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Comparative Resistance of *Candida albicans* Clinical Isolates to Fluconazole and Itraconazole In Vitro and In Vivo in a Murine Model

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Relationships between azole susceptibility and in vivo response to antifungal therapy in a murine model of candidiasis were investigated for *Candida albicans* isolates sampled from human immunodeficiency virus type 1-positive patients with oropharyngeal candidiasis. The susceptibilities of seven clinical isolates and two reference strains to fluconazole (FCZ) and itraconazole (ITZ) were determined in vitro by the broth microdilution method. Four isolates were resistant to FCZ and ITZ, two were susceptible to both azoles, and three were resistant to FCZ and susceptible to ITZ (dissociated resistance). CD1 mice were inoculated with each isolate and treated with either FCZ or ITZ (drug regimen, 5 mg/kg of body weight twice daily for 5 days). Quantitative cultures of kidneys were performed at the end of the treatment. On the other hand, the survival rates of the mice were followed daily. These two parameters were clearly correlated with in vitro susceptibility. Thus, the phenomenon of a dissociation of resistance to FCZ and ITZ may be found in vivo as well as in vitro.

Oropharyngeal candidiasis (OPC) caused by *Candida albicans* is one of the most frequent opportunistic infections occurring in human immunodeficiency virus type 1 (HIV-1)-infected patients (4, 7); most of these patients (probably >90%) present with candidiasis during the last stage of HIV-1 infection (6). Among the azole drugs, fluconazole (FCZ) and itraconazole (ITZ) exhibit satisfactory safety and efficacy. However, the emergence of clinical resistance during long-term therapy has recently been reported (3, 4, 7, 13). Studies have assessed the importance of in vivo antifungal susceptibility testing in order to discriminate microbiological resistance from host factors that could interfere with failure of the antifungal therapy (8, 15). In order to analyze the FCZ-ITZ cross-resistance of *C. albicans* isolates, we have selected isolates from HIV-1-positive patients with recurrent OPC, despite the administration of large doses of FCZ or ITZ. In vitro antifungal susceptibility testing was performed by a broth microdilution method. The isolates exhibited various susceptibilities to the two azoles, with, in some cases, dissociated resistance to FCZ and ITZ. They were then tested in a hematogenous model of candidiasis in mice in order to analyze these differential resistances to FCZ and ITZ in vivo.

MATERIALS AND METHODS

***C. albicans* isolates.** Seven clinical *C. albicans* isolates obtained from HIV-1-positive patients with recurrent OPC were selected during the clinical course of candidiasis while the patients were under treatment with an azole, either FCZ or ITZ. The treatment and clinical observations on the sampling days and 10 days later are summarized in Table 1. Three isolates were obtained from three different patients with differences in their OPC symptoms: isolate B (from a patient presenting with OPC on the sampling day and cured with FCZ at 100 mg/day), isolate C (from a patient presenting with OPC under therapy with FCZ at 600 mg/day and cured with ITZ at 200 mg/day), and isolate D) from a patient presenting with OPC under therapy with FCZ at 200 mg/day and cured with intravenous amphotericin B deoxycholate at 50 mg/day). Two pairs of isolates (isolates A1 and A2 and isolates E1 and E2) were obtained from two pairs of patients. It was demonstrated elsewhere, by using multilocus enzyme electrophoresis of 12 genetic loci, that each pair of isolates was genetically identical (13). Two strains, referred to as Rr (in vivo and in vitro azole-resistant strain obtained from J. Van Cutsem and referred to as NCPF 3363 [16]) and Sr (azole-susceptible strain ATCC 44858 [18]), were used as resistant and susceptible controls, respectively.

