

## Effect of Fluconazole on Viability of *Candida albicans* over Extended Periods of Time

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**The treatment of chronic mycoses may expose the infecting organisms to antimicrobial agents for extended periods of time. It is possible that an azole antifungal drug such as fluconazole, with primarily fungistatic activity in standard in vitro susceptibility tests, might be able to damage the fungal cells and reduce their viability over prolonged incubations under nonproliferating conditions. To test this possibility, *Candida albicans* yeast cells were exposed to various concentrations of fluconazole in RPMI 1640 tissue culture medium for 4 h at 37°C, washed free of the drug, and then incubated at 37°C for a 28-day period; enumeration of the remaining CFU at various times during this period revealed no increased loss of viability for the fluconazole-exposed organisms. However, when fluconazole was added to the organisms maintained in distilled water (with or without pretreatment with the drug), a marked reduction of viability was found. At 14 days of incubation with two strains of *C. albicans*, negative cultures were found for 7 of 10 and 10 of 11 samples, respectively, containing 1.0 µg of fluconazole per ml versus 0 of 10 and 1 of 11 control samples (*P* of <0.01 and 0.001, respectively). The effect of fluconazole on fungal viability under these conditions became noticeable at approximately 7 days and was greater when the samples were incubated at 37°C rather than 25°C. These findings suggest that fluconazole may have fungicidal effects on fungal cells during prolonged exposures under conditions in which the organisms are prevented from proliferating by lack of nutrients.**

Fluconazole and other agents of the azole class of antifungal drugs are generally considered to be fungistatic rather than fungicidal in standard in vitro susceptibility tests (2,9,15). When susceptibility to rapidly growing organisms such as *Candida albicans* is evaluated, these tests generally employ a relatively short exposure period of 24 to 48 h (12,15). Although there does seem to be a general relationship between in vitro susceptibility to the drug and its effectiveness in vivo, a variety of differences in test conditions influence the in vitro activity of fluconazole against *C. albicans* (7,15). Physicochemical and pharmacokinetic properties may also be important in determining whether good in vitro activity will translate into clinical effectiveness with drugs of this class (17). Therefore, it may be difficult to relate the results of standard in vitro susceptibility tests to the effect of a particular antimicrobial agent on the target organisms in vivo.

The treatment of chronic fungal infections is generally carried out for long periods. For example, courses of azole antifungal agents of at least 6 months are recommended for the treatment of the endemic mycoses (3,5,8). Similarly, very long courses of antifungal therapy may be required to treat certain *Candida* infections such as chronic disseminated candidiasis (2). Studies of experimental bacterial endocarditis have demonstrated that the infecting organisms may exist in a relatively dormant state (6). The relatively slow growth of *C. albicans* pseudohyphae has also been shown in experimental infections in mice (16). If the organisms in chronic fungal infections are not proliferating rapidly, then it is possible that individual fungal cells may be exposed to antifungal drugs for prolonged

periods. Therefore, the short exposure times used in standard susceptibility tests may not relate to what actually happens in vivo during treatment of this type of infection.

On the basis of the above factors, we considered the possibility that antifungal drugs with predominantly fungistatic activity in standard susceptibility tests could damage the target organisms and exert killing activity during longer incubations. Therefore, we tested fluconazole against *C. albicans* yeast cells during prolonged incubations under conditions in which the organisms could not proliferate because they lacked the nutrients needed for growth. The results of these studies demonstrated that this agent did markedly accelerate loss of viability of the organisms under these conditions.

### MATERIALS AND METHODS

**Organisms.** Two *C. albicans* strains were used in these studies, including strain ATCC 26310 and the local clinical isolate TW. The organisms were maintained for 3 to 5 days on Sabouraud dextrose agar slants before being used in the studies so that they would be in the stationary phase when the incubations began.

