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Resistance of *Candida albicans* Biofilms to Antifungal Agents In Vitro

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Biofilms formed by *Candida albicans* on small discs of catheter material were resistant to the action of five clinically important antifungal agents as determined by [^{3}H]leucine incorporation and tetrazolium reduction assays. Fluconazole showed the greatest activity, and amphotericin B showed the least activity against biofilm cells. These findings were confirmed by scanning electron microscopy of the biofilms.

Insertion of prosthetic devices such as catheters and joint replacements into patients provides pathogenic microorganisms with a substratum on which they can form adherent biofilms consisting of layers of cells embedded in a matrix material (2, 3, 7–10). Bacterial biofilms of this type, including those produced by *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, are relatively insensitive to antibiotic treatment (1, 3), and eradication of implant-associated infections often involves removal of the infected prosthesis. Investigations into the mechanisms of antibiotic resistance suggest that the recalcitrance of biofilm cells may be due to reduced drug penetration into biofilms, nutrient limitation, a low bacterial growth rate, or the production of antibiotic-degrading enzymes (1).

The majority of implant infections attributable to fungi are caused by the pathogenic *Candida* species, especially *Candida albicans*. Recently, we described a model system for studying *Candida* biofilms growing on the surface of small discs of catheter material (11). Biofilm growth was monitored quantitatively by dry weight, colorimetric, and radioisotope assays and was visualized by scanning electron microscopy. In the present study, we have used the model system to determine the susceptibility of *Candida* biofilms to five clinically important antifungal agents.

C. albicans GDH 2346, a denture stomatitis isolate (13), was used in most of these experiments. Other *C. albicans* strains and *Candida* species were from a collection described previously (11). MICs of antifungal agents for *C. albicans* GDH 2346 were determined, courtesy of F. C. Odds, at the Janssen Research Foundation, Beerse, Belgium, by a microplate modification of the National Committee for Clinical Laboratory Standards protocol (14) with broths containing 2% glucose in RPMI 1640 medium. For biofilm studies, the organism was grown in yeast nitrogen base medium (Difco) containing 50 mM glucose or 500 mM galactose for 24 h as reported elsewhere (11, 13), and washed cell suspensions were adjusted to an optical density of 0.8 at 520 nm. Biofilms were formed on small discs (surface area, 0.5 cm^2) cut from polyvinyl chloride central venous catheters (Rüsch UK Ltd., High Wycombe, United Kingdom) and sterilized with ethylene oxide as described previously (11). Briefly, the discs were placed in wells of 24-well Nunclon tissue culture plates, and a standardized cell suspension (80 μl) was applied to the surface of each one. Initially, incubation lasted 1 h at 37°C (adhesion period). Nonadherent organisms were removed by washing, and the discs were then incubated in the wells for 48 h at 37°C, submerged in 1 ml of growth medium (biofilm formation).

Amphotericin B (Sigma), flucytosine (5-fluorocytosine; Sigma), fluconazole (Pfizer), itraconazole (Janssen), and ketoconazole (Janssen) were all tested against 48-h biofilms in the model system. Stock solutions of the drugs in dimethyl sulfoxide (amphotericin B, itraconazole, and ketoconazole), sterile distilled water (flucytosine), and dimethylformamide (fluconazole) were prepared immediately prior to use and diluted in growth medium buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). Following incubation of catheter discs for 48 h at 37°C, the growth medium was removed from each well and replaced with 1 ml of buffered medium containing the test antifungal agent at concentrations ranging from 0 to 250 μg/ml. The biofilms were incubated for a further 5 h at 37°C and then washed gently in 5 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.2. Biofilm activity was assessed by [^{3}H]leucine incorporation and tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT]) (Sigma) reduction assays described previously (11). For comparison, the effects of antifungal agents on planktonic (free-floating) organisms in wells containing biofilm discs were also determined. Following drug treatment, growth medium and planktonic cells were removed from the wells. The cells were harvested, washed twice in PBS, and resuspended in PBS (1 ml) containing [^{3}H]leucine or MTT, and [^{3}H]leucine incorporation or MTT-formazan formation was assayed as before (11). The effect of an antifungal agent was measured in terms of the percentage inhibition of [^{3}H]leucine incorporation or MTT-formazan formation by biofilms or planktonic cells compared with values obtained for control biofilms or planktonic cells incubated in the absence of the agent. These results, in the form of dose-response curves, were then used to calculate the drug concentration that caused 50% inhibition of [^{3}H]leucine incorporation (IL_{50}) or 50% inhibition of MTT-formazan formation (IF_{50}).