Antifungal susceptibility testing. The susceptibilities of the isolates to FCZ and ITZ were determined by a broth microdilution method derived from the technique of Anaissie et al. (2). RPMI medium (Sigma Chemical Co., St. Louis, Mo.) with L-glutamine and without $NaHCO_3$ was used. The powder was dissolved in 1 liter of deionized water, and medium was buffered with morpholinepropanesulfonic acid (MOPS; 34.53 g/liter; Sigma). The medium was adjusted to a final pH of 7.3, filter sterilized, and stored at 4°C. Stock solutions of FCZ (Pfizer Central Research, Sandwich, United Kingdom) and ITZ (Janssen Pharmaceutica, Beerse, Belgium) were prepared from reagent-grade powders by using deionized water as a solvent for FCZ and 100% dimethyl sulfoxide (DMSO; Sigma) as a solvent for ITZ; initial stock concentrations were 1 mg/ml. Drugs were diluted 10-fold in RPMI medium to provide a working solution for each drug of 100 μg/ml; a second working solution of ITZ (3.12 μg/ml) was also prepared. Broth microdilution susceptibility testing in 96-well microtiter trays was then performed as follows. Microtiter plates were inoculated with 150 μl of RPMI medium. Ten twofold serial dilutions were made: the concentrations ranged from 50 to 0.097 μg/ml for FCZ and from 50 to 0.097 and 1.56 to 0.003 μg/ml for ITZ. Microtiter plates were inoculated with 10 μl of a yeast inoculum of 5×10^5 CFU/ml. Twenty microliters of 2% formaldehyde solution was dispensed into column 1 (blank control). Drug-free medium in column 12 served as a growth control. Plates were incubated at 28°C for 48 h. Spectrophotometric readings were performed with a UV Max Kinetic Microplate Reader (Molecular Devices) at 490 nm after 5 min of agitation. The MIC endpoint was defined as the first drug concentration at which turbidity in the well was more than 50% less than that in the growth control well (MIC). All isolates were tested in duplicate on each plate, and each experiment was repeated twice. On the other hand, the susceptibilities of the isolates to the antifungal agents were also tested by using the M27-P guideline of the National Committee for Clinical Laboratory Standards (14).

Animals and in vivo experimentation. Female CD1 mice (Charles Rivers, Cléon, France) were used; they were 6 to 8 weeks old and weighed 30 g (average). Two days before inoculation, the *C. albicans* isolates were plated onto Sabouraud-chloramphenicol agar (Sanofi Diagnostic Pasteur, Marnes-La-Coquette, France), and the plates were incubated at 28°C. The yeasts were then sterilely collected from the agar, numbered, and adjusted to 5×10^7 blastospores per ml in sterile normal saline. The yeast inoculum (200 μl of sterile normal saline containing 10^7 blastospores) was injected into the lateral tail vein of the

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TABLE 1. Clinical data

Isolate	Treatment BS (drug, dosage [mg/day])[a]	OPC BS	Treatment (drug, dosage [mg/day])[b]	OPC AS[c]
A1 (IVP 1454-15H)	FCZ, 400	+	ITZ, 200	−
A2 (IVP 1454-22H)	ITZ, 400	+	AmB,[d] 50	−
B (IVP 1714)	0	+	FCZ, 100	−
C (IVP 1639-6)	FCZ, 600	+	ITZ, 200	−
D (IVP 1680-3)	FCZ, 100	+	AmB, 50	−
E1 (IVP 2045-11B)	ITZ, 400	+	ITZ, 600	+
E2 (IVP 2045-13D)	ITZ, 600	+	ITZ, 600	+

[a] BS, before sampling.
[b] This treatment was introduced on the sampling day.
[c] AS, registered 10 days after sampling.
[d] AmB, intravenously administered amphotericin B deoxycholate.

mouse. Groups of 45 mice each were injected with one of the *C. albicans* isolates. Fifteen mice received FCZ orally, 5 mg/kg of body weight twice a day, solubilized in sterile normal saline. Fifteen mice received ITZ orally, 5 mg/kg twice a day, solubilized in DMSO and dispersed in polyethylene glycol (final DMSO concentration, 5%; preliminary results demonstrated the innocuousness of this dose in our model). The remaining 15 mice, considered controls, received no treatment. Treatments were initiated 12 h after inoculation and were repeated twice daily for 5 days. On the day following the last treatment, five mice in each group were anesthetized and sacrificed by cervical dislocation, and their left kidneys were isolated, weighed, and homogenized with 20 ml of sterile normal saline (Ultra-Turrax; Janke & Kunkel, Ita Werk, Germany). The samples were then serially diluted and plated onto Sabouraud-chloramphenicol agar; after 48 h of incubation at 28°C, the numbers of CFU per gram of kidney were determined. For the remaining mice, survival times were monitored for 4 weeks.