**Short-term susceptibility tests.** In order to choose appropriate drug concentrations for monitoring the experiments, susceptibility tests in 96-well tissue culture plates were first performed. For these assays an inoculum of 10<sup>4</sup> *C. albicans* yeast cells in 0.1 ml of RPMI 1640 tissue culture medium was used. Various concentrations of fluconazole from 0.1 to 50.0 µg/ml were added, and the plates incubated for 24 h at 37°C. At the end of the incubation period the wells were observed for visible growth. The contents of the negative wells were removed and plated on Sabouraud dextrose agar to determine the number of CFU remaining from the original inoculum. The MICs of fluconazole were also determined for inocula from 24-h cultures (i.e., non-stationary-phase organisms) by National Committee on Clinical Laboratory Standards method M27-T (13).

**Extended incubations in tissue culture plates.** *C. albicans* yeast cells (strain ATCC 26310) were suspended at 10<sup>6</sup>/ml in RPMI 1640 tissue culture medium and incubated at 37°C for 4 h in media containing fluconazole. For these experiments the fluconazole concentrations were 0, 1.0, and 5.0 µg/ml. After this incubation the cells were washed three times and resuspended in distilled water. The treated organisms were added in an inoculum of 10<sup>2</sup> in 0.1 ml of distilled water and placed into tissue culture plate wells, with half of the samples receiving fluconazole at 1.0 µg/ml. Therefore, in some cases, fluconazole was present

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TABLE 1. Effects of fluconazole on growth and viability of two *C. albicans* strains during short incubations

Fluconazole concn ( $\mu\text{g/ml}$ ) <sup>a</sup>	CFU remaining ( $10^4$ ) <sup>b</sup> (mean $\pm$ SD)	
	Strain ATCC 26310	Strain TW
0.5	1.9 $\pm$ 0.5	2.6 $\pm$ 0.4
5.0	1.3 $\pm$ 0.3	2.0 $\pm$ 0.4
50.0	1.1 $\pm$ 0.3	2.1 $\pm$ 0.5

<sup>a</sup> The MIC of fluconazole, the last dilution at which no growth was visible, was  $0.5 \pm 0.0 \mu\text{g/ml}$  for both strains.

<sup>b</sup> Incubations were carried out in RPMI 1640 medium for 24 h at 37°C; at the end of this time the wells were observed for visible growth, and those without growth were plated in appropriate dilutions to determine the number of CFU remaining from the original inoculum of  $10^4$  cells. The data are for a total of four experiments.

during the entire prolonged incubation period under non-growth conditions (i.e., in distilled water). In addition, the inoculum was  $10^2$  instead of  $10^4$  organisms; this reduction in the number of fungal cells present gave greater sensitivity in detecting loss of viability. The plates were incubated in a moist chamber at 37°C and then sampled on days 0, 3, 7, 14, 21, and 28 for viability of the cells after the addition of 0.1 ml of Sabouraud dextrose broth and incubation at 37°C for 24 h by examining each well for visible growth. The data were expressed as the number of wells positive for each experimental point (of four experiments).

**Quantitative assessment of fluconazole effects on viability of *C. albicans*.** For these experiments *C. albicans* yeast cells (both strains) were placed at an inoculum of  $10^4/0.1$  ml of distilled water in tissue culture plate wells containing fluconazole in concentrations of 0, 0.1, 1.0, and 5.0  $\mu\text{g/ml}$ . The plates were incubated in a moist chamber for 28 days at either 25 or 37°C. On days 0, 3, 7, 14, 21, and 28, the contents of the wells were removed, plated on Sabouraud dextrose agar plates, and incubated at 37°C for 24 h, at which time the numbers of colonies produced were counted. The data were expressed as the number of organisms remaining from the original inoculum of  $10^4$  cells.

**Statistics.** Summary descriptive statistics were calculated for each treatment group in the quantitative assessment system. Analysis of variance was used to compare the data between groups, with the Dunnett's procedure being carried out to compare each treatment group to the control group for that experiment. The chi-square test was used to evaluate data for the numbers of negative wells in treated versus untreated samples. Results were considered significant at  $P < 0.01$ .

