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Fluconazole versus *Candida albicans*: A Complex Relationship

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A murine model of systemic candidiasis was used to assess the virulence of serial *Candida albicans* strains for which fluconazole MICs were increasing. Serial isolates from five patients with 17 episodes of oropharyngeal candidiasis were evaluated. The MICs for these isolates exhibited at least an eightfold progressive increase from susceptible (MIC < 8 μg/ml; range, 0.25 to 4 μg/ml) to resistant (MIC ≥ 16 μg/ml; range, 16 to ≥128 μg/ml). Virulence of the serial isolates from three of five patients showed a more than fivefold progressive decrease in the dose accounting for 50% mortality and was associated with development of fluconazole resistance. Low doses of fluconazole prolonged survival of mice infected with susceptible yeasts but failed to prolong survival following challenge with a resistant strain. In addition, a decreased burden of renal infection was noted in mice challenged with two of the three resistant strains. This was consistent with reduced virulence. Fluconazole did not further decrease the level of infection. In the isolates with a decrease in virulence, two exhibited overexpression of *CDR*, which encodes an ABC drug efflux pump. In contrast, serial isolates from the remaining two patients with the development of resistance did not demonstrate a change in virulence and fluconazole remained effective in prolonging survival, although significantly higher doses of fluconazole were required for efficacy. Resistant isolates from both of these patients exhibited overexpression of *MDR*. This study demonstrates that decreased virulence of serial *C. albicans* isolates is associated with increasing fluconazole MICs in some cases but not in others and shows that these low-virulence strains may not consistently cause infection.

Antifungal susceptibility testing has been recently standardized for yeasts. The currently used National Committee for Clinical Laboratory Standards macrobroth and microbroth methods allow for comparison of identical yeast isolates in different laboratories and also allow for serial testing of isolates recovered from one patient in one laboratory (17, 18). The ultimate value of such testing lies both in facilitating communication among laboratories and in determining whether test results offer some correlation with clinical outcome from antifungal therapy. There is some correlation of fluconazole in vitro susceptibility with fluconazole clinical response (1, 13, 14, 19). In oropharyngeal candidiasis, an in vitro MIC of ≤8 μg/ml is associated with clinical responses of more than 90% to a daily dose of 100 mg. MICs of 16 and 32 μg/ml are associated with responses to higher doses of fluconazole, while a MIC of >64 μg/ml may be associated with failures at doses exceeding 400 mg per day. However, some patients with in vitro resistant organisms respond to lower doses of fluconazole. In a population of 43 patients, Revankar et al. (16) observed the development of fluconazole-resistant *Candida albicans* isolates in 7 of 15 patients treated with continuous suppressive therapy and in 11 of 28 patients treated with fluconazole only for active thrush. Although 20 of 43 patients required increasing doses for treatment of thrush, only 2 patients developed fluconazole refractory thrush. Higher fluconazole doses were frequently, but not always, required in patients with less-susceptible yeasts. These findings suggest that factors other than in vitro susceptibility may be important in response to therapy, including changes in host immune response, virulence of the organisms, or other factors not yet defined.

In an initial study of two serial isolates with identical DNA patterns obtained from a patient with first responsive and then refractory thrush, we found that the isolate obtained from the patient with refractory thrush was resistant both in vitro and also in an animal model of candidemia (6). However, it was not clear whether this response could be generalized to other patients. Accordingly, in the present study, we expanded murine studies of virulence and response to fluconazole for serial *C. albicans* isolates from five human immunodeficiency virus-positive patients with recurrent thrush.

MATERIALS AND METHODS

Selection of isolates. Fungal isolates were chosen from five patients. Isolates were chosen at various times during a course in which increasing doses of fluconazole were required to achieve clinical response. Serial isolates obtained from each patient reflected the majority population of *Candida* isolated from the mouth of each patient at the time of culture. All five patients responded to fluconazole at doses of 800 mg/day or less. Strain identity was determined by pulsed-field gel analysis of chromosomal DNA and by restriction fragment length polymorphisms as described previously (15).

In vitro testing. The National Committee for Clinical Laboratory Standards procedure for fluconazole susceptibility testing was used, with macrobroth MICs at both 24 and 48 h of incubation reported (16, 17).