All of the antifungal agents tested showed much less activity against 48-h biofilms than against planktonic cells (Table 1). IL_{50}s and IF_{50}s for biofilms were 5 to 8 times higher than the corresponding values for planktonic cells and 30 to 2,000 times higher than the relevant MICs. Results for biofilms grown in medium containing 50 mM glucose as the carbon source were very similar to those for biofilms grown in medium with 500

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TABLE 1. Comparison of IL_{50}s and IF_{50}s for 48-h biofilms and planktonic cells of *C. albicans* GDH 2346[a]

Antifungal agent	IL_{50} (μg/ml)				IF_{50} (μg/ml)				MIC (μg/ml)
	Biofilms		Planktonic cells		Biofilms		Planktonic cells		
	Glc	Gal	Glc	Gal	Glc	Gal	Glc	Gal	
Amphotericin B	54.2	57.5	10.6	11.2	48.5	50.0	9.4	9.6	1.3
Fluconazole	11.5	11.8	2.2	2.6	14.5	15.0	2.6	3.0	0.4
Flucytosine	41.5	42.0	6.3	6.1	20.7	23.5	4.6	4.8	0.2
Itraconazole	44.5	56.0	6.2	6.5	35.5	40.0	5.9	6.0	≤0.025
Ketoconazole	42.2	46.5	6.0	6.1	23.5	27.5	4.8	4.9	≤0.025
Amphotericin B + flucytosine[b]	13.6	14.0	ND	ND	13.3	15.0	ND	ND	
Fluconazole + flucytosine[b]	6.3	7.5	ND	ND	9.2	10.2	ND	ND	

[a] The data are means for three independent experiments carried out in triplicate. Cells were grown in medium containing 50 mM glucose (Glc) or 500 mM galactose (Gal). ND, not determined.
[b] The concentrations of the two drugs were equal.

mM galactose. Fluconazole was the most effective agent against biofilms, with IL_{50}s and IF_{50}s five and three times lower, respectively, than those for amphotericin B, the least effective agent (Table 1). When combinations of drugs were used, the greatest synergism was observed with amphotericin B and flucytosine. Interestingly, although the lowest MICs were obtained for itraconazole and ketoconazole (≤0.025 μg/ml), the activities of these azoles against biofilms were similar to that of amphotericin B (Table 1). An analogous lack of correlation between MICs and the effectiveness of different cephalosporins for *Escherichia coli* biofilms was reported by Dix et al. (5). These investigators originally devised the [^{3}H]leucine incorporation assay used here and were able to demonstrate a close relationship between inhibition of [^{3}H]leucine uptake and decreased viability of *E. coli* biofilm cells. Unfortunately, such a demonstration was not possible in the present study because viable counts of *C. albicans* biofilms do not give reproducible results, presumably because of the mixed yeast and hyphal morphologies present (11).

In a separate series of experiments, biofilm inocula (used for the initial adhesion phase of biofilm development) were exposed to antifungal agents prior to biofilm formation. Drugs were added to yeast suspensions (80 μl of suspension in 900 μl of buffered growth medium) at final concentrations ranging from 0 to 100 μg/ml. After incubation for 1 h at 37°C, suspensions were centrifuged at 2,500 × *g* for 5 min and the cells were washed twice with 1 ml of 0.15 M PBS, pH 7.2, to remove antifungal agents. Cell suspensions were then applied to the surface of catheter discs in wells of tissue culture plates and incubated for 1 h at 37°C (adhesion period). Nonadherent organisms were removed by washing, and the discs were incubated for a further 48 h at 37°C in 1 ml of growth medium without drugs (biofilm formation). The effects of antifungal agents were determined by the [^{3}H]leucine incorporation and tetrazolium reduction assays as before and expressed as drug concentrations that caused a 50% inhibition of biofilm or planktonic cell activity (IL_{50} or IF_{50}) following preincubation with inocula.

This treatment of inocula resulted in IL_{50}s and IF_{50}s for biofilms (Table 2) that were significantly lower (often by a factor of 2) than those recorded for treated, 48-h biofilms (Table 1); however, they were still substantially higher than the corresponding values for planktonic cells or the relevant MICs. With the drug combinations tested, differences in IL_{50}s and IF_{50}s for biofilms were much smaller. As before, fluconazole was the most effective agent against biofilms, and amphotericin B was the least effective agent (Table 2). IL_{50}s and IF_{50}s for flucytosine, itraconazole, and ketoconazole were reasonably similar and indicated that these compounds are typically three to four times less effective than fluconazole at inhibiting biofilm development when added to inocula under the conditions of this experiment. The activities of all of the antifungal agents were again largely unaffected by the nature of the carbon source (glucose or galactose) used.