Statistical analysis. Differences in the numbers of CFU per gram of kidney were analyzed by the Student *t* test.

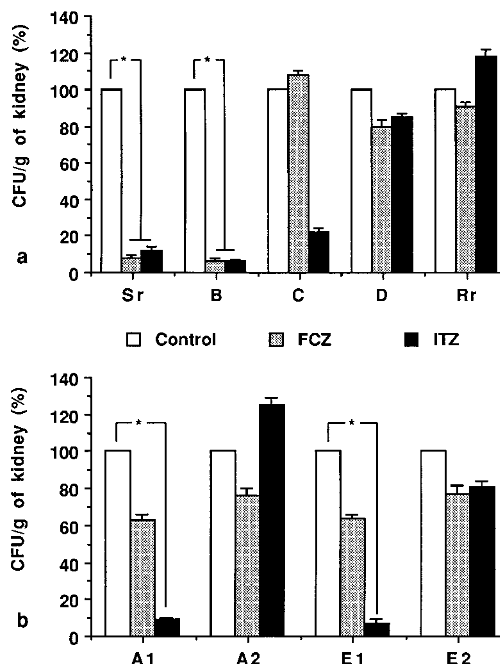

FIG. 1. Development of disseminated candidiasis on the day following the last treatment, analyzed as the numbers of CFU of *C. albicans* per gram of kidney. Results are presented as the percentages of the values obtained from five kidneys sampled on the day following the last treatment (control without treatment was referred to as 100%). *, significant differences ($P < 0.01$). FCZ, fluconazole-treated mice; ITZ, itraconazole-treated mice.

RESULTS AND DISCUSSION

In vitro susceptibility to antifungal agents. The MICs obtained for the seven clinical isolates and the two reference strains are presented in Table 2. The breakpoint retained for the distinction between resistance and susceptibility were those described elsewhere (11, 13) (Table 2). The isolates were then classified as either resistant (FCZ and ITZ resistant), susceptible (FCZ and ITZ susceptible), or dissociated (FCZ resistant and ITZ susceptible). The patterns in terms of the clinical success or failure of FCZ and ITZ therapy and in vitro susceptibility or resistance to FCZ and ITZ were similar, as described elsewhere (8). Moreover, resistance to FCZ and ITZ was dissociated for some isolates (namely, isolates A1, C, and E1). Because the treatment for OPC was FCZ and then ITZ (except for isolate D), we probably selected only isolates resistant to FCZ and susceptible to ITZ. The results obtained by the micromethod did not differ from those obtained by the method of the National Committee for Clinical Laboratory Standards, at least for FCZ; for ITZ, slight differences were observed, mainly for the ITZ-resistant isolates (Table 2).

TABLE 2. MICs for the *C. albicans* isolates tested

Isolate	MIC (μg/ml)[a]	
	FCZ	ITZ
Sr	0.39/NT	0.012/NT
Rr	25/25	1.56/0.78
A1	>50/>50	0.097/0.78
A2	>50/>50	1.56/0.39
B	0.78/0.195	0.012/0.195
C	>50/>50	0.097/0.195
D	12.5/>50	1.56/0.195
E1	50/>50	0.195/0.39
E2	>50/>50	1.56/0.78

[a] For values to the right of the slash, antifungal susceptibility was tested by the method of the National Committee for Clinical Laboratory Standards. For values to the left of the slash, antifungal susceptibility was tested by the broth microdilution method. NT, not tested.