Animal model. ICR outbred male mice weighing approximately 30 g each were used. Groups of five mice were caged together and given food and water ad libitum. For survival experiments, unmanipulated mice were initially infected intravenously with *C. albicans* at 10^6 CFU/mouse and observed for up to 30 days for survival. As it became apparent that there was a wide range of survival, even among isolates obtained from the same patient, we further characterized each isolate for virulence using the LD_{50}, defined by probit analysis as the dose which accounted for 50% mortality. We measured LD_{50} at 8 days after infection in order to assess acute mortality. For these studies, groups of five mice for each dose were infected with *C. albicans* intravenously at 5×10^4, 1×10^5, 5×10^5, 1×10^6, 5×10^6, 1×10^7, or 5×10^7 CFU of *C. albicans*/mouse.

Because some isolates did not kill mice by 8 days after infection, even with an infecting dose of 5×10^7 CFU/mouse, we elected to compare responses to

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TABLE 1. Characterization of *C. albicans* isolates obtained from patients 7, 14, 43, 9, and 40 treated with fluconazole

Patient and isolate	Sample collection date (mo/day/yr)	FLU dose[a] (mg/day)	MIC (μg/ml)[b]		LD_{50} (10^6 CFU/mouse) at 8 days	DNA strain identity
			24 h	48 h		
Patient 7						
412	2/15/95	100	<0.125	0.5	9	A
1907	9/13/95	200	4	8	6	A
2307	11/22/95	400	>128	>128	50	A
Patient 14						
580	3/13/95	100	<1	4	7	B
649	3/20/95	200	4	16	0.7	B
2438	1/3/96	200	16	32	>50	B
Patient 43						
1649	7/19/95	100	0.25	0.25	2.4	C
1831	9/1/95	100	4	8	>50	C
2183	10/27/95	100 (F)[c]	32	>128	>50	C
Patient 9						
437	2/16/95	100	0.5	1	0.5	D
1002	4/18/95	100	0.25	0.25	3.4	D
1442	6/15/95	100	≤0.125	≤0.125	0.3	D
2271	11/6/95	100	8	16	1.9	D
2823	4/2/96	800	32	16	0.6	D
Patient 40						
1622	7/10/95	100	0.5	0.5	1.3	E
2225	11/2/95	200	4	4	0.9	E
2512	12/21/95	800	32	32	2.1	E

[a] FLU dose, fluconazole dose required to suppress thrush.
[b] MICs of fluconazole in macrobroth culture after 24 or 48 h of incubation.
[c] (F), failed 100 mg of fluconazole/day when this isolate was obtained.

fluconazole with immune suppressed mice given a standard inoculum of 10^6 CFU/mouse. For these experiments, we pretreated mice with 5-fluorouracil (150 mg/kg of body weight) given intravenously 1 day before infection. This reduces the peripheral blood neutrophil count to <100 CFU per μl for about 10 days. We considered that immune suppression would reduce the contribution of host defense to outcome and provide a further measure of virulence of the fungal isolates. Mice were infected intravenously. One day after infection, mice were randomly assigned to groups of 10 for treatment with either 0.3% Noble agar (control) or fluconazole at 1, 3, or 5 mg/kg twice daily by gavage in a 0.2-ml volume. Separate controls were used for each experimental group. Mice found moribund were sacrificed, and death was recorded as occurring on the next day. Treatment of mice was continued from day 1 through 10, and observation for survival was continued through day 30.

For study of renal tissue burden, immune competent mice were infected intravenously with approximately 10^6 CFU of *C. albicans* per mouse and treated from days 1 through 7 with fluconazole at 5 mg/kg twice daily by gavage. Controls received 0.3% Noble agar. On day 8, mice were sacrificed. Both kidneys were removed aseptically and homogenized in 2 ml of sterile saline, and tissue burden was determined by colony count dilutions using 0.1-ml aliquots. Where there were no counts in the initial culture dilution, the entire 2-ml volume was cultured to determine total organ tissue sterility.