## RESULTS

The two strains of *C. albicans* were tested in 24-h susceptibility tests against fluconazole to determine the MICs of the drug and also to evaluate fluconazole's effects on viability of the fungal cells under these conditions. As shown in Table 1, the MICs of fluconazole against both strains were 0.5  $\mu\text{g/ml}$ . The values obtained by National Committee for Clinical Laboratory Standards method M27-T (13) were 1.0  $\mu\text{g/ml}$  for both strains. The numbers of CFU remaining at higher concentrations of the drug, up to 100 times the MICs, were not reduced below the initial inoculum.

In the next series of experiments, we tested fluconazole's effects on the viability of organisms either preexposed to fluconazole or not exposed and incubated for up to 28 days under non-growing conditions (i.e., in distilled water alone, with or without 1.0  $\mu\text{g}$  of fluconazole per ml). As shown in Table 2, preincubation with either 1.0 or 5.0  $\mu\text{g}$  of fluconazole per ml did not reduce the viability of the organisms over a subsequent 28-day incubation period. However, when 1.0  $\mu\text{g}$  of fluconazole per ml was added to the distilled water for the incubations, viability during the later stages of the incubation period (from 7 days onward) was reduced. It did not seem to make any difference whether the organisms had been exposed to fluconazole beforehand; only the drug in the prolonged cultures affected the viability of the organisms.

In the next series of experiments, fluconazole's effects on fungal viability during prolonged incubations were assessed quantitatively by determining the number of CFU of the re-

TABLE 2. Effects of fluconazole exposure on viability of *C. albicans* during extended incubations

Incubation period (days)	No. of experiments with visible growth of organisms pretreated with indicated amt of FL ( $\mu\text{g/ml}$ ) and incubated without or with FL (1 $\mu\text{g/ml}$ )					
	Without FL <sup>a</sup>			With FL		
	0 <sup>b</sup>	1	5	0	1	5
0	4	4	4	4	4	4
3	4	4	4	4	3	4
7	3	4	4	1	0	1
14	4	4	4	0	0	1
21	3	3	3	1	1	0
28	3	3	3	0	0	0

<sup>a</sup> Incubations were carried out at 37°C with  $10^2$  organisms per well with or without 1  $\mu\text{g}$  of fluconazole for the indicated times.

<sup>b</sup> Preincubations with 0, 1, or 5  $\mu\text{g}$  of fluconazole (FL) per ml were carried out in RPMI 1640 medium for 4 h, and the organisms washed three times afterwards. The data are the number of experiments (four total) in which the particular well showed visible growth after the addition of Sabouraud dextrose broth and incubation at 24 h at 37°C.

maining organisms in the samples. As shown in Fig. 1 and 2, presence of fluconazole during the prolonged incubations at 37°C under the non-growing conditions markedly decreased the viability of the exposed organisms of each strain. The effect was present at concentrations of 1.0 and 5.0  $\mu\text{g/ml}$  (and at 0.1  $\mu\text{g/ml}$  for strain ATCC 26310). The numbers of wells with no growth on subculture were also found to be greater for samples containing fluconazole, as shown in Table 3. On the other hand, the effect of fluconazole in decreasing fungal viability was reduced when the incubations were carried out at 25°C, as shown in Fig. 3 and 4.