In vivo susceptibility to antifungal agents in the mouse model. The susceptibilities of the different isolates to azoles were tested in a mouse model of disseminated candidiasis. On the day following the last treatment day, the development of disseminated candidiasis was analyzed by determining the numbers of CFU per gram of kidney (Fig. 1a and b). This analysis showed that for the isolates defined to be resistant and susceptible in vitro, the doses and regimens for the treatments were convenient, since significant differences (greater than 1 $\log_{10}$) in the numbers of CFU per gram of kidney were observed between control and treated mice inoculated with the susceptible isolates (Fig. 1a). For isolate C, the results were similar to the results obtained in vitro, since treatment with ITZ led to lower numbers of CFU per gram of kidney, while FCZ treatment resulted in numbers of CFU per gram of kidney similar to those obtained in control mice (Fig. 1a). For the two pairs of genetically identical isolates which were classified as dissociated (isolates A1 and E1) and resistant (isolates A2 and E2) after in vitro analysis, the results obtained with the mouse model corroborated the in vitro data (Fig. 1b). Such a result, already observed in vitro, was thus confirmed in vivo; it could be explained by the fact that although the isolates were very similar from a genetic point of view (as determined by multilocus enzyme electrophoresis), the isolates were phenotypically different.

The survival of the mice was monitored for 4 weeks after *C. albicans* inoculation (Fig. 2). Death of the mice in the control groups began after the 6th to the 9th days following inocula-