Analysis. The Wilcoxon test of life tables and log rank tests were used for comparison of survival times. Tukey's standardized range test was used for comparisons of tissue counts. A P of <0.05 was required for confirmation of statistical significance. Probit analysis with best fit was used to determine the LD_{50} of *C. albicans*. A rise of LD_{50} was defined as decreased virulence. We arbitrarily defined an LD_{50} of $<5 \times 10^6$ CFU/mouse as a highly virulent isolate.

RESULTS

Patients were selected on the basis of initially responding to a dose of 100 mg of fluconazole per day, then failing this dose, and requiring higher doses, up to 800 mg/day, for successful treatment of thrush. Isolates from each patient were characterized; these data are presented in the tables. Table 1 characterizes the isolates from each patient according to the date of collection of each isolate, dose of fluconazole needed to achieve clinical response, and DNA subtype of the isolate (12). In addition, virulence of the isolate by LD_{50} (see Table 1), response of neutropenic mice to 1 or 5 mg/kg twice daily, as measured by prolonged survival compared with that of untreated controls (see Table 2), and response of immune competent mice to fluconazole at 5 mg/kg twice daily, as measured by reduction of renal tissue counts (see Table 3), are shown. Additional experiments studying the effect of decreasing the dose on survival were done; the dose was decreased to a minimum of 0.1 mg/kg given twice daily for isolates from two patients (see Table 2).

As assessed by DNA typing of *Candida* isolates obtained at different dates from the same patient, the same unique isolate was recovered serially from each patient. Five patients and the 17 isolates from these patients were studied. Three patients (patients 7, 14, and 43) showed a marked rise in LD_{50} to $>5 \times 10^7$ CFU/mouse in four isolates (Table 1). In each of these patients, low-virulence isolates correlated with increasing MICs. The 48-h MICs for the initial isolates were 0.5, 4, and 0.25 μg/ml, while the MICs of serial isolates with decreased virulence rose to >128, 32, and >128 μg/ml, respectively.

In patients 7 and 14, the in vivo response to fluconazole correlated with the in vitro MIC. Both the 1- and 5-mg/kg doses of fluconazole prolonged survival in the susceptible isolates but low-dose fluconazole at 1 mg/kg failed to prolong survival of neutropenic mice with the resistant isolate from patient 7 and at both 1 and 5 mg/kg in the resistant isolate from patient 14 (Table 2). In addition, fluconazole at 5 mg/kg did not significantly reduce the renal tissue counts of immune competent mice infected with fluconazole-resistant isolates from patients 7 and 14 (Table 3). Notably, these isolates from both patients 7 and 14 produced renal counts in untreated control mice which were significantly lower than for the earlier,

TABLE 2. Survival of neutropenic mice infected with isolates from different patients[a]

Patient and isolate	Mean survival (no. of days) ± SEM of mice given the following FLU[b] doses (mg/kg):					
	0 (control)	5	1	0.5	0.25	0.1
Patient 7						
412	5 ± 0.3	17 ± 3[c]				
	5 ± 0.5		24 ± 1.7[c]			
1907	8 ± 3	18 ± 3[c]				
2307	8 ± 0.7	28 ± 2.3[c]	7 ± 0.4			
	10 ± 2		9 ± 1			
Patient 14						
580	7 ± 2	23 ± 4[c]				
	9 ± 3		13 ± 2[c]			
649	9 ± 1	24 ± 3[c]				
2438	20 ± 4	23 ± 4				
	16 ± 4		17 ± 3			
Patient 43						
1649	15 ± 3	28 ± 4[c]				
	7 ± 1		30 ± 1[c]			
1831	11 ± 2	26 ± 3[c]				
2183	13 ± 3	29 ± 1[c]				
	6 ± 1		6 ± 1			
Patient 9						
437	3 ± 0.3	14 ± 3[c]				
	2 ± 0.2		6 ± 1[c]			
1002	6 ± 3	10 ± 3[c]				
1442	3 ± 0.3	13 ± 3[c]				
	5 ± 0.2			12 ± 2[c]	13 ± 3.9[c]	4 ± 0.4
2271	3 ± 0.2		18 ± 5[c]			
2823	4 ± 0.4	10 ± 2[c]				
	7 ± 0.7		11 ± 0.7[c]			
	4 ± 0.3			5 ± 0.6	5 ± 0.5	4 ± 0.2
Patient 40						
1622	5 ± 0.4	19 ± 4[c]				
	5 ± 0.4		21 ± 2[c]			
	5 ± 0.5			13 ± 2.9[c]	8 ± 1.2[c]	6 ± 0.4
2225	4 ± 0.5	8 ± 2[c]				
2512	5 ± 0.6	17 ± 2[c]				
	17 ± 2		12 ± 3[c]			
	5 ± 0.3			4 ± 0.4	5 ± 0.2	4 ± 0.5