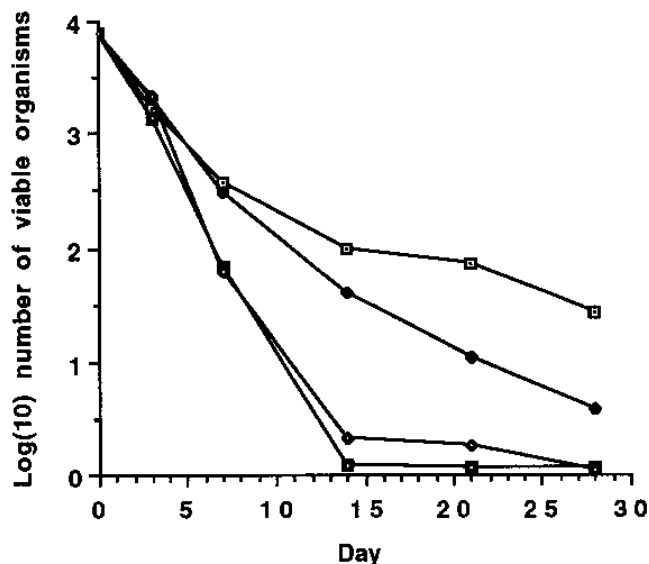


FIG. 1. Effect of fluconazole on viability of *C. albicans* ATCC 26310 ( $\square$ ) over prolonged incubation periods at 37°C. The organisms were placed into tissue culture plate wells in inocula of  $10^4$  fungal cells per 0.1 ml of distilled water containing various concentrations of fluconazole and then incubated for up to 28 days. Viability was determined by the number of CFU remaining after culture of the wells' contents on Sabouraud dextrose agar. At 28 days, the numbers of viable organisms were significantly less for the samples containing fluconazole at 0.1 ( $\circ$ ), 1.0 ( $\blacksquare$ ), and 5.0 ( $\bullet$ )  $\mu\text{g/ml}$  than for controls by analysis of variance and the Dunnett's test ( $P < 0.01$  in each case).

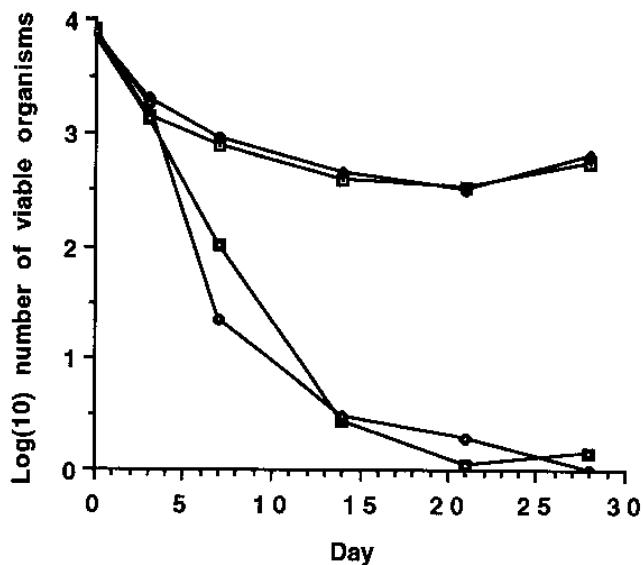


FIG. 2. Effect of fluconazole on viability of *C. albicans* TW (□) over prolonged incubation periods at 37°C. The organisms were placed into tissue culture plate wells in inocula of  $10^4$  fungal cells per 0.1 ml of distilled water containing various concentrations of fluconazole and then incubated for up to 28 days. Viability was determined by the number of CFU remaining after culture of the wells' contents on Sabouraud dextrose agar. At 28 days, the numbers of viable organisms were significantly less for samples containing fluconazole at 1.0 (■) and 5.0 (●) µg/ml than for controls by analysis of variance and the Dunnett's test ( $P < 0.01$  in each case). ◆, 0.1 µg of fluconazole per ml.

## DISCUSSION

The present studies evaluated possible fungicidal effects of fluconazole on *C. albicans* during prolonged incubations under conditions in which the organisms could not proliferate. Fluconazole and other azole drugs inhibit 14- $\alpha$ -demethylase, the enzyme responsible for conversion of lanosterol to ergosterol, the predominant component of fungal cell membranes (10,19). The resulting effect on cell membranes causes a selective inhibition of the uptake of precursors of RNA, DNA, and mucopolysaccharide (18). The original expectation in beginning this work was that a brief (4-h) exposure to fluconazole under growing conditions (RPMI 1640 tissue culture medium) might damage the organisms so that they would not last as long during prolonged incubation as would untreated organisms. Such an effect would be similar to the post-antifungal effect that has previously been described for fluconazole under certain conditions (12). However, the results from the first series of experiments demonstrated that such was not the case. Exposures to fluconazole for 4 h did not affect the subsequent viability during the 28-day period of incubation. On the other hand, continuous exposure to fluconazole during the long incubations under non-growing conditions did significantly reduce the subsequent viability of the organisms.

