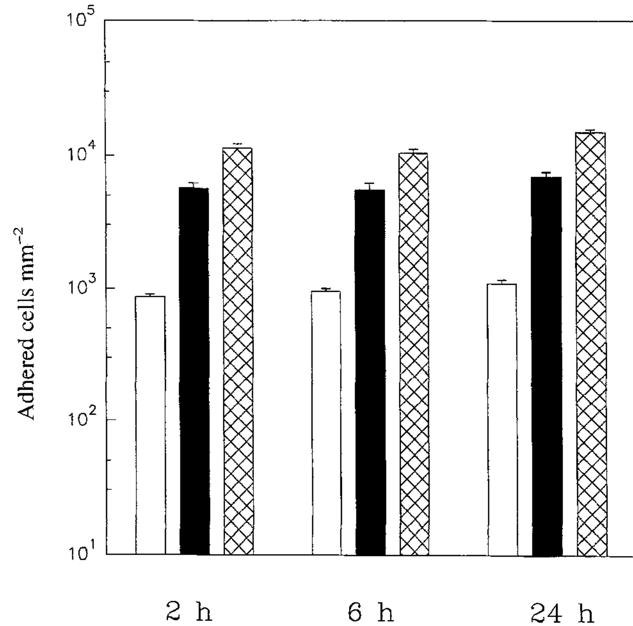

Fig 1. Adherence of *C. albicans* 30 □. *C. parapsilosis* 32 ■, and *C. tropicalis* ▨ to silicone after 2, 6, and 24 h exposure to cell suspensions in saline ($n = 3$). Inocula of 5×10^5 cells.

Adherence experiments were repeated with *C. albicans* 30 with catheter sections which were sliced longitudinally. The MICs' and MFCs' results were similar to those obtained with whole catheter sections (data not shown). When catheter sections were introduced into the inoculated antibiotic assays, the MICs and MFCs for *C. albicans* 30 were the same or within one dilution of the values for non-adhered cells.

TABLE 2

MFCs (μg ml^{-1}) of selected antifungals for non-adhered (N) and adhered (A) cells of *Candida* spp. exposed to antifungals for 24 h[a]

Species	Antifungals[b]									
	AmB		Ket		Mic		Flu		Itr	
	N	A	N	A	N	A	N	A	N	A
C. albicans 30	1.6[c]	6.3	1.6	12.5	0.8	12.5	6.3	>25	1.6	25
C. albicans 44	1.6	6.3	0.8	6.3	0.4	12.5	3.1	>25	0.8	>25
C. albicans 9	1.6	6.3	1.6	12.5	0.8	3.2	6.3	25	0.4	12.5
C. parapsilosis 32	0.4	12.5	0.8	12.5	6.3	25	6.3	>25	0.4	12.5
C. parapsilosis 39	0.8	12.5	1.6	12.5	0.8	12.5	6.3	>25	0.4	12.5
C. tropicalis 224	1.6	12.5	1.6	25.0	0.8	25	6.3	>25	1.6	6.3
C. tropicalis 772	3.2	12.5	1.6	25.0	0.8	12.5	12.5	>25	1.6	6.3

[a] Inocula: non-adhered cells, $2–5 \times 10^5$; adhered-cells, $10^3–10^4$.
[b] AmB, amphotericin B; Ket, ketoconazole; Mic, miconazole; Flu, fluconazole; Itr, itraconazole.
[c] μg ml^{-1} (determined from subcultures incubated for 48 h).

DISCUSSION

Defined MICs and MFCs of the polyene amphotericin B were obtained for representative isolates of *Candida* species, whereas the MICs and MFCs for most of the azoles were defined arbitrarily. The endpoints for azoles (except fluconazole) were possible with our subculture procedure, but by 72 h some growth at higher concentrations usually occurred. Arbitrary end points such as fifty percent inhibitory concentrations therefore are often employed to establish in vitro MICs for the azoles, partially because the endpoints vary with inocula, media composition and pH. Our MIC results are in general agreement with those reported in the literature [6,7,18,19,20].

Comparisons of non-adhered cells and cells adhered to catheters indicated that adhered cells generally were more resistant to antifungals. The number of cells adhering to silicone catheter sections, as determined with radiolabeled cells suspended in saline for 2, 6, and 24 h, were similar, ranging from about $10^3–10^4$ CFU mm^{-2} depending upon the strain. The washing step prior to introduction of the catheter-bound cells into the antifungals reduced the inocula further (data not given). The equivalent susceptibilities of cells adhered to catheters at 6 and 24 h suggested that the possible lateral multiplication of the biofilm within this time span was negligible.

The various mechanisms described for bacterial adherence and antibiotic insensitivity also may play a role in the increased resistance to antifungals of adhered yeast cells. Extracellular polymer with high mannose content that promotes adherence has been isolated from culture supernatants of yeasts, especially *C. albicans* grown on several carbon sources (glucose, sucrose, galactose) [16]. Alterations in the mannoproteins can change the hydrophobic and hydrophilic properties of the cell wall and hence the adherence capabilities of the cell [13]. The clumping noted herein with strains of *C. tropicalis* and to a lesser extent with *C. parapsilosis*, in the presence of azoles may be attributed to such changes in cell surface properties. Mannoproteins have been indicated to mediate properties like adherence to epithelial cells and immunomodulation [13,15].

During the processing of this report, Hawser and Douglas [12] reported on the in vitro development of biofilms, dense networks of yeasts and hyphae, by *Candida* spp. on the surfaces of catheter materials. These investigators found that biofilm formation of *C. albicans* was increased slightly on latex or silicone elastomer in growth medium compared with polyvinyl chloride and that biofilm formation on 100% silicone (the material used in this study) was substantially reduced. Preliminary scanning electron microscopy of the silicone-adhered cells in our study indicate that the cells adhered from saline have not yet coalesced into a biofilm.

This study indicates that adhered yeasts are able to survive exposure to concentrations of antifungals exceeding those inhibitory for non-adhered cells. The enhanced resistance of adhered cells was observed even for the normally biocidal antifungal amphotericin B. The increased resistance of adhered cells may help explain the lack of correlation of in vitro susceptibilities of non-adhered cells with certain therapeutic results. Catheter and prosthetic device-associated infections are often chronic in spite of rigorous chemotherapy [24]. Techniques that include adhered cell susceptibilities may be necessary before antifungal regimens for yeast infections associated with prosthetic devices are appropriately defined.

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