[a] Each mouse was infected with 10^6 CFU of *C. albicans* isolate.
[b] FLU, fluconazole.
[c] Significantly different from the control value ($P < 0.05$).

more-virulent isolates. In isolates from patient 43, which were identical by DNA typing, decreased virulence was also observed with the resistant strain and fluconazole at 1 mg/kg twice daily failed to prolong survival. However, the resistant strain produced a significant renal burden in controls, which was reduced with fluconazole therapy at 5 mg/kg/dose.

In isolates 1907 (MIC = 8 μg/ml) and 2307 (MIC > 128 μg/ml) from patient 7 and isolates 1831 (MIC = 8 μg/ml) and 2183 (MIC > 128 μg/ml) from patient 43, overexpression of *CDR*, encoding an ABC drug efflux pump, was detected but none exhibited overexpression of *MDR* (12). Decreased virulence as assessed by LD_{50} was seen in three of those four strains. In isolate 2438 (MIC = 32 μg/ml) from patient 14, overexpression of known resistance genes was not detected (12), although virulence was decreased.

Isolates from patients 9 and 40 also showed a late rise in fluconazole MICs with serial isolates (Table 1). However, isolates from both of these patients remained virulent to mice and fluconazole was effective at 1- and 5-mg/kg doses in prolonging survival (Table 2). Doses of fluconazole as low as 0.25 mg/kg were effective in prolonging survival in susceptible strains, whereas only the 1- and 5-mg/kg doses were effective in the resistant strains. The renal burden of *Candida* was reduced with fluconazole at 5 mg/kg in the susceptible and resistant strains from these two patients (Table 3). Resistant isolates from both of these patients exhibited overexpression of *MDR* (12).

DISCUSSION

The results of this study indicate that the relationship of pathogen, host, and antifungal therapy is complex. Patients with thrush refractory to 100 mg of fluconazole per day usually have oropharyngeal *C. albicans* for which MICs are above 8 μg/ml (17, 18). *Candida* spp. other than *C. albicans* may cause thrush, but this is less common. The role of fluconazole-resistant *C. albicans* is thus unquestionably important in clinically refractory disease. However, not every patient with even highly resistant *C. albicans* fails fluconazole treatment. Rex et al. (19) found that more than half of human immunodeficiency virus-infected patients with resistant organisms responded to fluconazole therapy. Revankar et al. (16) found that development of fluconazole resistance in oropharyngeal *C. albicans* isolates during the course of chronic fluconazole preventive therapy is common. However, few of these patients had relapses despite the presence of resistant isolates, as contrasted with patients who were treated only intermittently for episodes of thrush and had relapses more frequently. This murine model of systemic candidiasis was used to evaluate the response to fluconazole and virulence of these isolates.

The aim of this study was to determine host-pathogen outcome in a setting in which host variables could be controlled.

TABLE 3. Total kidney burdens of mice[a]