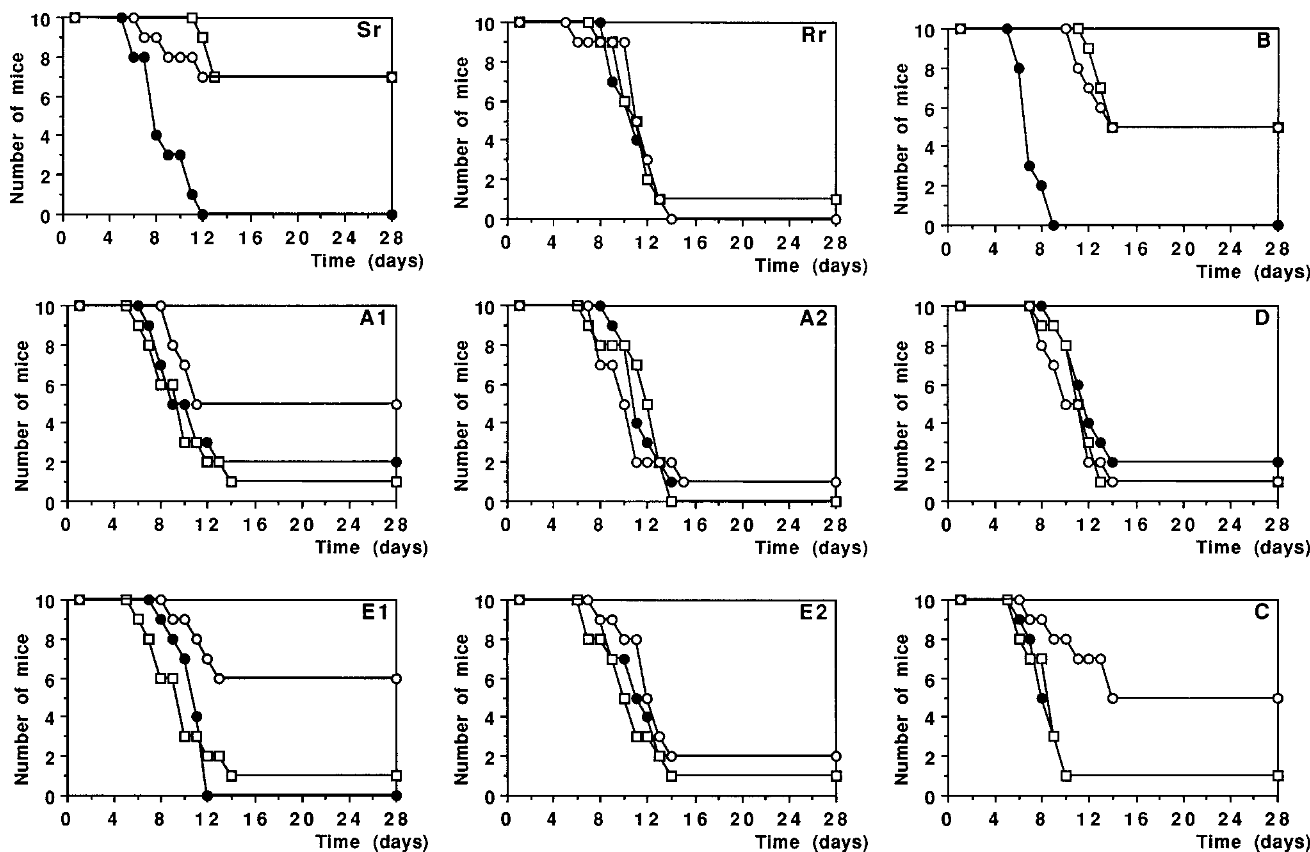

FIG. 2. Survival of mice inoculated with the different isolates. Rr, resistant reference isolate; Sr, susceptible reference isolate; B, FCZ- and ITZ-susceptible isolate; A1, C, and E1, FCZ-resistant, ITZ-susceptible isolates; A2, D, and E2, FCZ- and ITZ-resistant isolates. ●, no treatment; □, FCZ at 5 mg/kg twice daily for 5 days; ○, ITZ at 5 mg/kg twice daily for 5 days.

tion, depending on the isolate tested. Such differences in the pathogenicities (measured as the beginning of mouse deaths in the control groups of mice) of the isolates have already been described (11). Recently reported studies conducted with mutants of the same strains showed that resistant mutants had lower pathogenicities (5, 9); in our study, it appeared that the survival times of mice inoculated with susceptible isolates are shorter than those of mice inoculated with resistant isolates (Fig. 2; survival time of isolate B, ≤8 days; survival time of isolate D, ≤13 days). Despite these differences, the treatment effects clearly appeared for susceptible fungi; the survival rates for mice inoculated with strain Sr and isolate B were 7 of 10 and 5 of 10 mice treated with ITZ and FCZ, respectively (Fig. 2). For resistant isolates, the treatment had no effect; survival rates were 0 of 10, 1 of 10, 2 of 10, and 0 of 10 for mice inoculated with the resistant isolates A2, E2, D, and the Rr strain, respectively (Fig. 2). For the three dissociated isolates, ITZ led to survival rates of 5 of 10 (isolate A1), 5 of 10 (isolate C), and 6 of 10 (isolate E1), while FCZ was not efficient in preventing mouse death compared with the control treatment (Fig. 2).

The present study analyzed the susceptibilities of clinical isolates to antifungal agents in a disseminated model of antifungal activity (1). The drug regimens were close to those described in other experiments (1, 10, 19) and were efficient at limiting the deaths of mice caused by the susceptible strains but not the resistant strains. This result confirmed the validity of the mouse model for testing the in vivo susceptibilities of clinical isolates, despite differences in the pathogenicities of the isolates or at least the number of surviving mice after challenge (Fig. 2) (12). The good survival obtained in vivo after treatment with ITZ could be explained by the twice-daily regimen chosen for this antifungal drug. Several studies have assessed the importance of in vivo testing of antifungal agents after in vitro testing. In vitro and in vivo correlations have been established (17) and, more recently, have been documented for FCZ, amphotericin B, and flucytosine (1). Moreover, an in vivo dissociation in resistance to FCZ and ITZ was also observed among the isolates tested. A dissociation of resistance between FCZ and ITZ was reported in vitro (3). Most of the analysis concerning this dissociation was performed in vitro, and evidence of such a dissociation in vivo is reported herein. The mouse model confirmed the dissociation of resistance found in vitro, even with some isolates that were very similar in terms of their genotypes (13).

Drug resistance has been identified as a major cause of treatment failure among patients treated with azoles (20). In the present study, we found a link between clinical observations and the in vitro susceptibilities of clinical isolates to antifungal agents, even when isolates were resistant to only one azole. The presence of a dissociation in resistance between azoles (namely, FCZ and ITZ), which has already been described in vitro, has also been confirmed in vivo. This dissociation in resistance for the same isolate could, however, disappear among isolates from the same patient during the course of the antifungal therapy and lead to FCZ-ITZ cross-resistance.

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