These studies suggest that fluconazole may inhibit some ongoing process that the organisms need to survive during long incubations. Some metabolism no doubt proceeds even when the microorganisms are held under conditions in which they cannot proliferate. It is possible that the fungal cell membranes may begin to deteriorate over prolonged cultures and need repair. Inhibition of this kind of maintenance by fluconazole might have been responsible for the reduction of viability seen during the long in vitro incubations in these experiments. Alternatively, it may be that low levels of proliferation may have occurred in the distilled water medium even in the absence of

TABLE 3. Sterilization of samples containing *C. albicans* yeast cells during extended incubations in the presence or absence of fluconazole

Incubation period (days)	No. of negative wells/total no. of wells <sup>a</sup>			
	Strain ATCC 26310		Strain TW	
	With FL	Without FL	With FL	Without FL
0	0/11	0/11	0/10	0/10
3	0/11	0/11	0/10	0/10
7	2/11	0/11	1/11	0/11
14	9/11	1/11	7/10	0/10
21	10/11	2/11	8/10	0/10
28	10/11	4/11	7/10	1/10

<sup>a</sup> Incubations were carried out in distilled water for up to 28 days at 37°C; at the end of this time the wells were plated in appropriate dilutions on Sabouraud dextrose agar to determine the number of CFU remaining from the original inoculum. Wells with no CFU were considered negative. At day 14, the numbers of negative wells were found to be significantly greater by the chi-square test for fluconazole (FL [1.0 µg/ml])-containing samples versus controls ( $P$  of  $<0.01$  for strain ATCC 26310 and  $P$  of  $<0.001$  for strain TW).

nutrients; fluconazole might have inhibited this process and prevented the emergence of new progeny with longer lifetimes. Finally, azoles may have additional effects on the target fungal cells (2); it is possible that one of these effects may relate to loss of viability during prolonged exposures, whereas the inhibition of ergosterol biosynthesis is responsible for growth inhibition under usual conditions. The fact that fluconazole reduced survival of the organisms more at 37°C than at 25°C suggests that the rate of fungal cell metabolism was somehow involved in these effects.

As noted above, the azole class of antifungal agents is generally considered to be fungistatic rather than fungicidal. Our

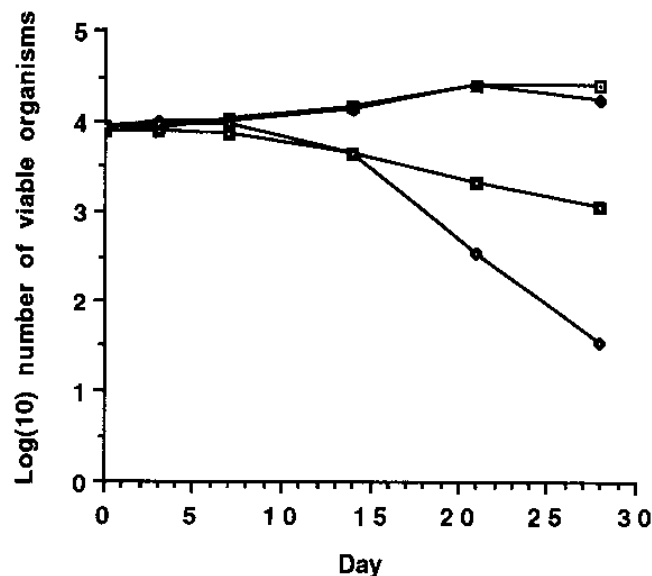


FIG. 3. Effect of fluconazole on viability of *C. albicans* ATCC 26310 (□) over prolonged incubation periods at 25°C. The organisms were placed into tissue culture plate wells in inocula of  $10^4$  fungal cells per 0.1 ml of distilled water containing various concentrations of fluconazole and then incubated for up to 28 days. Viability was determined by the number of CFU remaining after culture of the wells' contents on Sabouraud dextrose agar. At 28 days, the numbers of viable organisms were significantly less for samples containing fluconazole at 1.0 (■) and 5.0 (●) µg/ml than for controls by analysis of variance and the Dunnett's test ( $P$  of  $<0.01$  in each case). ◆, 0.1 µg of fluconazole per ml.