Patient and isolate	Median total kidney burden (10^5 CFU/mouse) (range) of mice	
	Control	Treated with FLU[b]
Patient 7		
412	260 (79–800)	0.04[c] (0.01–3.1)
1907	230 (100–730)	4.4[c] (1.1–8)
2307	0.005 (<0.001–0.003)	0.0003 (<0.001–0.002)
Patient 14		
580	24 (7.1–120)	0.8[c] (0.34–4.6)
649	5.5 (0.008–55)	2.3[c] (0.086–7.5)
2438	0.15 (0.44–120)	0.5 (0.11–4.1)
Patient 43		
1649	6.6 (2–21)	0.03[c] (0.002–5.4)
1831	47 (27–170)	11[c] (1.8–29)
2183	57 (10–320)	3[c] (0.92–9.4)
Patient 9		
437	4.9 (1.9–7.8)	0.03[c] (0.012–0.34)
1002	2.6 (1.3–10)	0.018[c] (0.009–0.06)
1442[d]	3.0 (0.6–9.6)	0.054[c] (0.015–10)
2271	11 (7.1–52)	0.022[c] (0.011–0.5)
2823	3.2 (0.7–51)	0.14[c] (0.09–0.8)
Patient 40		
1622	9.9 (0.02–291)	0.01[c] (0.01–3.1)
2225	6.6 (0.8–609)	0.1[c] (0.03–0.7)
2512	10.4 (02.7–43)	0.07[c] (0.01–0.35)

[a] Total kidney burdens of mice on day 8 after intravenous infection of immune competent mice with 10^6 CFU of *C. albicans* isolate per mouse.
[b] Mice were treated with 5 mg of fluconazole (FLU) per kg.
[c] Significantly different from the control value ($P < 0.05$).
[d] An error was made for mice infected with isolate 1442; the infecting dose was 10^7 CFU/mouse.

This was done by the following: (i) using DNA typing in *C. albicans* strains serially isolated, (ii) determining which isolates were associated with resistance in distinct strains, and (iii) controlling variables such as growth phase of the organism, size of inoculum, the host immune status, and uniformity of both schedule and dose of therapy (4–6). An initial study (5) suggested that this was feasible, and in that study, the in vitro susceptibility of *C. albicans* and in vivo outcome were closely related (5). However, when we expanded our focus of study to a library of 17 isolates collected serially from five patients, the results were surprising.

First, the isolates from all patients required (by definition) increasing fluconazole MICs and these patients all had a clinical requirement for increasing doses of fluconazole. The need for higher fluconazole doses in all five patients was associated with rising in vitro MIC, measured at either 24 or 48 h of incubation. The 48-h MIC was ≥4 times higher than the 24-h MIC for only 4 of the 17 isolates, and for isolates from two of these patients (patients 7 and 14), the 24-h MIC was 16 μg/ml, indicating decreased fluconazole susceptibility. Therefore, for these patients, discordance of 24- and 48-h MIC results (trailing end points) was not observed.

In three patients (patients 7, 14, and 43), the isolates requiring higher fluconazole MICs also had high LD_{50}s, indicating a progressive decrease in virulence. In patients 7 and 14, the high LD_{50} in one isolate from each patient was associated with a decreased fluconazole response of mice treated with either the 1- or 5-mg/kg dose. However, in patient 43, (who had two low-virulence isolates), all three isolates were successfully treated in mice with 5 mg/kg. The lower 1-mg/kg dose was ineffective in the resistant isolate 2183. Notably, despite the decreased virulence of isolates 1831 and 2183 from patient 43, the burden of infection in kidney tissue of untreated mice was greater than that seen in the baseline isolate from patient 43. The mechanisms of this disparity are unclear.

Therefore, only the isolates which had low virulence and had high fluconazole MICs were associated with fluconazole failure (at 1 and 5 mg/kg/doses) when they were tested in mice with candidal infections. Mice infected with the fluconazole-susceptible isolates from these three patients had a good response to fluconazole both in survival and reduction of renal tissue counts. The primary mechanism of resistance in isolates from these patients did not involve overexpression of *MDR*, which encodes an efflux pump with apparent selectivity for fluconazole (12). Overexpression of *CDR* was detected in three of the four isolates with decreased virulence from patients 7, 14, and 43.

Two additional patients (patients 9 and 40) had an increase in the in vitro MIC and required higher doses of fluconazole for clinical response. However, all isolates were of high virulence. Mice infected with eight isolates obtained from these patients responded well to fluconazole, at a dose as low as 1 mg/kg. Only when fluconazole doses were extended down to a minimum of 0.1 mg/kg in survival experiments did a difference between susceptible and resistant isolates emerge. In isolates from patients 9 and 40, the mechanism of resistance appeared to involve overexpression of *MDR*. Three isolates from patient 40 required an MIC of ≥8 μg/ml, but only one of these isolates was tested in vivo.