The effect of amphotericin B on 48-h biofilms was also investigated by scanning electron microscopy using procedures described previously (11). Biofilms treated with the drug at a concentration of 15 μg/ml (11 times the MIC) for 5 h were quite similar in appearance to untreated (control) biofilms, consisting of a dense mixture of yeasts, pseudohyphae, and

TABLE 2. Comparison of IL_{50}s and IF_{50}s of antifungal agents added to inocula of *C. albicans* GDH 2346[a]

Antifungal agent	IL_{50} (μg/ml)				IF_{50} (μg/ml)			
	Biofilms		Planktonic cells		Biofilms		Planktonic cells	
	Glc	Gal	Glc	Gal	Glc	Gal	Glc	Gal
Amphotericin B	23.8	26.5	5.2	5.4	23.5	24.8	5.5	5.7
Fluconazole	4.9	5.3	1.1	1.2	5.5	6.0	1.3	1.3
Flucytosine	16.5	18.0	3.3	3.4	16.6	18.5	3.2	3.4
Itraconazole	20.4	21.8	3.9	3.8	17.5	18.5	3.6	3.5
Ketoconazole	18.5	20.2	3.6	3.7	14.8	16.0	3.0	3.2
Amphotericin B + flucytosine[b]	12.5	13.0	ND	ND	10.4	13.4	ND	ND
Fluconazole + flucytosine[b]	5.0	5.3	ND	ND	3.7	4.8	ND	ND

[a] The data are means for three independent experiments carried out in triplicate. Cells were grown in medium containing 50 mM glucose (Glc) or 500 mM galactose (Gal). ND, not determined.
[b] The concentrations of the two drugs were equal.