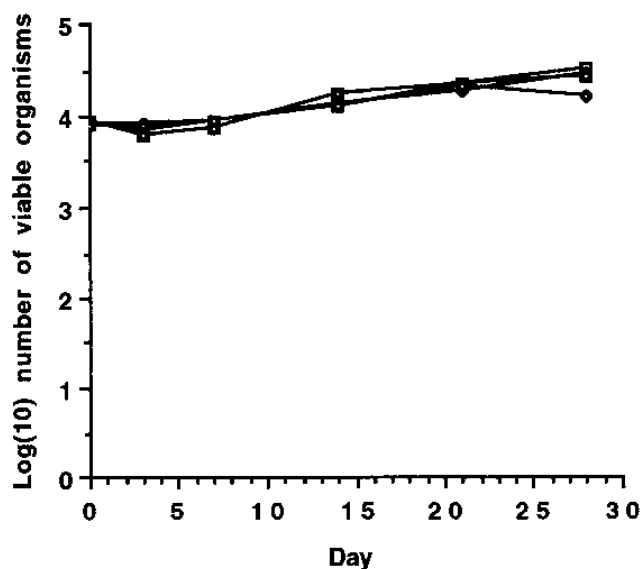


FIG. 4. Effect of fluconazole on viability of *C. albicans* TW (□) over prolonged incubation periods at 25°C. The organisms were placed into tissue culture plate wells in inocula of  $10^4$  fungal cells per 0.1 ml of distilled water containing various concentrations of fluconazole and then incubated for up to 28 days. Viability was determined by the number of CFU remaining after culture of the wells' contents on Sabouraud dextrose agar. At 28 days, the number of viable organisms was significantly less for the samples containing 5.0 µg of fluconazole per ml (◆) than for the controls by analysis of variance and the Dunnett's test ( $P < 0.01$ ). ◆ and ■, 0.1 and 1.0 µg of fluconazole per ml, respectively.

results with the two *C. albicans* strains used in these experiments indicated that fluconazole had primarily growth inhibitory rather than killing effects when tested over short incubation periods in RPMI 1640 tissue culture medium. However, at higher doses the azole class of drugs does produce fungicidal effects, even during short incubations (4,10). It is possible that the type of damage required to kill the organisms merely takes longer to occur at lower drug concentrations. Alternatively, the effect of fluconazole on fungal viability over the prolonged incubations in the present studies may be due to an entirely different mechanism.

The results from this study raise the possibility that azole antifungal drugs effect clearance of chronic fungal infections by reducing viability of the infecting organisms over long periods rather than by suppressing their growth during short ones. As discussed above, interference of these drugs with low rates of proliferation during prolonged exposures could suppress emergence of new daughter cells that are younger and therefore would live longer. Otherwise, these agents may interfere with certain maintenance functions that nondividing cells need for prolonged survival. In any event, it may be that antimicrobial agents with only microbistatic activity might hasten clearance of infections in the absence of help from the host defense system. Indeed, long courses of fluconazole have been effective in treating certain patients with chronic disseminated candidiasis even after failure with amphotericin B therapy (1,11). On the other hand, successful therapy of invasive fungal infections in severely immunosuppressed patients generally requires restoration of adequate host defenses.

In summary, prolonged exposures to relatively low concentrations of fluconazole were found to reduce the viability of

*C. albicans* yeast cells over a prolonged incubation period during which the organisms were kept from proliferating by lack of nutrients. On the other hand, short-term exposure of the organisms to the drug under conditions in which they could undergo cell division did not appear to reduce viability during a subsequent, long incubation. The exact mechanism by which fluconazole reduced fungal viability during the conditions used is unclear at the present time. However, the above findings raise the possibility that microbistatic antimicrobial drugs might effect the elimination of infecting microorganisms over extended periods without help from the host defenses.

#### ACKNOWLEDGMENTS

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