Therefore, there appear to be two patterns of fluconazole response in these patients. In the first patients (patients 7, 14, and 43), the emergence of fluconazole resistance in vitro and clinically was associated with a decrease in the virulence of the isolates and a reduced response to fluconazole therapy in mice. In the second pattern, mice responded to rather low doses of fluconazole, despite the high fluconazole MICs for some isolates. These isolates were all virulent. This suggests a link in some isolates between fluconazole resistance and virulence. One might anticipate that a mutation could affect both virulence and fluconazole susceptibility. However, a single mutation did not explain all findings. For example, patient 9 had identical DNA typing results for five isolates, but there was no loss of virulence with in vitro resistance, and in vivo responses showed resistance only at very low doses of fluconazole.

Fluconazole resistance is mediated by multiple mechanisms. These mechanisms may include overexpression of the target enzyme, mutation of the target enzyme, or exclusion of fluconazole from fungal cells (11, 19). The last may be the most important mechanism and may be mediated by one or more proteins which serve to pump fluconazole out of fungal cells (9). Increased efflux of drug is mediated by multidrug pumps belonging to two different families, the major facilitators and the ATP-binding cassette (ABC) transporters (7, 9, 20, 21). The genes coding for several ABC transporters in *C. albicans* have been identified, including some *CDR* genes (20). These ABC transporters, which have been associated with drug resistance in a variety of eukaryotic cells, include a membrane pore of transmembrane segments and two ATP-binding cassettes on the cytosolic side of the membrane, which provide the energy source for the pump (3, 7). The *MDR1* gene (also called *BEN*^{r}) is the only gene coding for a major facilitator that has been identified in *C. albicans*, and its overexpression leads to fluconazole resistance exclusively among azole drugs (20, 21). The major facilitators contain a transmembrane pore but use proton motive force as the energy source (7). These pumps may normally serve to translocate lipids and hydrophobic compounds across cell membranes (7). However, they also serve to facilitate resistance to multiple unrelated pharmaceutical agents and are thus designated MDR (for multidrug resistance). One such pump in *C. albicans* is the product of the *MDR1* gene. It is not essential for survival of *Candida*, in that homozygous disruptants are viable (7).

In addition, in a recent study, Becker et al. (7) created heterozygous and homozygous disruptants of a *C. albicans mdr1*/*mdr1* clinical isolate. However, the disruptant (*mdr1*/*mdr1*) was much less virulent to mice than the parent isolate (*MDR1*/*MDR1*). If the *MDR1* gene confers virulence on *C. albicans*, this gene is not likely to be involved in our isolates from patients 7, 14, and 43, in which virulence decreased as fluconazole resistance emerged. This may argue for the presence of another pump (we demonstrated CDR gene overexpression) or a completely different mechanism affecting both fluconazole resistance and virulence, as was demonstrated in these isolates (12). Changes in ligands for phagocyte receptors, potential to elicit cytokine production, morphotype, phospholipases, or other factors could account for altered virulence of some isolates (2, 3, 8, 10, 13).

In the second pattern of resistance, although the fluconazole MIC increased for patients 9 and 40 and these patients required higher doses of fluconazole, the isolates remained virulent and the mice responded to low doses of fluconazole. It is surprising and unexplained that the patients would require more fluconazole for clinical response and yet show fluconazole failure only at extremely low drug doses for these "resistant" isolates. In these isolates, overexpression of *MDR* did not alter virulence or ability to infect tissue.

One other potential reason for this may be that the *C. albicans* clinical infections occurred in patients with defective cell-mediated immunity and thrush, while we studied a less relevant model of disseminated infection in mice with neutropenia, an immune deficiency not specifically related to thrush in AIDS.

In summary, the responses seen in our experiments are more

varied than we had earlier thought and suggest that fluconazole resistance in some patients may be linked to decreasing virulence. The mechanism(s) for such a relationship at present remains unknown but may relate to hyperexpression of a pump in the CDR family, a group which mediates a variety of transport processes.

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