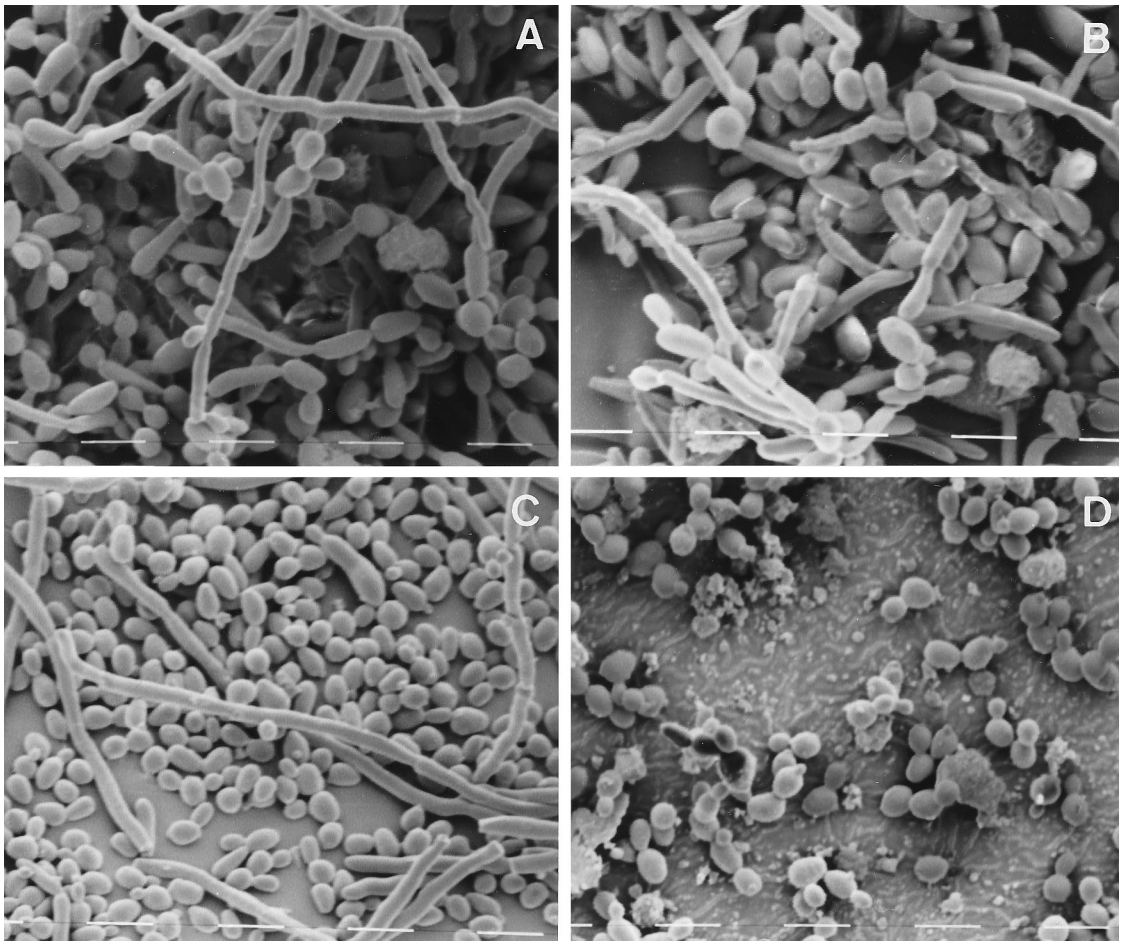

FIG. 1. Scanning electron micrographs of 48-h biofilms of *C. albicans* GDH 2346 treated for 5 h with amphotericin B at 15 (B), 50 (C), or 100 (D) μg/ml. (A) Control. Bars, 10 μm.

hyphae (Fig. 1A and B); several of the cells appeared swollen, but the overall structure of the biofilm remained intact. Treatment with amphotericin B at 50 μg/ml (38 times the MIC), which approximates the IL_{50}s and IF_{50}s for this drug (Table 1), caused a large reduction in cell numbers and in the thickness of the biofilm (Fig. 1C). An even higher drug concentration, 100 μg/ml (77 times the MIC), produced large amounts of cell debris, and relatively few intact cells remained (Fig. 1D).

Overall, our results indicate that biofilms of *C. albicans*, like those of bacteria, are highly resistant to antimicrobial agents. Although the data presented in Tables 1 and 2 were obtained with a single strain of the organism, we have tested a total of 13 different isolates of *C. albicans*. All gave similar results; for example, experiments with fluconazole produced IL_{50}s and IF_{50}s for biofilms ranging from 9.6 to 14.4 and from 9.9 to 17.6 μg/ml, respectively (Table 3). Analogous figures were also obtained with other pathogenic *Candida* species. Biofilms of *C. tropicalis* (two strains), *C. parapsilosis* (two strains), and *C. pseudotropicalis* gave IL_{50}s and IF_{50}s of 10.0 to 13.8 and 10.3 to 14.5 μg/ml, respectively, with fluconazole. However, *C. glabrata*

TABLE 3. Fluconazole IL_{50}s and IF_{50}s for 48-h biofilms and planktonic cells of *Candida* species[a]

Species (no. of strains)	IL_{50} (μg/ml)				IF_{50} (μg/ml)			
	Biofilms		Planktonic cells		Biofilms		Planktonic cells	
	Glc	Gal	Glc	Gal	Glc	Gal	Glc	Gal
C. albicans (13)	9.8–13.2	9.6–14.4	2.0–2.8	2.3–2.9	9.9–14.6	14.9–17.6	2.1–2.9	2.4–3.0
C. tropicalis (2)	10.0–11.4	13.3–13.8	2.6–3.3	2.7–3.4	10.4–10.6	10.3–10.9	2.5–3.2	2.5–3.4
C. parapsilosis (2)	10.2–10.6	13.0–13.3	2.8–3.4	2.7–3.3	10.6–10.8	13.3–14.5	2.4–2.9	2.6–3.1
C. pseudotropicalis (1)	10.5	13.2	3.1	3.5	10.8	13.6	3.0	3.6
C. glabrata (1)	32.8	39.8	30.4	34.2	39.6	44.4	35.2	38.8
C. krusei (1)	120.4	126.5	110.2	117.2	133.5	145.5	121.2	133.3

[a] The data are means for three independent experiments carried out in triplicate. Cells were grown in medium containing 50 mM glucose (Glc) or 500 mM galactose (Gal).

and *C. krusei*, two species known to be resistant to fluconazole even when grown in planktonic culture (4), had IL_{50}s of 39.8 and 126.5 μg/ml, respectively, and IF_{50}s of 44.4 and 145.5 μg/ml, respectively (Table 3).

A variety of mechanisms have been proposed to explain the recalcitrance of bacterial biofilms to antibiotics, including exclusion of the drug by the biofilm matrix (1). The mechanisms by which *Candida* biofilms resist the action of antifungal agents are unknown. When *C. albicans* biofilms are formed under conditions of static incubation, as described here, very little matrix material is evident. However, we have recently found that gentle shaking to produce a flow of liquid over the catheter disc significantly increases the synthesis of matrix material by biofilm cells so that, in some cases, the cells are completely enveloped (12). Such conditions probably resemble those present in vivo more closely than does static incubation and may increase fungal resistance still further. Additional studies are required to investigate this possibility, perhaps with a flow system of the type described by Domingue et al. (6), which incorporates National Committee for Clinical Laboratory Standards guidelines into the testing of biofilm bacteria for sensitivity to antibiotics.

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